Neutrophil Involvement in Autoimmune Rheumatic Disease: The Modulatory Roles of Hypoxia and Autoimmune Immunoglobulin G

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Declaration

I, Akif Ahmed Khawaja, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Signature:

Date:

Abstract

Neutrophil dysfunction has been described in various inflammatory and autoimmune rheumatic diseases (ARDs), including rheumatoid arthritis (RA), systemic lupus erythematosus (SLE) and antiphospholipid syndrome (APS). These ARDs are typically characterised by circulating autoantibodies, which contribute to immunopathology. Studies have also reported low oxygen levels, or hypoxia, in association with disease manifestations.

One mechanism of neutrophil activation, results in the release of a meshwork of chromatin fibres decorated with antimicrobial proteins, called neutrophil extracellular traps (NETs), which promote pathogen killing. Whilst NETs are important in fighting infection, if unchecked severe damage to host organs can be caused. Aberrant NETosis has been described in ARDs. Integrin engagement modulates several aspects of neutrophil activation including NETosis, cytokine production and reactive oxygen species (ROS) generation, which are associated with pathology in certain ARDs. Therefore, my PhD aimed to examine the effects of hypoxia and purified ARD-IgG on integrin activation, ROS generation and NETosis.

Isolated neutrophils were cultured under normoxia (21% oxygen) or hypoxia (1% oxygen) and integrin expression, adhesion, ROS generation and NETosis examined. Hypoxic neutrophils had higher α_M and α_X expression and increased adhesion to endothelial cells. Trans-endothelial migration was also enhanced under hypoxia. Whilst hypoxia did not have an effect on ROS generation, NETosis was higher in hypoxic cells. IgG was purified from the serum of RA, SLE and APS patients. The effects of purified IgG upon neutrophil adhesion, ROS generation and NETosis were examined. ARD-IgG had a differential effect upon integrin activation, with RA- and SLE-IgG promoting $\alpha_M\beta_2$ (Mac-1)-mediated adhesion whilst APS-IgG enhanced β_1-integrin mediated adhesion. Moreover, RA- and SLE-IgG increased rates of hydrogen peroxide generation and NETosis.

The results obtained in this thesis demonstrate that hypoxia modulates neutrophil function. Purified ARD-IgG was also identified as having differential effects on neutrophil integrin activation, ROS generation and NETosis.

Acknowledgements

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Abbreviations and Definitions

aCL	anti-cardiolipin antibodies
ACPA	anti-citrullinated peptide antibody
ACR	American college of rheumatology
AIA	antigen-induced arthritis
ANCA	anti-neutrophil cytoplasm antibodies
APC	antigen presenting cell
aPL	antiphospholipid antibodies
APS	antiphospholipid syndrome
ARD	autoimmune rheumatic disease
ARNT	aryl hydrocarbon receptor nuclear translocator protein
BAL	bronchoalveolar lavage
BCECF-AM	2', 7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein acetoxymethyl ester
BCR	B cell receptor
bHLH	basic helix-loop-helix
Breg	regulatory B cell
BSA	bovine serum albumin
C-TAD	C-terminal transactivation domain
CAPS	catastrophic APS
CBP	CREB-binding protein
CCP	cyclic citrullinated peptide
CD	cluster of differentiation
CD99L2	CD99-like protein 2
CIA	collagen-induced arthritis
COX	cyclooxygenase
CR	complement receptor
CRP	C-reactive protein
CTL	cytotoxic T lymphocytes
CTLA-4	cytotoxic T-lymphocyte-associated protein 4
DAPI	4'-6-diamidino-2-phenylindole
DAS28	disease activity score in 28 joints
DC	dendritic cell
DMARD	disease-modifying anti-rheumatic drug
DMOG	dimethyloxalyglycine

dsDNA	double stranded DNA
EBM-2	endothelial basal medium 2
EC	endothelial cell
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
EGM-2	endothelial growth medium 2
ELISA	enzyme-linked immunosorbent assay
EPAS1	endothelial PAS domain protein 1
ERK	extracellular signal-regulated kinase
ESR	erythrocyte sedimentation rate
EULAR	European League Against Rheumatism
FcR	Fc receptors
FCS	foetal calf serum
FIH1	factor inhibiting HIF-1α
FLS	fibroblast-like synoviocytes
fMLP	formyl-methionyl-leucyl-phenylalanine
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GlyCAM-1	glycosylation-dependent cell adhesion molecule 1
GM-CSF	granulocyte/macrophage colony-stimulating factor
HBSS	Hank's balanced salt solution
HC	healthy control
HCQ	hydroxychloroquine
hEGF	human epidermal growth factor
HIF	hypoxia-inducible factor
HLA	human leukocyte antigen
HMGB	high mobility group box
HRE	hypoxia responsive element
HRP	horseradish peroxidase
HUVEC	human umbilical cord vein endothelial cell
I-domain	inserted domain
I-EGF	integrin epidermal growth factor
I/R	ischemia/reperfusion
ICAM	intercellular adhesion molecule
IFN	interferon
Ig	immunoglobulin
IgSF	immunoglobulin super family
IL	interleukin

ILD	interstitial lung disease
IPAS	inhibitory PAS domain protein
ITAM	immunoreceptor tyrosine-based activating motif
iTreg	induced regulatory T cell
JAM	junctional adhesion molecule
LA	lupus anticoagulant
LA-1	leukadherin-1
LDG	low-density granulocyte
LFA-1	lymphocyte function-associated antigen 1
LMWH	low molecular weight heparin
LPAM-1	lymphocyte Peyer patch adhesion molecule-1
LPS	lipopolysaccharide
M-199	medium-199
Mac-1	macrophage-1 antigen
MAdCAM-1	mucosal vascular addressin cell adhesion molecule 1
MAPK	mitogen-activated proteins kinases
MFI	mean fluorescence intensity
MHC	major histocompatibility complex
MIDAS	metal ion-dependent adhesion site
mmHg	millimetre of mercury
MMP	matrix metalloproteinase
MPO	myeloperoxidase
mTOR	mammalian target of rapamycin
mTORC	mTOR complex
N-TAD	N-terminal transactivation domain
NADPH	nicotinamide adenine dinucleotide phosphate
NE	neutrophil elastase
NEPAS	neonatal and embryonic PAS protein
NET	neutrophils extracellular trap
NF-κB	nuclear factor-κB
NK	natural killer
NKT	natural killer T
NSAID	non-steroidal anti-inflammatory drug
OA	osteoarthritis
OD	optical density
PAD	peptidyl arginine deiminase
PAF	platelet-activating factor

PAR-2	protease-activated receptor-2
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
pDC	plasmacytoid DC
PDGF	platelet-derived growth factor
PECAM-1	platelet-endothelial cell adhesion molecule 1
PER	period circadian protein
PFA	paraformaldehyde
PHA	phytohaemagglutinin
PHD	prolyl hydroxylase
PI3K	phosphatidylinositol-3-kinase
PKB	protein kinase B
PKC	protein kinase C
PMA	phorbol 12-myristate 13-acetate
PMN	polymorphonuclear leukocytes
PR3	proteinase 3
PSGL-1	P-selectin glycoprotein 1
PSI	plexin/semaphoring/integrin
PVDF	polyvinylidene difluoride
pVHL	von Hippel-Lindau protein
R^3-IGF-1	recombinant insulin-like growth factor 1
RA	rheumatoid arthritis
RANK	receptor activator of NF-κB
RANKL	receptor activator of NF-κB ligand
RBC	red blood cell
RhF	rheumatoid factor
ROS	reactive oxygen species
RPMI	Roswell Park Memorial Institute medium
SD	standard deviation
SDL	specificity-determining loop
SF	synovial fluid
SFK	Src family kinase
SIM	single-minded protein
SLE	systemic lupus erythematosus
sLe^x	Lewis x blood group
Syk	spleen tyrosine kinase
TAD	transactivation domain

TBS	tris buffered saline
TCA	tricarboxylic acid
TCR	T cell receptor
TF	tissue factor
TFG-β	transforming growth factor β
Th	T helper cell
TLR	toll-like receptor
TNF-α	tumour necrosis factor-α
Treg	regulatory T cell
TSC	tumor suppressor complex
VCAM-1	vascular cell adhesion molecule 1
VEGF	vascular endothelial growth factor
VLA	very late antigen
VNTR	variable number of tandem repeats
β_2GP1	β_2-glycoprotein 1

Chapter One:
Introduction

1.1 Cells of the immune system

The immune system is composed of an intricate network of cells, humoral factors, cytokines and lymphoid organs, all of which are important in maintaining host health and wellbeing. Immune defence can be divided into two categories: innate immunity, which is present and active at all times; and adaptive immunity, which provides long-lasting immune protection. All immune cells are derived from haematopoietic stem cells and can be referred to as leukocytes. As haematopoietic stem cells differentiate into specialised immune cells, they can be further categorised as either lymphocytes or myeloid cells (Figure 1.1).

1.1.1 Lymphocytes

Lymphocytes largely form part of the adaptive immune system and confer long-lasting immune protection. The common lymphoid progenitor cell is capable of differentiating into natural killer (NK) cells, dendritic cells (DCs) or small lymphocytes. Small lymphocytes further differentiate into either B lymphocytes (B cells) or T lymphocytes (T cells), depending on the compartmental microenvironment and cytokines present during development.

1.1.1.1 B lymphocytes

B cells are produced within the bone marrow, but mature in the Bursa of Fabricus (Ribatti et al., 2006). On leaving the bone marrow, each B cell expresses a unique antigen-binding B cell receptor (BCR) on its cell membrane. This BCR is a membrane-bound antibody molecule. When a naïve B cell first recognises an antigen via its BCR, the binding of antigen induces rapid cell division. B cells can differentiate into memory B cells or plasma cells. Regulatory subsets of B cells (Bregs) are also produced, which can suppress and regulate B cell activity (Fillatreau et al., 2002). Memory B cells have a longer life span than naïve B cells and express the same BCR as their parent B cell. Plasma cells produce antibodies in a form that can be secreted and have little or no membrane-bound antibody (Delves and Roitt, 2000, Goldsby et al., 2003).

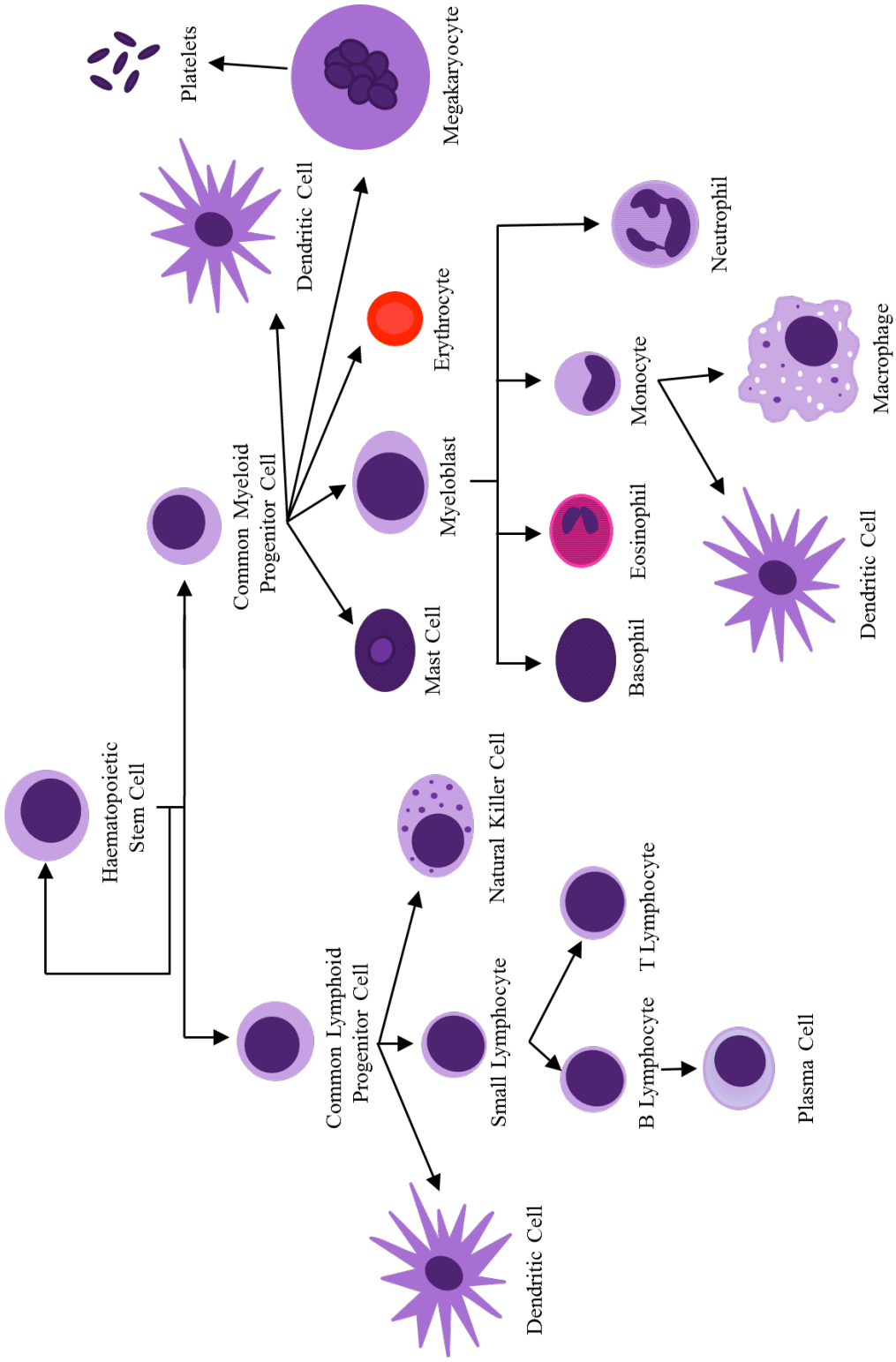


Figure 1.1: Immune cell differentiation. This schematic briefly depicts the stages of differentiation that haematopoietic stem cells undergo to produce the immune cells that form the human immune system.

Antibodies are glycoproteins that consist of two identical heavy and light polypeptide chains. Each heavy chain is joined to a light chain by a disulphide bond, with additional disulphide bonds holding the two pairs together (Edelman, 1973) (Figure 1.2). The N-terminal variable ends of the heavy and light chains form a cleft that binds antigen. The C-terminal domains of the heavy and light chains form the constant region, which define the class and subclass of the antibody. There are five classes of immunoglobulin (Ig) (IgG, IgA, IgM, IgD and IgE), four subclasses of IgG (IgG1-4) and two subclasses of IgA (IgA1-2). These classes and subclasses have different functions (Janeway, 1993, Delves and Roitt, 2000, Goldsby et al., 2003), which are outlined in Table 1.1.

When a microorganism is coated with antibodies, or opsonised, it can be eliminated in several ways. For example, antibodies can cross-link several antigens to form clusters that are more readily ingested by phagocytic cells. Binding of antibody to antigen can also activate the complement system, resulting in lysis of the foreign organism. Antibodies can also neutralise toxins or viral particles by coating them, which prevents binding to host cells (Janeway, 1993).

1.1.1.2 T lymphocytes

T cells are also produced in the bone marrow but migrate to the thymus to mature (Kruisbeek, 1999). During maturation, T cells develop a unique antigen-binding molecule on their membrane: the T cell receptor (TCR). Unlike the BCR that can directly recognise antigen, TCRs can only recognise antigens that are presented by major histocompatibility complex (MHC) molecules on the surface of antigen presenting cells (APCs), such as macrophages, B cells and DCs, which are described in further detail in section 1.1.2.1. There are two types of MHC molecules: class I MHC molecules, which are expressed by nearly all nucleated cells and class II MHC molecules that are only expressed by APCs. When naïve T cells encounter antigen presented by MHC, they proliferate and differentiate into memory or effector T cells (Ellmeier et al., 1999, Delves and Roitt, 2000, Goldsby et al., 2003).

There are two well-defined subpopulations of T cells: T helper (Th) cells and cytotoxic T lymphocytes (CTL), which are distinguished from one another by surface expression of either

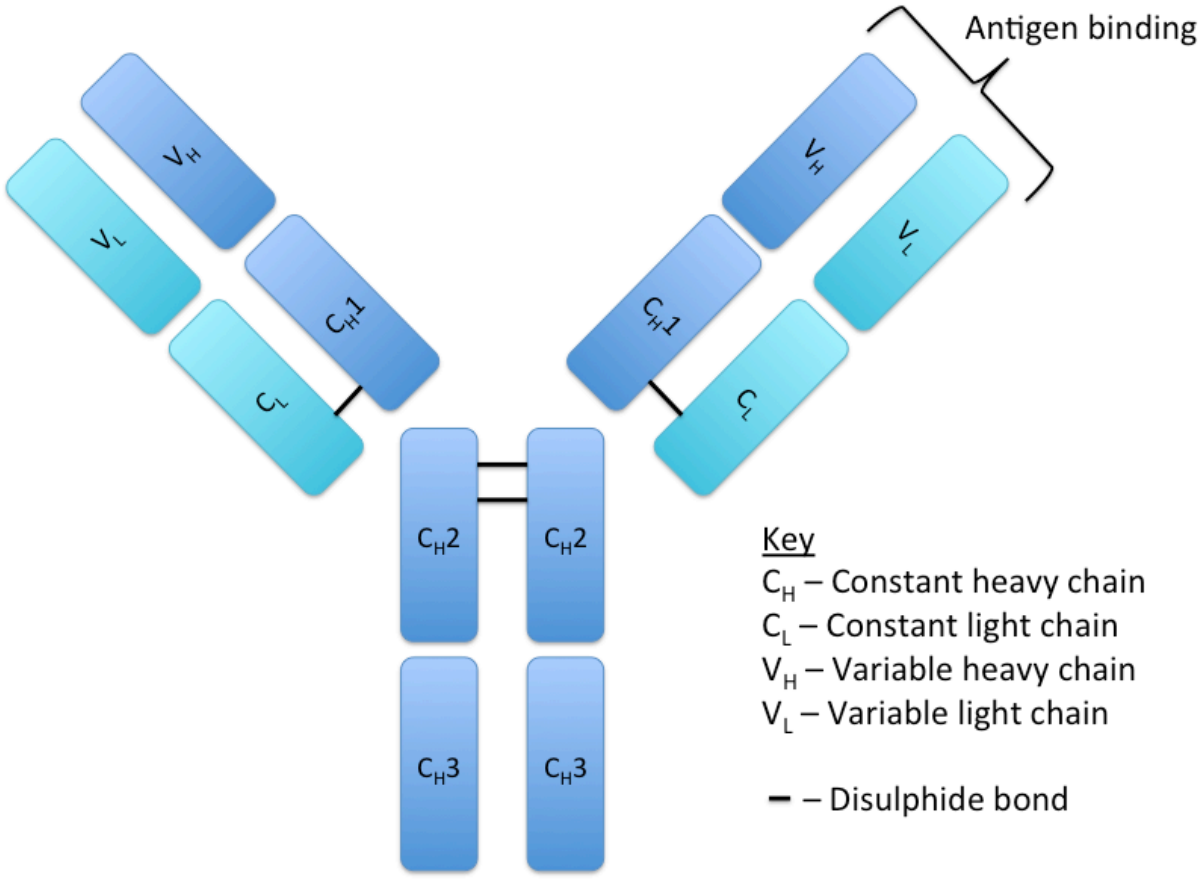


Figure 1.2: Schematic of antibody structure. This diagram illustrates the basic structure of an antibody, highlighting the constant region, which determines the class and subclass of the antibody, and the variable domains, which bind antigen.

Class	Subclass	Function
IgA	IgA1 IgA2	IgA accounts for 10-15% of total serum Ig. The predominant Ig class in external secretions, such as breast milk, saliva, tears and mucus of the bronchial, genitourinary and gastrointestinal tracts, IgA has important effector functions at mucus membrane surfaces.
IgD	None	IgD constitutes ~0.2% of total serum Ig. No biological effector function has been identified for IgD, however, together with IgM, it is the major membrane-bound Ig expressed by mature B cells.
IgE	None	Found in very low levels in serum, IgE mediate the immediate hypersensitivity reaction that are responsible for the symptoms of hay fever, asthma, hives and anaphylactic shock.
IgG	IgG1 IgG2 IgG3 IgG4	IgG constitutes ~80% of total serum Ig. IgG antibodies are produced by B cells when the body is attacked by the same pathogen in a subsequent invasion (i.e. part of the adaptive immune response). IgG can activate the complement system and is frequently involved in opsonisation.
IgM	None	IgM Accounts for 5-10% of total serum Ig. IgM is the first Ig class produced in a primary response to an antigen and is the first Ig synthesised by neonates. IgM can also activate complement.

Table 1.1: Immunoglobulin class, subclass and function. The above table details the different classes and subclasses of the five Ig produced by humans with an overview of their function.

cluster of differentiation (CD)4 or CD8. $CD4^+$ T cells recognise antigens bound to class II MHC molecules, whereas $CD8^+$ T cells recognise antigen bound to class I MHC molecules. The classification of $CD4^+$ MHC class II restricted cells as Th cells and $CD8^+$ MHC class I restricted cells as CTLs is not absolute and there is some overlap between functional activities (Goldsby et al., 2003, Delves and Roitt, 2000).

Naïve $CD4^+$ T cells can differentiate into several Th cell lineages, defined by their pattern of cytokine production and function, including Th1, Th2, Th17 and induced regulatory T cells (iTregs). Other types of $CD4^+$ T cells have been described, such as natural killer T (NKT) cells and natural Tregs (nTregs), however, these cells are not derived from naïve $CD4^+$ T cells, but develop in parallel from small lymphocytes within the thymus (Zhu et al., 2010).

1.1.2 Myeloid cells

Myeloid cells form part of the innate immune system and can directly recognise and kill pathogens. The common myeloid progenitor cell can differentiate into several immune cells, including DCs, erythrocytes, megakaryocytes, mast cells and myeloblasts. Myeloblasts can differentiate into basophils, eosinophils, neutrophils and monocytes, which can further differentiate into either macrophages or monocyte-derived DCs (Ma et al., 2012).

Microbial killing is facilitated by phagocytosis, aided by opsonisation with Ig or the complement product iC3b. Most myeloid cells express Fc receptors (FcR) that bind the constant region of Ig, as well as complement receptor (CR)1, CR3 and CR4, which bind complement products. Successful phagocytosis is achieved by the synergistic activation of these receptors. When APCs phagocytose pathogens, they are able to degrade and present pathogen-derived antigens to lymphocytes (Delves and Roitt, 2000).

1.1.2.1 Antigen presenting cells

APCs are specialised cells, including macrophages, B cells and DCs, which express class II MHC molecules and are able to deliver the co-stimulatory signals required for $CD4^+$ T cell activation. APCs internalise antigen, either by phagocytosis or endocytosis, and then display antigen-derived peptides in a complex with class II MHC molecules that are recognised

by $CD4^+$ T cells. Additional co-stimulatory signals, provided by the APC, are then required for full $CD4^+$ T cell activation (Goldsby et al., 2003). In the absence of these co-stimulatory signals, cells can undergo anergy.

Most leukocytes can be found circulating within the vasculature. These leukocytes are endowed with the vital ability to migrate from the circulation into the underlying tissues, where they can mediate their immunological roles. The process whereby leukocytes migrate into tissues is referred to as leukocyte extravasation.

1.2 Leukocyte adhesion and transmigration

Leukocyte extravasation is achieved via an orchestrated series of reversible and transient adhesive events. The most extensively studied form of leukocyte extravasation is migration across the endothelium, which represents the first barrier for immune surveillance. Trans-endothelial migration can be divided into three stages: attachment and rolling, activation-dependent firm adhesion and transmigration.

1.2.1 Attachment and rolling

Leukocytes can roll along activated endothelial cells (ECs), primarily mediated by selectins (Figure 1.3). Selectins are specialised receptors that recognise proteins modified with sialyated fucosylated carbohydrate residues belonging to the sialyl Lewis X family (sLe^X). These interactions are known for their fast on/off rates, with cell contacts being rapidly formed and broken. To date, three members of the selectin family have been described: E-selectin (endothelial; CD62E) (Bevilacqua et al., 1989), L-selectin (leukocyte; CD62L) (Gallatin et al., 1983) and P-selectin (platelet; CD62P) (Hsu-Lin et al., 1984), each named after the cell in which they were first described. Activated ECs also express P-selectin (Bonfanti et al., 1989).

The most characterised selectin ligand is P-selectin glycoprotein ligand (PSGL)-1, which is expressed on leukocytes (McEver and Cummings, 1997). On activation, ECs upregulate E-selectin and externalise P-selectin within Weibel-Palade bodies, both of which bind PSGL-1. Leukocytes can facilitate cell rolling via the redistribution of PSGL-1 to protruding pseudopods (Bruehl et al., 1997). ECs can also enhance interactions by modifying

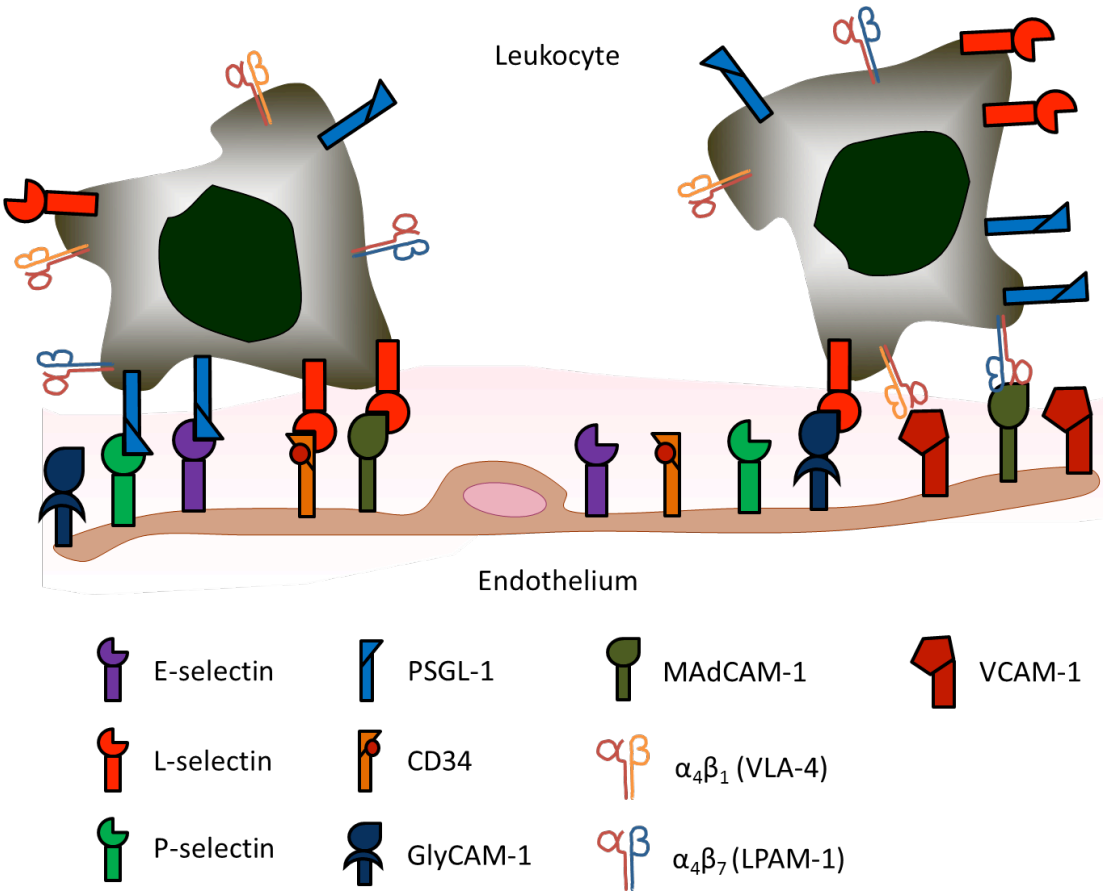


Figure 1.3: Leukocyte rolling is mediated by L-selectin, PSGL-1 and VLA-4. This schematic details some of the key adhesive molecules involved in initial tethering and rolling of leukocytes within the vasculature. Both E-selectin and P-selectin expressed on endothelial cells bind PSGL-1. L-selectin expressed by leukocytes can interact with modified CD34, GlyCAM-1 and MAdCAM-1 found on endothelial cells. VLA-4 and LPAM-1 can interact with VCAM-1 and MAdCAM-1 respectively. Abbreviations: GlyCAM-1, glycosylation-dependent cell adhesion molecule-1; LPAM-1, lymphocyte Peyer patch adhesion molecule; MAdCAM-1, mucosal vascular addressin cell adhesion molecule-1; PSGL-1, P-selectin glycoprotein ligand-1; VCAM-1, vascular cell adhesion molecule-4; VLA-4, very late antigen-4.

surface CD34 with the sLe^x group, transforming CD34 into a L-selectin ligand (Hiraoka et al., 1999). L-selectin has also been shown to bind glycosylation-dependent cell adhesion molecule (GlyCAM)-1 (Imai et al., 1991) and mucosal vascular addressin cell adhesion molecule (MAdCAM)-1 (Bargatze et al., 1995), which further promotes leukocyte rolling.

There is also evidence that α_4 integrins contribute to leukocyte rolling. Integrins are a family of adhesive molecules, which will be discussed in section 1.3. Studies using cultured T cells found very late antigen (VLA)-4 (CD49d/CD29, $\alpha_4\beta_1$) and lymphocyte Peyer patch adhesion molecule (LPAM)-1 ($\alpha_4\beta_7$) mediate rolling via interactions with vascular cell adhesion molecule (VCAM)-1 (CD106) and MAdCAM-1 respectively, independent of selectins (Berlin et al., 1995, Alon et al., 1995). Work using freshly isolated T cells however, found VLA-4 and P-selectin initiated rolling together. Furthermore, *ex vivo* transmigration assays found VLA-4 to initiate rolling in cooperation with L-selectin in both freshly isolated monocytes (Luscinskas et al., 1994) and eosinophils (Sriramarao et al., 1994).

The discrepancy between freshly isolated and cultured cells suggests that selectin dependency may be linked to leukocyte activation, with resting cells having a higher degree of selectin-independent integrin-mediated tethering. Given that T cells are easily cultured over longer periods of time, whilst eosinophils and monocytes cannot be extensively cultured without cell death or differentiation, interpreting these observations is difficult. As VLA-4 has been implicated in both freshly isolated and cultured cells, it is reasonable to conclude that selectins and α_4 integrins work cooperatively to mediate leukocyte attachment and rolling.

1.2.2 Activation-dependent firm adhesion

Firm adhesion is predominantly mediated by endothelial intercellular adhesion molecule (ICAM)-1 (CD54) binding to lymphocyte function-associated antigen-1 (LFA-1; CD11a/CD18; $\alpha_L\beta_2$) and macrophage-1 antigen (Mac-1; CD11b/CD18; $\alpha_M\beta_2$) expressed on leukocytes. Following initial adhesion, ECs can redistribute ICAM-1, enriching the areas beneath leukocytes to further stabilise interactions with LFA-1 and Mac-1 (Shaw et al., 2004). This observation demonstrates the importance of ICAM-1 in leukocyte firm adhesion.

ICAM-2 (CD102) has also been implicated in extravasation, with inhibition of neutrophil transmigration being reported in both ICAM-2 knockout mice and wild-type mice administered ICAM-2 blockade (Huang et al., 2006, Woodfin et al., 2009). These results have not been replicated *in vitro*, which may suggest that whilst ICAM-2 may be important in murine leukocyte extravasation, it mediates a subtler role in human leukocyte transmigration.

Interestingly, a similar pattern of adhesion molecule clustering has been documented with endothelial VCAM-1 around VLA-4 on adherent leukocytes (Barreiro et al., 2002, Carman and Springer, 2004). Taken together, these results indicate that firm adhesion is achieved by the collaborative action of the β_1 and β_2 integrins binding VCAM-1 and ICAM-1, and possibly ICAM-2, rich regions on ECs (Figure 1.4). These stronger interactions allow for the cessation of leukocyte rolling and enable cells to migrate out of the vasculature into the underlying tissues.

1.2.3 Transmigration

Following firm adhesion, leukocytes initiate diapedesis and migrate across the endothelium. This process is mediated by junctional adhesion molecule (JAM)-A, JAM-B and JAM-C, all of which are highly expressed at EC borders. There is also evidence for the contribution of platelet-endothelial cell adhesion molecule (PECAM)-1 (CD31) and CD99.

JAM proteins preserve the integrity of resting endothelial monolayers. JAM-A engages in homophilic interactions with other JAM-A molecules, whereas JAM-B and JAM-C partake in both homophilic and heterophilic binding. During inflammation, JAM proteins can bind leukocyte integrins and facilitate extravasation. LFA-1 can bind JAM-A (Ostermann et al., 2002), however conflicting results arise from JAM-A inhibition. Whilst some groups report reduced transmigration (Martin-Padura et al., 1998, Woodfin et al., 2009), others found JAM-A blockade had no significant effect (Liu et al., 2000, Schenkel et al., 2004b). Schenkel et al. did describe a 10% decrease in transmigration on JAM-A inhibition, but suggested that this effect was insignificant. Taken together, the evidence suggests that JAM-A contributes to leukocyte transmigration. JAM-B has been shown to interact with VLA-4, promoting leukocyte rolling and adhesion *in vitro* (Ludwig et al., 2009), however the effects on migration were not explored.

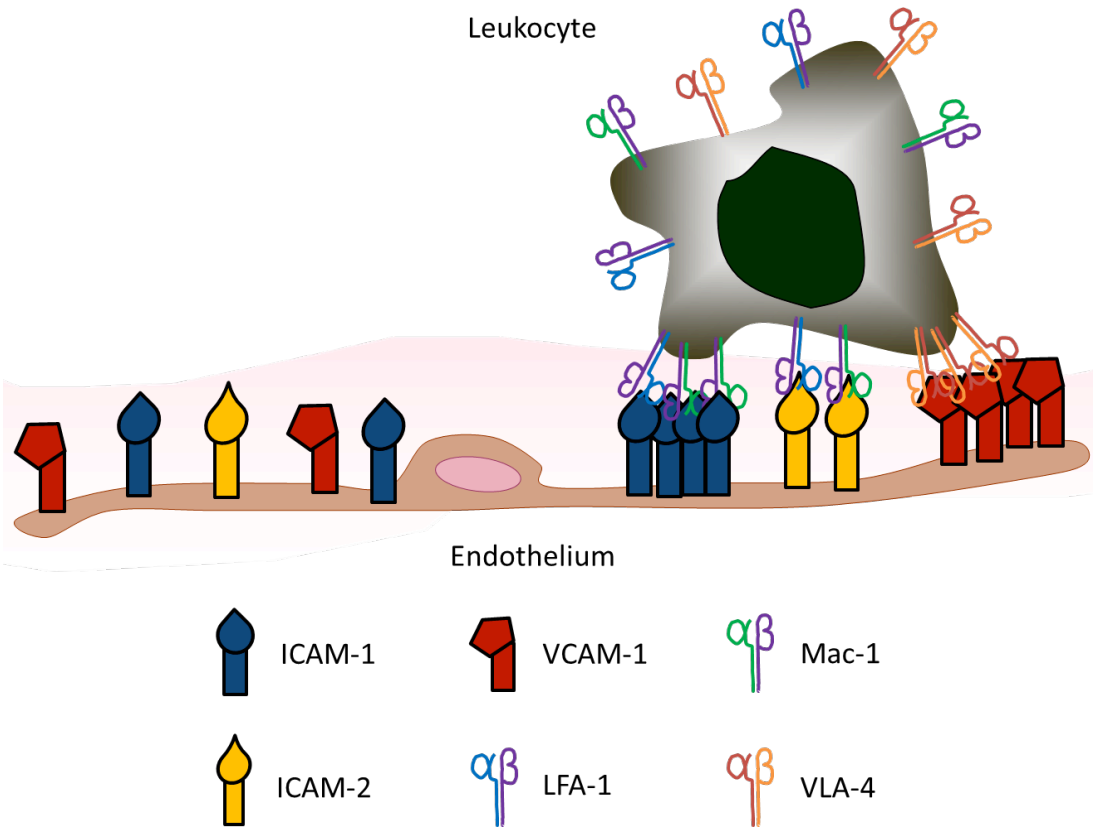


Figure 1.4: Firm adhesion of leukocytes is mediated by LFA-1, Mac-1 and VLA-4. This schematic depicts the key adhesive molecules mediating activation-dependent firm adhesion. The key interactions mediating firm adhesion are those between endothelial ICAM-1 and LFA-1 and Mac-1 expressed on leukocytes. ICAM-2 also interacts with both LFA-1 and Mac-1, whilst VLA-4 binds to VCAM-1. Endothelial cells are able to redistribute cell surface ICAM-1 and VCAM-1 underneath adherent cells to further facilitate adhesion. Abbreviations: ICAM-1, intercellular adhesion molecule-1; LFA-1, lymphocyte function-associated antigen-1; Mac-1, macrophage-1 antigen; VCAM-1, vascular cell adhesion molecule-1; VLA-4, very late antigen-4.

Mac-1 has been shown to interact with JAM-C, allowing for transmigration both *in vitro* and *in vivo* (Johnson-Leger et al., 2002, Chavakis et al., 2004).

PECAM-1 is a member of the Ig superfamily (IgSF) and is expressed in a variety of cell types including platelets, monocytes, neutrophils and some T cell subsets. PECAM-1 is also found on EC borders, where it binds leukocyte-expressed PECAM-1 and mediates the beginnings of diapedesis. PECAM-1 blockade both *in vitro* and *in vivo* does not inhibit adhesion to the endothelium, however leukocytes are unable to transmigrate (Muller et al., 1993, Bogen et al., 1994, Mamdouh et al., 2003, Schenkel et al., 2004a, Schenkel et al., 2004b). PECAM-1 stimulation has been reported to increase leukocyte adhesion via LFA-1 (Piali et al., 1993, Berman et al., 1996), Mac-1 (Berman and Muller, 1995, Berman et al., 1996), VLA-4 (Leavesley et al., 1994, Chiba et al., 1999) and $\alpha_v\beta_3$ (Chiba et al., 1999). These observations suggest that PECAM-1 engagement may enhance β_1, β_2 and β_3 integrin affinity. In addition, studies in which PECAM-1 was transfected into cells that do not express it, found that transfected cells supported leukocyte transmigration, a phenomenon that has not been replicated with any other adhesion molecule (Dasgupta et al., 2009).

CD99 is an evolutionary conserved protein, believed the earliest cell adhesion molecule (Suh et al., 2003). As well as being expressed by ECs, CD99 expression has also been found on monocytes (Schenkel et al., 2002) and neutrophils (Lou et al., 2007). Homophilic CD99 interactions are essential for transmigration and mediate the final stages of diapedesis. In agreement with previous work, both studies found that with PECAM-1 blockade, monocytes and neutrophils could adhere to the endothelium but remained at the cell membrane. Following CD99 blockade, leukocytes could partially migrate across the endothelium, with cell protrusions visible between ECs, but were unable to achieve complete transmigration (Schenkel et al., 2002, Lou et al., 2007). A schematic of diapedesis can be seen in Figure 1.5.

To conclude, leukocyte extravasation is a highly regulated process, mediated by the sequential activation of adhesion molecules and integrins. Moreover, experimental evidence highlights the importance of the β_1 and β_2 integrins in extravasation, given their roles throughout the stages of transmigration.

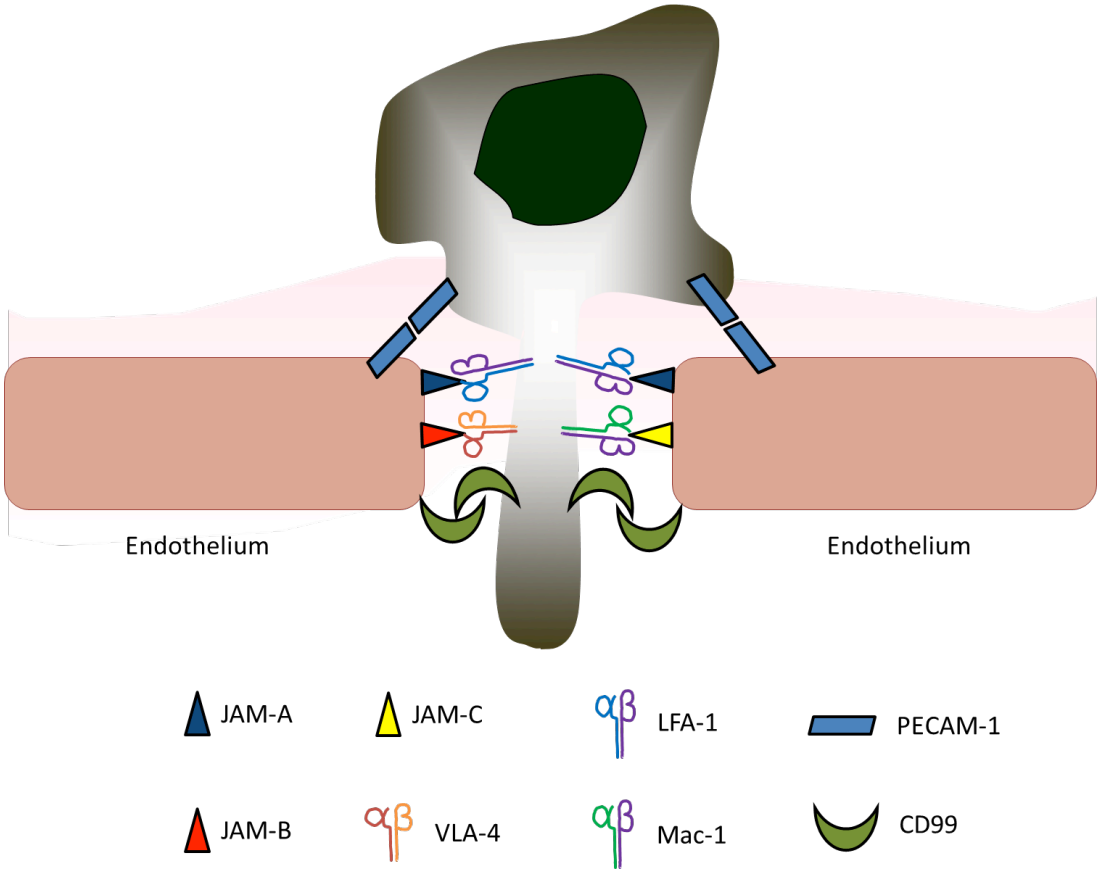


Figure 1.5: Leukocyte diapedesis is mediated by junctional adhesion molecules, PECAM-1 and leukocyte integrins. Depicted above are the key adhesion molecules that regulate the final step of extravasation. Homophilic PECAM-1 interactions are believed to be one of the first steps of leukocyte diapedesis across the endothelium. JAM-A interacts with LFA-1, JAM-B with VLA-4 and JAM-C with Mac-1, all of which allow the leukocyte to move between endothelial cells. CD99 mediates the final step of transmigration, which undergoes homophilic associations and enables the leukocyte to successfully cross the endothelial monolayer. Abbreviations: JAM, junctional adhesion molecule; LFA-1, lymphocyte function-associated antigen; Mac-1, macrophage-1 antigen; PECAM-1, platelet-endothelial adhesion molecule; VLA-4, very late antigen-4.

1.3 Integrins

The term 'integrin' was first used in 1986 to describe transmembrane protein complexes integral to leukocyte interactions with the extracellular matrix (ECM) (Tamkun et al., 1986). As homologous proteins were identified, the term integrin was adopted to describe the family of structurally related cell surface receptors. Integrins are expressed by all multicellular organisms, with differential integrin expression being observed between phyla (Hynes, 2002). The immune system is highly dependent on integrins for orchestrating leukocyte migration. Integrin function also facilitates immune synapse formation, phagocytosis and cell signalling transduction. It is therefore vital that leukocytes regulate integrin expression and activity.

Integrin ligands can be divided into three major groups: basal ECM (e.g. laminins, collagens), provisional ECM (e.g. fibrinogen, fibronectin) and cell surface adhesion molecules. The majority of the cell surface adhesion molecules that function as integrin ligands are IgSF proteins, which are characterised by multiple Ig-like domains. Members of the IgSF, such as ICAM-1 and VCAM-1, are expressed by numerous cell types and are upregulated on cell activation (Marui et al., 1993, Lee et al., 2001, Chiu et al., 2004, Min et al., 2005, Hortelano et al., 2010), which promotes leukocyte extravasation.

1.3.1 Leukocyte integrins

Integrins are heterodimeric type I transmembrane glycoprotein receptors, composed of non-covalently associated α and β subunits. To date, 18 α subunits and 8 β subunits have been described within the human integrin family, which through combinational variation give rise to 24 different heterodimeric αβ integrin molecules (Figure 1.6). Integrin expression varies between cell types and can be restricted to specific cell lineages or subsets. For example, β_2 integrins are only expressed by haematopoietic cells and the $\alpha_E\beta_7$ integrin is expressed by mucosal T cells (Kilshaw, 1999).

Electron microscopy demonstrates that integrin subunits are composed of a globular N-terminal ligand-binding 'head', representing a critical interface between the α and β subunits. The extracellular domain is attached to a 'leg' domain that connects the extracellular head to the

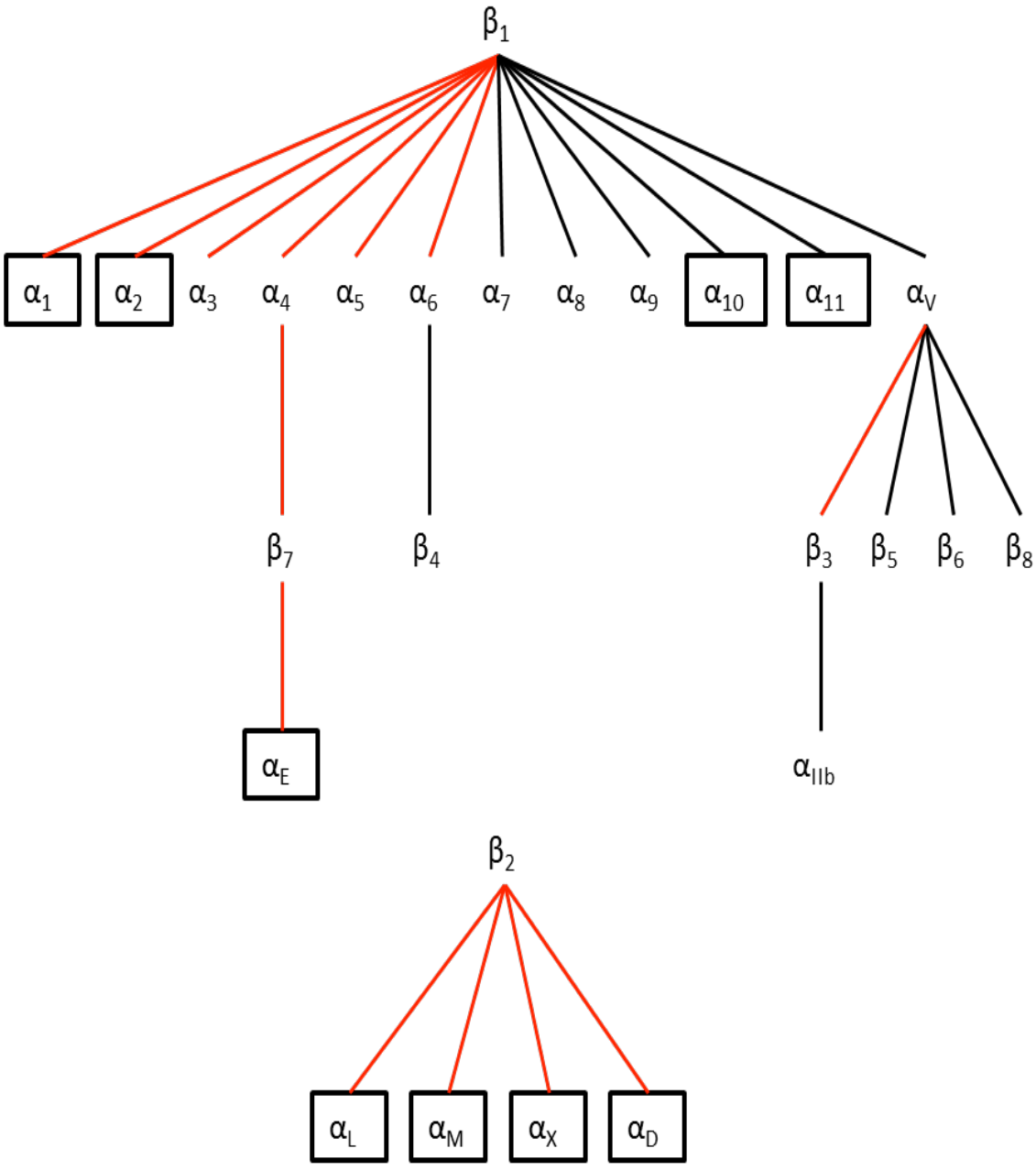


Figure 1.6: All 24 αβ integrins found in human cells. Depicted above are all of the integrin pairs found within human cell types. Integrin pairs connected with a red line are expressed on leukocytes. The α subunits boxed represent those subunits that contain the I-domain.

single spanning transmembrane domain. With the exception of the β_4 subunit, integrin subunits possess a short cytoplasmic tail (Nermut et al., 1988). Debate arose when X-ray crystallography of the $\alpha_V\beta_3$ integrin found the 'legs' in a bent topology, with the extracellular 'head' juxtaposed to the cell membrane (Xiong et al., 2001, Xiong et al., 2002). Electron microscopy and Förster resonance energy transfer analysis confirmed the existence of two conformations (Takagi et al., 2002b, Takagi et al., 2003, Kim et al., 2003), with the bent conformation representing a low-affinity state and the extended conformation being a high-affinity state with the ligand-binding site exposed.

X-ray crystallography of the α subunit found seven repeated domains, numbered I-VII, which forms a seven-bladed β-propeller motif (Xiong et al., 2001, Xiao et al., 2004). Of the 18 α subunits, half contain an inserted (I)-domain, residing between the second and third β-sheets of the β-propeller motif. Multiple interactions are formed between extracellular ligands and the I-domain, functioning as either the exclusive or major ligand-binding site (Emsley et al., 2000, Shimaoka et al., 2003, Song et al., 2005). *In vitro* expression of the I-domain, independent of the parent integrin, found high retention of ligand-binding activity, emphasising the functional importance of the I-domain. This phenomenon has been demonstrated in I-domains of LFA-1 (Randi and Hogg, 1994), Mac-1 (Ueda et al., 1994, Zhou et al., 1994) and VLA-2 (CD49b/CD29, $\alpha_2\beta_1$) (Kamata and Takada, 1994).

Crystallography studies also highlighted a β subunit I-domain, similar in structure to the α subunit I-domain but containing two extra segments, termed the I-like domain. Mutations in the I-like domain result in defective binding of immobilised ligands (Bilsland et al., 1994, Huang et al., 2000). Microscopic analysis and functional adhesion assays found that one of the extra segments forms an interface with the β-propeller of the α subunit, whilst the other mediates ligand binding (Takagi et al., 2002a, Tsuruta et al., 2003). This observation suggests that whilst the I-domain may be directly involved in binding, the I-like domain may play an auxiliary role by ensuring the correct conformation is adopted to expose the ligand-binding site.

Divalent cations are essential for integrin-mediated adhesion, demonstrated by the inhibition of adhesion following cation chelation by ethylenediaminetetraacetic acid (EDTA)

(Dransfield et al., 1992). Divalent cations act as coordination centres, which interact with and stabilise the I-domain. Due to the dependency of cations, the region surrounding the I-domain is termed the metal ion-dependent adhesion site (MIDAS), which contains several key Mg^{2+} and Ca^{2+} binding sites that aid molecular coordination and support integrin structure (Lee et al., 1995). The MIDAS structure has also been identified in the I-like domain, which adopts a similar fold as the α subunit counterpart (Xiao et al., 2004).

The 'leg' domain connects the extracellular 'head' to the transmembrane domain and is composed of three β-sandwich motifs, referred to as the thigh, calf-1 and calf-2 domains. A Ca^{2+} binding site lies in between the thigh and calf-1 domains to form a flexible region, often called the 'genu', which is a key pivotal point. The β subunit 'leg' domain is organised differently, with the I-like domain inserted into a hybrid domain, forming part of the upper β subunit 'leg' domain. The hybrid domain is inserted within a plexin/semaphoring/integrin (PSI) domain, which completes the upper β subunit 'leg'. The lower portion of the β subunit 'leg' is composed of four integrin epidermal growth factor-like (I-EGF) domains and a β-tail domain. Electron microscopy has found that the α subunit 'genu' and the β subunit 'leg' I-EGF-1 and I-EGF-2 interface are within close geometric proximity of one another (Takagi et al., 2002b, Nishida et al., 2006). The conformational changes during integrin activation are achieved by rotations around the axis generated by the region formed by the α and β subunit 'leg' domains. A basic schematic of the primary structure of integrins can be seen in Figure 1.7.

1.3.2 Integrin activation

Integrin activation is vital in enabling leukocyte interactions and is achieved by either inside-out or outside-in signalling. Inside-out signalling induces a conformational change that exposes the ligand-binding site and adopts a high-affinity state (Anthis and Campbell, 2011, Springer and Dustin, 2012, Zhang and Chen, 2012, Zhu and Springer, 2013).

When in a low-affinity state, the integrin extracellular 'head' sits in close proximity to the cell surface (Takagi et al., 2002b). On activation, cytoskeletal proteins talin and kindlin bind the cytoplasmic tail of the integrin β subunit cytoplasmic tail, causing the dissociation of the α

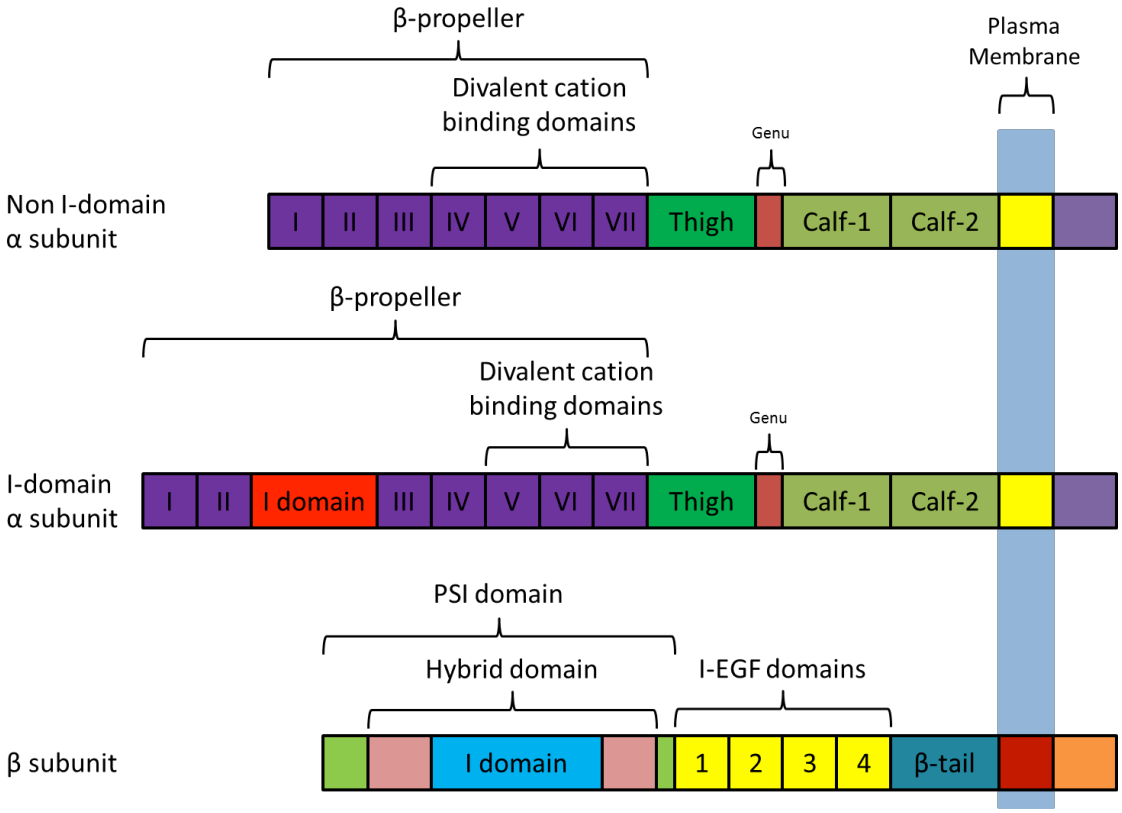


Figure 1.7: Schematic of the primary structure of leukocyte integrins. Structures for both non I-domain and I-domain containing α subunits are shown in this diagram. The α subunit is non-covalently linked to the β subunit on the cell membrane.

and β subunit cytoplasmic tails (Vinogradova et al., 2002). A cascade of molecular movements is subsequently initiated, dissociating the transmembrane helices. Extension of the 'leg' domains followed by the rotation of the 'head' domain, described as a 'switchblade motion', allows greater access to ligands. An intermediate state with extended 'leg' domains but closed 'head' has also been described (Lefort et al., 2012, Cheng et al., 2013). This intermediate conformational state may represent a mechanism whereby surroundings are surveyed without full integrin activation and signalling induction. Inside-out signalling is a dynamic process, where integrins exist in equilibrium between low- and high-affinity states. This conformational equilibrium allows leukocytes to constantly survey their surroundings. A schematic overview of inside-out integrin activation can be seen in Figure 1.8.

Outside-in signalling induces integrin clustering at the cell membrane and high-avidity interactions, typically occurring following intracellular signalling via the guanine nucleotide-binding protein Gα13 (Gong et al., 2010). On activation, Gα13 phosphorylates Src family kinases (SFKs), initiating numerous downstream signalling events (Klinghoffer et al., 1999, Obergfell et al., 2002, Inoue et al., 2003, Gong et al., 2010). One of these events is the activation p115RhoGEF and phosphorylation of Rho (Gong et al., 2010). Rho can activate formin homology proteins, referred to as formins, which mediate actin polymerisation (Pruyne et al., 2002). Formin activation promotes stress fibre formation (Kitzing et al., 2010), resulting in cytoskeletal rearrangement and integrin clustering (Vega et al., 2011). Whilst p115RhoGEF activation is one mechanism induced by outside-in signalling, several other pathways are also involved (Shen et al., 2012).

Divalent cations are essential for integrin function, stabilising structure and modulating integrin-ligand interactions in both enhancing and suppressive manners (Kunicki et al., 1981, Ginsberg et al., 1986, Staatz et al., 1989, Steiner et al., 1991, Dransfield et al., 1992). Leukocytes are typically exposed to 1mM Ca^{2+} and 1mM Mg^{2+} in the vasculature, with each cation conferring opposing effects. Whilst evidence suggests Ca^{2+} suppresses integrin activation (van Kooyk et al., 1993, Hu et al., 1995, Mandeville and Maxfield, 1997), Mg^{2+} has been shown to induce integrin-mediated binding (Staatz et al., 1989). Interestingly, these same studies found

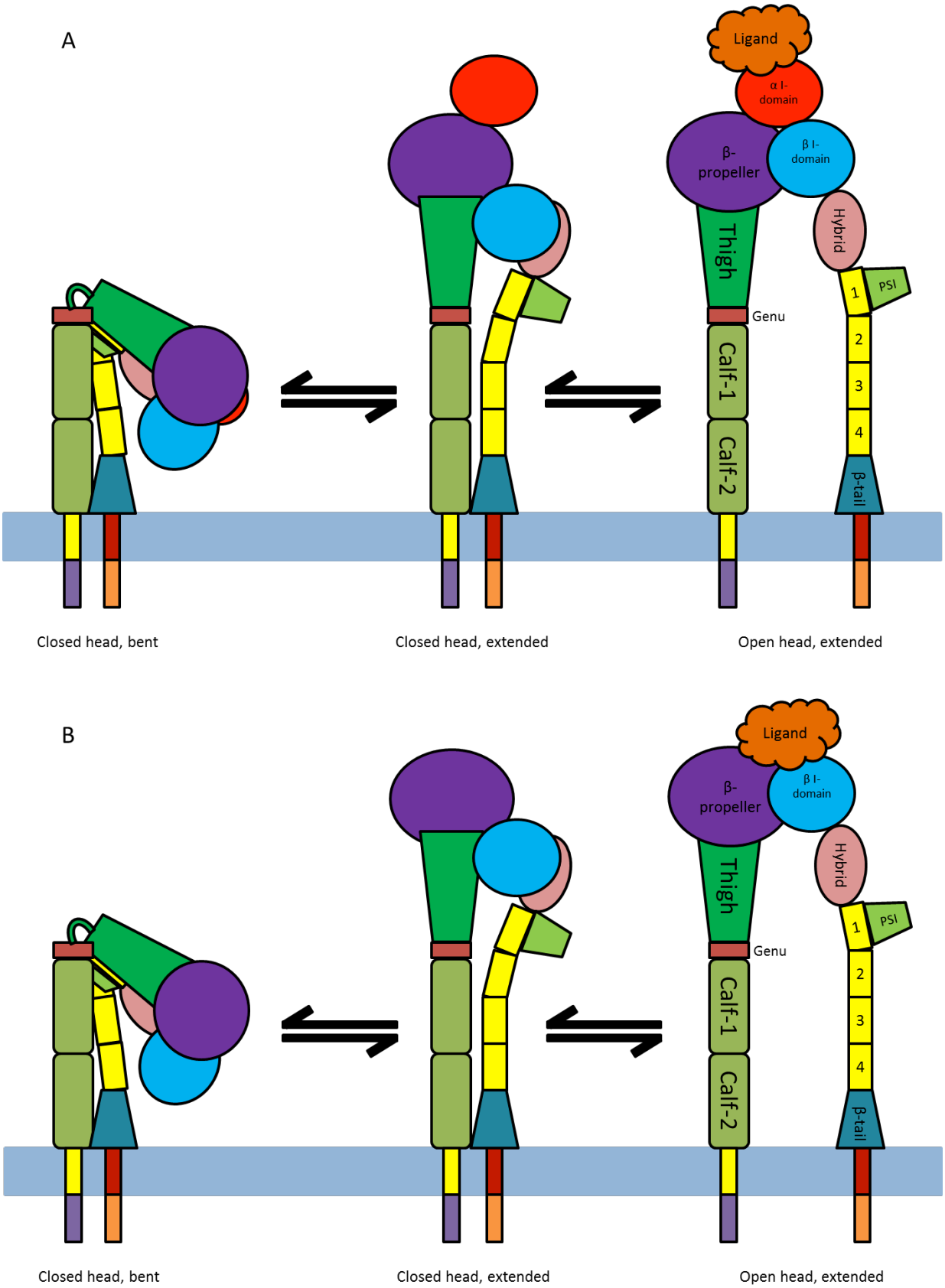


Figure 1.8: Inside-out signalling is mediated by a series of conformational changes. Integrin activation involves a series of molecular movements and conformational changes that exposes the ligand-binding site. Schematics for these molecular movements can be seen for both **(A)** I-domain containing αβ integrins and **(B)** non-I-domain αβ integrins.

that micromolar ranges of Ca^{2+} could synergise with suboptimal levels of $Mg^{2+}$to increase integrin affinity, suggesting dynamic interactions between integrins and divalent cations.

1.3.3 Integrin ligands

Integrin ligands can be divided into three major groups: basal ECM, provisional ECM and cell surface adhesion molecules. Many of these cell surface adhesion molecules are IgSF proteins, which are upregulated upon cellular activation. The most studied IgSF integrin ligands include ICAM-1, ICAM-2, ICAM-3 (CD50), VCAM-1 and MAdCAM-1.

ICAM-1 is the most ubiquitously expressed ICAM isotype and is weakly expressed on both resting leukocytes and endothelium and is upregulated on cell activation (Chiu et al., 2004, Min et al., 2005). ICAM-1 has been shown to bind both LFA-1 and Mac-1 (Diamond et al., 1991, Stanley et al., 1994). LFA-1 and Mac-1 also bind ICAM-2 (de Fougerolles et al., 1991, Xie et al., 1995). ICAM-2 has constitutively high expression on ECs, but has also been described in bronchial epithelial cells, eosinophils, monocytes, T cells, B cells and platelets (de Fougerolles et al., 1991, Diacovo et al., 1994, Porter and Hall, 2009). When initially characterised, ICAM-2 was not identified on neutrophils (de Fougerolles et al., 1991), however more recently ICAM-2 expression has been reported in bone marrow and peripheral blood neutrophils (Sundd et al., 2012). Initial ICAM-2 characterisation by de Fougerolles et al. used human cells, whilst Sundd and colleagues examined murine neutrophils, so differential ICAM-2 expression between human and mouse leukocytes may explain this discrepancy.

ICAM-3 is only expressed by leukocytes and is thought to mediate the early phases of antigen presentation via interactions with LFA-1 (de Fougerolles and Springer, 1992). ICAM-4 expression is restricted to erythrocytes and erythroid precursors, where it interacts with $\alpha_{IIb}\beta_3$ on platelets (CD41/CD61, also referred to as glycoprotein IIb/IIIa) (Hermand et al., 2003), and p150,95 (CD11c/CD18, $\alpha_X\beta_2$) expressed on monocytes and macrophages (Ihanus et al., 2007). The final ICAM isotype, ICAM-5 is only found in the grey matter of the telencephalon. ICAM-5 interacts with LFA-1 (Tian et al., 2000, Zhang et al., 2008) and VLA-5 (CD49e/CD29, $\alpha_5\beta_1$) (Ning et al., 2013) expressed by T cells, implicating a role in T cell cerebral migration.

ECs also express VCAM-1, which rapidly increases on activation (Marui et al., 1993, Lee et al., 2001, Min et al., 2005, Hortelano et al., 2010). VCAM-1 is a ligand for the α_4 integrins VLA-4 and $\alpha_4\beta_7$ expressed on circulating leukocytes (Elices et al., 1990, Chan et al., 1992). VCAM-1 has also been described as a novel ligand for the β_2 integrins Mac-1 and $\alpha_D\beta_2$ (Yakubenko et al., 2006). MAdCAM-1 is a member of both the IgSF and the mucin family of proteins and is selectively expressed on the venules involved in lymphocyte trafficking to mucosal tissues. MAdCAM-1 is the physiological ligand for $\alpha_4\beta_7$ and mediates recruitment of T cells to mucosal Peyer's patches and the lamina propria (Berlin et al., 1993).

In conclusion, cells express a multitude of adhesion molecules and integrin ligands to interact with circulating leukocytes. Integrins can either be highly specific for their ligand or display a degree of promiscuity in their binding partners. Common physiological integrin ligands are listed in Table 1.2. The expression of integrins and their ligands are influenced by inflammatory and infective signals. This regulation means that leukocytes can preferentially migrate into infected or inflamed tissue, where they mediate their immune function. One such leukocyte that relies on integrins to exert immune surveillance is the neutrophil.

1.4 The role of neutrophils in the innate immune system

Innate immunity is maintained through the combined function of multiple cell types including DCs, monocytes, mast cells, NK cells and granulocytes. Granulocytes, often referred to as polymorphonuclear leukocytes (PMNs), are immune cells that possess microscopic granules containing proteolytic enzymes. Basophils, eosinophils and neutrophils are all granulocytes. Basophils are the smallest population of granulocytes, which mediate hypersensitivity and allergic inflammation (Mukai et al., 2005, Obata et al., 2007, Tsujimura et al., 2008). Eosinophils were traditionally considered to be proinflammatory effector cells involved in parasitic defence, however emerging work implicate roles for eosinophils in innate and adaptive immune system regulation (Travers and Rothenberg, 2015). Neutrophils are the predominant granulocyte subset, which contribute to immune surveillance and microbial killing.

Integrin Subunits		Ligands & Counter-Receptors
β_1	α_1	Collagen (Types I, IV and VI), Laminin
	α_2	Collagen (Types I, IV and VI), Laminin, Tenascin
	α_3	Collagen (Type I), Entactin, Epiligrin, Fibronectin
	α_4	Invasin, Fibronectin, VCAM-1
	α_5	Denatured Collagen, Fibronectin
	α_6	Laminin
	α_7	Laminin
	α_8	Fibronectin, Tenascin
	α_9	Tenascin
	α_{10}	Collagen (Type II)
	α_{11}	Collagen (Type I)
	α_V	Fibronectin, Vitronectin
β_2	α_L	ICAM-1, ICAM-2, ICAM-3, ICAM-4, ICAM-5
	α_M	Factor X, Fibrinogen, iC3b, ICAM-1, ICAM-2
	α_X	Fibrinogen, iC3b, ICAM-4,
	α_D	ICAM-3
β_3	α_{IIb}	Fibrinogen, Fibronectin, Thrombospondin, Vitronectin, von Willebrand Factor
	α_V	Collagen, Fibrinogen, Fibronectin, Osteopontin, Tenascin, Thrombospondin, Vitronectin, von Willebrand Factor
β_4	α_6	Laminin
β_5	α_V	Vitronectin
β_6	α_V	Fibronectin
β_7	α_4	Fibronectin, MAdCAM-1, VCAM-1
	α_E	E-cadherin
β_8	α_V	Vitronectin

Table 1.2: Physiologically relevant integrin ligands. Listed in the above table are key physiological integrin ligands. Integrins expressed by leukocytes are indicated by red α subunits.

Neutrophils are crucial for the resolution and clearance of a multitude of pathogens. Circulating neutrophils have an estimated lifespan of 4-10 hours, however neutrophil survival can range between 1-2 days in response to cytokines and proinflammatory stimuli (Faurschou and Borregaard, 2003). Neutrophils fight infection via three main mechanisms: phagocytosis, degranulation and the release of neutrophil extracellular traps (NETs).

1.4.1 Immunoglobulin- and complement-mediated phagocytosis

Microbial killing is a critical function mediated in part by neutrophils, which is facilitated by opsonisation with Ig and iC3b. Neutrophils express FcRs that recognise IgG (FcγRI, FcγRIIA, and FcγRIIIB), IgA (FcαRI) and in some circumstances IgE (FcεRI and FcεRII). The most important neutrophil FcγRs are considered to be FcγRIIA and FcγRIIIB (Bruhns, 2012). Engagement of both these low-affinity FcγRs is required for cellular activation (Walker et al., 1991, Brennan et al., 1991, Naziruddin et al., 1992, Strohmeier et al., 1995, Jakus et al., 2008). Studies show that FcγRIIIB makes initial contact and tethers the immune complex (IC) (Coxon et al., 2001), with activation being induced by the synergistic ligation of FcγRIIA and FcγRIIIB (Zhou et al., 1995). Activated neutrophils have also been shown to bind IgG via the high-affinity FcγRI (Guyre et al., 1990, Repp et al., 1991, McKenzie and Schreiber, 1998).

The cytoplasmic domain of FcγRIIA contains an immunoreceptor tyrosine-based activating motif (ITAM) (Sanchez-Mejorada and Rosales, 1998), which enables intracellular signalling. FcγRI and FcγRIIIB lack this motif, however associate with a dimeric ITAM-containing γ-subunit that allows for intracellular signalling (Sanchez-Mejorada and Rosales, 1998). On binding IgG, FcγR-associated ITAMs are phosphorylated by SFK members (Fitzer-Attas et al., 2000), which recruit spleen tyrosine kinase (Syk) (Agarwal et al., 1993, Ghazizadeh et al., 1995). Syk is subsequently phosphorylated and activated (Greenberg et al., 1994, Darby et al., 1994). Early reports found that macrophages lacking Syk were unable to phagocytose IgG-opsonised particles, demonstrating the importance of Syk (Matsuda et al., 1996, Crowley et al., 1997, Kiefer et al., 1998). Whilst Syk is critical for FcγR-mediated phagocytosis, the downstream signalling events are unclear. FcγR-mediated signalling induces actin remodelling

and the production of pseudopods that surround and engulf opsonised microbes, which are then taken up into a specialised structure, the phagosome, and degraded by proteolytic enzymes stored in cytosolic granules (Nordenfelt and Tapper, 2011).

Alternatively, when opsonised with complement fragment iC3b, neutrophils can engage microbes via CRs. Neutrophils express CR1, CR3 (Mac-1) and CR4 (p150,95), all of which have been implicated in phagocytosis (Gordon et al., 1987, Myones et al., 1988, Brown, 1991, Ueda et al., 1994). CR1 is a transmembrane protein that binds opsonised pathogens (Brown, 1991, Fallman et al., 1993). Mac-1 (CR3) and p150,95 (CR4) mediate phagocytosis of iC3b-opsonised pathogens (Allen and Aderem, 1996a). Whilst FcγR-mediated phagocytosis requires generation of pseudopods, CR-mediated phagocytosis occurs via a 'sinking' of the iC3b-opsonised particle that generates very little membrane protrusion (Roubey et al., 1991, Allen and Aderem, 1996b). Mac-1 mediated phagocytosis is dependent on serine phosphorylation of the β_2 integrin subunit by protein kinase C (PKC) (Roubey et al., 1991, Allen and Aderem, 1996b). Additional signalling differences between FcR- and CR-mediated phagocytosis have been highlighted. Rho guanosine triphosphatases, which act as a molecular switch and control cytoskeletal remodelling, are important in both forms of phagocytosis (Caron and Hall, 1998). Further molecular dissection found that Cdc42 and Rac mediate FcR-dependent phagocytosis, but CR-dependent phagocytosis is mediated by RhoA (Caron and Hall, 1998). As Mac-1 can form lateral associations with numerous membrane proteins, including FcγRs (Zhou et al., 1993, Huang et al., 2011b), the precise pathways that regulate CR-mediated phagocytosis remain unclear.

1.4.2 Neutrophil degranulation

As well as phagocytosis, neutrophils can secrete antimicrobial proteins, reactive oxygen species (ROS) and cytokines to combat microorganisms and recruit other leukocytes to the site of infection. The secretion of cytotoxic mediators via exocytosis is referred to as degranulation. To date, five different types of neutrophil granules have been identified: primary granules; secondary granules; tertiary granules; phosphasomes; and secretory vesicles.

Primary granules, or azurophilic granules, contain a multitude of antimicrobial and highly toxic compounds including myeloperoxidase (MPO), neutrophil elastase (NE), defensins, lysozyme, cathepsin G, proteinase (PR)-3 and various proteoglycans (West et al., 1974, Garcia et al., 1985, Egesten et al., 1994). Primary granules can either be secreted via exocytosis or fuse with internalised phagosomes to kill engulfed microbes.

Secondary granules, or specific granules, are comprised of lactoferrin, alkaline phosphatase, lysozyme and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase. The cell adhesion molecules Mac-1, VLA-4 and VLA-5 have also been identified in secondary granules (O'Shea et al., 1985, Stevenson et al., 1987, Suchard et al., 1992). Secondary granules are rapidly released (Borregaard et al., 1992, Tapper and Grinstein, 1997), promoting pathogen killing and cell adhesion.

Tertiary granules contain matrix metalloproteinase (MMP)-9 and heparanase (Mollinedo et al., 1991, Mollinedo et al., 1997). Adhesion molecules have also been reported within tertiary granules (Todd et al., 1984, Petrequin et al., 1987, Sengelov et al., 1993). Phosphasomes are similar in structure to secondary granules, but are not as dense and have been reported to only contain alkaline phosphatase (Smith et al., 1985, Sengelov et al., 1992).

Secretory vesicles are rich in Mac-1 (Sengelov et al., 1993), CR1 (Sengelov et al., 1994b), formyl-methionyl-leucyl-phenylalanine (fMLP) receptors (Sengelov et al., 1994a), the lipopolysaccharide (LPS) co-receptor CD14 and FcγRIIIB (Detmers et al., 1995). Exocytosis of secretory vesicles is also accompanied by shedding of L-selectin from the neutrophil cell surface (Borregaard et al., 1994).

1.4.3 Neutrophil extracellular trap formation

Early studies found that stimulation with the potent PKC activator, phorbol 12-myristate 13-acetate (PMA), initiated a form of neutrophil cell death distinct from both apoptosis and necrosis that was dependent on ROS generation (Takei et al., 1996). PMA stimulation decreased chromatin compactness and induced nuclear membrane degeneration. Stimulation for 3 hours increased cell membrane permeability, with cell death peaking after 4

hours incubation. Agarose and pulsed field gel electrophoresis confirmed DNA had not been degraded (Takei et al., 1996), demonstrating that PMA induces the release of intact DNA.

The term NET was first used in 2004 to describe the meshwork of chromatin fibres embellished with granule-derived antimicrobial peptides and proteases released by stimulated neutrophils (Brinkmann et al., 2004). Following this study, NETosis was recognised as an important neutrophil response to infection. *In vitro* stimulation of neutrophils with PMA or interleukin (IL)-8 induced NETosis via a PKC-dependent mechanism (Brinkmann et al., 2004). Further investigation from this group found that NETosis represents a form of cell death distinct from apoptosis and necrosis (Fuchs et al., 2007), supporting earlier work by Takei and colleagues. Live-cell imaging confirmed that PMA induced a succession of nuclear remodelling events prior to NETosis, with changes in nuclear shape and chromatin decondensation. The nuclear envelope and granule membranes dissolved, allowing for the mixing of NET components, before the cell membrane was disrupted and NETs released. This process required NADPH oxidase-dependent ROS generation (Fuchs et al., 2007). Human neutrophils release NETs in response to a wide range of stimuli (Urban et al., 2006, Clark et al., 2007). Exploration of the mechanisms underlying NETosis highlighted two forms of NETosis: vital NETosis and suicidal NETosis.

1.4.3.1 Vital NETosis

Vital NETosis describes a process whereby NETs are released without compromising cell viability, thus allowing neutrophils to mediate 'conventional' host defence functions. Whilst the concept of a cell functioning without a nucleus is controversial, examples of this phenomenon exist. Early investigations described anuclear granulocytes that retained degrees of cellular functionality. These anuclear and granular-free granulocytes were termed cytoplasts or cytokineplasts (Malawista and De Boisfleury Chevance, 1982, Dyett et al., 1985). Cytoplasts were found to migrate towards fMLP (Dyett et al., 1985, Huang et al., 1991), as well as adhering and transmigrating across endothelial monolayers (Huang et al., 1991). Cytoplasts have also been suggested to migrate to inflammatory sites *in vivo* (Malawista et al., 2006). As

the majority of this work was conducted before the recognition of NETs, these observations may represent neutrophils following vital NETosis. Whilst cytoplasts may be controversial, red blood cells (RBCs) and platelets are accepted examples of functional anucleate cells, demonstrating the capability of cells to function in the absence of a nucleus.

More recent work also provides evidence to support vital NETosis. LPS-stimulated neutrophils have been shown to produce NETs but restrict intracellular access of SYTOX® Green (Clark et al., 2007). SYTOX® Green is a high-affinity nucleic acid stain that penetrates cells with compromised plasma membranes but cannot permeate live cells. This observation indicates that neutrophils cell membranes remain intact following LPS-induced NETosis.

Similar observations were reported in granulocyte/macrophage colony-stimulating factor (GM-CSF) primed neutrophils stimulated with LPS or C5a (Yousefi et al., 2009). Interestingly, the authors found that NETs were composed of mitochondrial DNA and not nuclear chromatin. Moreover, stimulated neutrophil did not uptake ethidium bromide, indicating that the cells were not dead. These results suggest that GM-CSF primed neutrophils produce NETs with mitochondrial DNA, which does not compromise cell viability. In a further study, PMA primed neutrophils co-cultured with *Staphylococcus aureus* rapidly released NETs via vesicular exportation, which also maintained cell integrity (Pilsczek et al., 2010).

NETosis has been observed *in vivo* following culture with *S. aureus* and *Streptococcus pyogenes* (Yipp et al., 2012). Both toll-like receptor (TLR)-2 and complement C3 knockout mice were resistant to *S. aureus*- and *S. pyogenes*-induced NETosis, implicating a TLR-2 and complement C3-dependent mechanism. Following NETosis, the anuclear neutrophils displayed a novel crawling phenotype (Yipp et al., 2012), supporting vital NETosis. *Candida albicans* was also found to induce vital NETosis, which was independent of ROS generation, but required Mac-1 engagement and signalled via the mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) pathway (Byrd et al., 2013).

1.4.3.2 Suicidal NETosis

Suicidal NETosis is a form of neutrophil cell death, arising from the sequential progression of chromatin decondensation and cytoplasmic localisation of DNA to cell membrane perforation and externalisation of NETs. Early investigations provide evidence consistent with suicidal NETosis. PMA stimulation of neutrophils, but not lymphocytes, was found to induce ROS-dependent cell death (Tsan, 1980). PMA stimulation also decreases neutrophil size and granularity (Esaguy et al., 1991). Microscopic analysis also highlighted numerous empty vesicles, smoothing of cell surfaces, a decrease in cytoplasmic compactness and some cytological features of cell death (Esaguy et al., 1991), suggesting that PMA-induced NETosis results in neutrophil cell death.

Pilsczek et al. noted that whilst NETs were released without compromising neutrophil viability in their model, at later time points there was disruption of the nuclear membrane (Pilsczek et al., 2010). Following stimulation for an hour, nuclear envelope rupture and cytoplasmic DNA were observed, which was hypothesised to be the beginnings of lytic cell death and suicidal NETosis, however this finding was not further explored. This observation may suggest that there is a signalling overlap in the pathways regulating both forms of NETosis.

1.4.4 Mechanisms underlying neutrophil extracellular trap formation

Since the identification of NETs, substantial work has been conducted to determine the mechanisms underpinning their generation. This section will explore how neutrophil integrins contribute to NETosis, evaluate the cell signalling pathways implicated in NETosis and discuss the key molecular mechanisms involved during NET generation

1.4.4.1 Integrin involvement in neutrophil extracellular trap formation

Integrin engagement is thought to be crucial in initiating NETosis, however limited work has been conducted to further dissect their precise contribution. Several groups have shown that Mac-1 blockade suppresses NETosis (Neeli et al., 2009, Raftery et al., 2014, Rossaint et al., 2014, Yalavarthi et al., 2015). Inhibition of the β_1 integrins, in particular VLA-3 (CD49c/CD29; $\alpha_3\beta_1$) and VLA-5, has been shown to suppress neutrophil ROS generation

(Lavigne et al., 2007). Another group found that pan-β_1 integrin blockade suppressed NETosis (Gillenius and Urban, 2015), suggesting that the β_1 integrins may also regulate NET production.

Interestingly, recent work has proposed a novel mechanism of NETosis that focuses on the contribution of L-selectin (Mohanty et al., 2015). Mohanty et al. found that NETosis could be induced by saliva and identified the presence of NETs within the oral cavity. Saliva-induced NETosis was rapid and did not require ROS generation and had reduced contributions of NE and NADPH compared to 'conventional' NETosis (Mohanty et al., 2015). Molecular dissection found that saliva-induced NETosis is mediated by L-selectin-dependent signalling, induced by interactions with mucins containing sLe^X residues. The authors also found that saliva-induced NETosis was deficient in patients with Behçets disease, an inflammatory condition characterised by recurrent oral ulcers, suggesting that L-selectin-mediated signalling may represent a novel mechanism important to mucosal immunity. Moreover, blockade of PSGL-1 in mice has also been shown to suppress NETosis (Etulain et al., 2015), suggesting that PSGL-1 may also provide neutrophils with the stimulatory signals to induce NETosis. These recent observations demonstrate the complexity of the interactions and signalling involved in neutrophil biology and the induction of NETosis.

1.4.4.2 Cell signalling transduction in neutrophil extracellular trap formation

Limited work has addressed the signalling pathways involved in NETosis, which can be found summarised at the end of section in Figure 1.9. There are conflicting results regarding mammalian target of rapamycin (mTOR) involvement. McInturff et al. report that mTOR enhances NETosis through post-transcriptional regulation of hypoxia-inducible factor (HIF)-1α (McInturff et al., 2012). Inhibition of mTOR, HIF-1α, or both, reduced NETosis; thus demonstrating a role for mTOR and HIF-1α in the regulation of NETosis. In contrast, Itakura and McCarty propose that mTOR inhibits NETosis by suppressing autophagy (Itakura and McCarty, 2013). Autophagy is a process that mediates protein homeostasis by regulating intracellular protein turnover (Ravikumar et al., 2010). A feature of autophagy is the formation of autophagosomes that fuse with lysosomes to degrade proteins. Itakura and McCarty found

mTOR inhibition increased both autophagosome formation and NETosis (Itakura and McCarty, 2013).

Discrepant findings may be explained by the differing experimental stimuli, leading to activation of two different mTOR complexes (mTORC): mTORC1 and mTORC2. McInturff et al. stimulated neutrophils with LPS, binding TLR-4 and activating nuclear factor (NF)-κB, which is known to promote HIF-1α signalling. Hypoxia has been shown to activate tumor suppressor complex 1/2 (TSC1/2) (DeYoung et al., 2008). It is therefore possible that HIF-1α can activate TSC1/2, leading to the activation of mTORC2 whilst supressing mTORC1 (Huang et al., 2008). Activation of mTORC2 activates protein kinase B (PKB, also referred to as Akt), which subsequently activates NF-κB and HIF-1α. There is also evidence to suggest that LPS can bind TLR-2 (Yang et al., 1998b, Takeuchi et al., 1999, Sabroe et al., 2002, Good et al., 2012). LPS could therefore activate phosphatidylinositol-3-kinase (PI3K) and Akt, which suppresses TSC1/2 and promotes mTORC1 activity. Inhibition of either HIF-1α or mTORC1 would therefore inhibit NETosis, as observed by McInturff and colleagues.

In contrast, Itakura and McCarty used fMLP, which stimulates the Ras-Raf pathway and activates ERK (Worthen et al., 1994). Stimulation of ERK leads to the activation of RSK, which suppresses TSC1/2 and enhances mTORC1 activity. Reports have found that fMLP increases intracellular calcium concentrations and stimulate PKC (Carter et al., 1989, O'Flaherty et al., 1990). PKC activation initiates ROS generation, a prerequisite for NETosis, which has also been linked to autophagy. Therefore, mTORC1 may reduce NETosis by suppressing autophagy signals induced by fMLP stimulation.

Screening of small molecule inhibitors found a Raf inhibitor was a potent suppressor of NETosis (Hakkim et al., 2011), implicating the MAPK/ERK pathway. As mTORC1 is activated by ERK and Raf inhibition reduces NET release, observations by Hakkim et al. suggest that mTORC1 promotes NETosis. Recent reports found Akt inhibition reduced PMA-induced NETosis, implicating the PI3K-Akt pathway (Douda et al., 2014). Akt inhibition would also suppress mTORC1, suggesting mTORC1 promotes PMA-induced NETosis.

Autophagy-related signalling has been implicated in NETosis. PI3K signalling regulates autophagy in neutrophils, with PI3K inhibition disrupting autophagosome production (Mitroulis et al., 2010). Examination of neutrophils from patients with acute gout and healthy controls (HCs), found that PI3K inhibition reduced both autophagosome-lysosome fusion and NETosis (Mitroulis et al., 2011). Microtubule-associated protein light chain 3 (LC3), a marker for autophagy, has been reported in PMA-stimulated neutrophils (Remijsen et al., 2011b). This group also found that superoxide generation and autophagy were required to generate NETs, with inhibition of ROS production or autophagy reducing NETosis (Remijsen et al., 2011b).

Inhibition of p38 MAPK has also been shown to reduce NETosis in PMA-stimulated neutrophils (Riyapa et al., 2012). Studies have found that PMA-induced NETosis is mediated by the ROS-dependent activation of p38 MAPK and ERK (Keshari et al., 2013). NETosis induced by ICs has been shown to require both FcγRIIIB and Mac-1, which activate SFK and Syk (Behnen et al., 2014). Activation of these tyrosine kinases is known to regulate the PI3K/Akt, MAPK/ERK and p38 MAPK pathways. Figure 1.9 illustrates how these cell signalling pathways may interact.

1.4.4.3 Molecular mechanisms of neutrophil extracellular trap formation

Regarding the molecular mechanisms of NETosis, peptidyl arginine deiminase (PAD)-4 has been shown to translocate to the nucleus, where it citrullinates histones (Nakashima et al., 2002, Wang et al., 2004). Neutrophils isolated from PAD-4 knockout mice fail to produce NETs, which supports a PAD-4-dependent mechanism (Li et al., 2010a). Defective bacterial killing and increased susceptibility to sepsis following *Klebsiella pneumoniae* or *Escherichia coli* infection were reported in NE knockout mice (Belaaouaj et al., 1998). Increased susceptibility to infection was also reported in NE and cathespin G knockout mice following infection with *Aspergillus fumigatus* spores (Tkalcevic et al., 2000). Following the establishment of NETosis as an antimicrobial response, Papayannaopoulos et al. demonstrated that NE knockout mice failed to produce NETs (Papayannopoulos et al., 2010). Defective *C. albicans* killing was also observed in neutrophils isolated from MPO-deficient mice compared

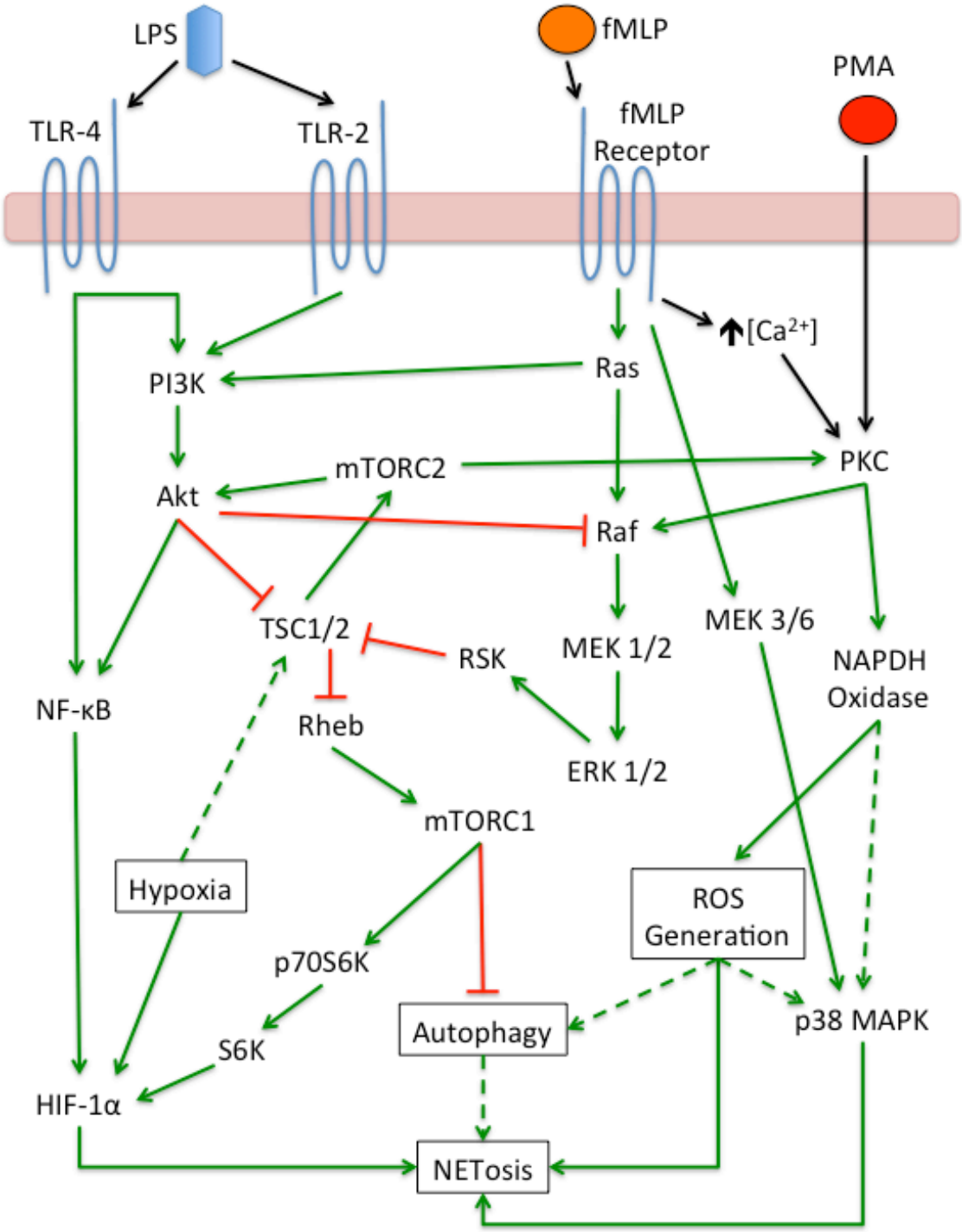


Figure 1.9: NETosis is regulated by PI3K/Akt, MAPK/ERK, mTOR and p38 MAPK signalling. Depicted are the signalling pathways implicated in NETosis. Green arrows represent activation signals, with solid lines being through known mechanism and dashed lines via unknown mechanisms. Red lines indicate suppressive signals. Abbreviations: Akt, protein kinase B; ERK, extracellular signal-related kinase; fMLP, N-formyl-methionine-leucyl-phenylalanine; HIF-1α, hypoxia inducible factor-1α; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; MEK, MAPK/ERK kinase; mTORC, mammalian target of rapamycin complex; NF-κB, nuclear factor κB; PI3K, phosphoinositide 3-kinase; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; Rheb, Ras homolog enriched in brain; RSK, ribosomal S6 kinase; S6K, S6 kinase; TLR, toll-like receptor; TSC, tumor suppressor complex.

to control (Aratani et al., 1999). Similar findings have been reported in humans, with MPO-deficient patients exhibiting delayed NETosis and neutrophils isolated from patients with a complete absence of MPO expression unable to produce NETs (Metzler et al., 2011).

Given these observations, NE and MPO are believed to be important in NETosis. Neutrophil extracts can promote *in vitro* chromatin decondensation (Papayannopoulos et al., 2010). NE was found to be both necessary and sufficient to degrade histones, whilst MPO could synergise with NE to enhance *in vitro* chromatin decondensation (Papayannopoulos et al., 2010). Papayannopoulos and colleagues therefore proposed that NE translocates to the nucleus first and begins to digest histones, with MPO subsequently trafficking to the nucleus and synergising with NE to enhance chromatin decondensation.

Papayannopoulos and colleagues subsequently examined NETosis in response to *C. albicans*, reporting impaired NETosis when hyphae morphology was disrupted (Branzk et al., 2014). Whilst wild-type hyphae induced NETosis, fragmented hyphae induced lower levels of NET release and promoted phagocytosis, suggesting neutrophils could modulate responses based on pathogen size. Phagocytosis inhibited NETosis by sequestering NE to phagosomes (Branzk et al., 2014). Therefore, an intracellular balance of NE and MPO may regulate NETosis. In this molecular model, when neutrophils encounter smaller pathogens and undergo phagocytosis, NE and MPO are trafficked to the phagosome and not the nucleus. If neutrophils encounter larger microbes that cannot be engulfed, there is a shift to nuclear localisation of both NE and MPO, where they facilitate nuclear decondensation and the generation of NETs.

In conclusion, integrin engagement, in particular the β_1 and β_2 integrins are thought to promote NETosis, however the precise signalling pathways are unknown. More recent work has also implicated selectin-mediated signalling, which further complicates the signalling pathways regulating NET generation. Various groups have presented evidence to support MAPK/ERK, PI3K/Akt, mTOR and p38 MAPK signalling pathways as regulators of NETosis. There is likely to be a fine balance between these signalling pathways *in vivo*, which determine the degree of ROS-dependency and whether neutrophils undergo vital or suicidal NETosis.

1.5 Disorders of immune regulation in autoimmune rheumatic disease

Autoimmunity arises when the immune system fails to recognise constituents of the host organism as 'self', in a process referred to as 'loss of tolerance', resulting in an immune response against self-antigens. Autoimmune diseases can be divided into either organ-specific or systemic diseases. Organ-specific autoimmunity is directed against a single organ or cell type, examples of which include Addison's disease, Hashimoto's thyroiditis, insulin-dependent diabetes mellitus and myasthenia gravis. In contrast, systemic autoimmune diseases target a broad range of tissues and can affect multiple organs. Autoimmune rheumatic disease (ARD) is a term used to describe a group of systemic autoimmune disorders associated with substantial morbidity and mortality, generally characterised by dysregulation of the immune system and the presence of circulating autoantibodies Three ARDs will be explored in this thesis: rheumatoid arthritis (RA), systemic lupus erythematosus (SLE) and antiphospholipid syndrome (APS). Neutrophil dysfunction and aberrant NETosis have been described in all three of these ARDs, which will be discussed in section 1.6.

1.5.1 Rheumatoid arthritis

RA is a chronic inflammatory autoimmune disease, which affects more women than men, and is characterised by the progressive destruction of synovial joints. Patients typically have circulating autoantibodies, which contribute to disease pathology. Multiple immune cells also contribute to the immunopathology underpinning RA.

1.5.1.1 Aetiology of rheumatoid arthritis

Recent reports estimate RA to affect between 18-32 men per 100,000 and 44-65 women per 100,000 in the UK (Humphreys et al., 2013). Early studies identified a class of autoantibodies termed rheumatoid factor (RhF), which bound the Fc portion of IgG and forms ICs, in RA patients (Rose et al., 1948). RhF was found to be 75% sensitive and 74% specific for RA (Bas et al., 2002). In contrast, autoantibodies recognising citrullinated antigens, anti-citrullinated protein antibodies (ACPA) (Schellekens et al., 1998), are highly specific for RA

(Schellekens et al., 2000, Vincent et al., 2002). Whilst ACPA are only 68% sensitive for RA, comparative analysis found ACPA to be 94% specific (Bas et al., 2002). ACPA can be detected many years prior to disease onset (van der Woude et al., 2010), making them a powerful diagnostic tool.

There is a complex interplay between genetic and environmental risk factors in RA. The human leukocyte antigen (HLA)-DR, a class II MHC molecule, is the strongest genetic determinant of RA. A strong association between the presence of a particular ACPA and HLA-DRB1*04 has been demonstrated, with HLA-DRB1*04 expressing the shared epitope motif found in 60-70% of patients (Gorman et al., 2004). Higher ACPA titres have been seen in patients with the non-shared epitope HLA-DRB1*15 allele, in particular among smokers with RA (Laki et al., 2012). An association with HLA-DR3 has been reported in ACPA negative RA patients (Verpoort et al., 2005). These reports highlight the complex relationship between HLA haplotype and RA.

Genome-wide association studies have implicated polymorphisms in *PADI4*, the gene encoding PAD-4, with an increased risk of RA in several Asian populations (Kang et al., 2006, Cheng et al., 2012, Suzuki et al., 2013). Some studies show that this association exists in some Caucasian communities (Iwamoto et al., 2006, Hoppe et al., 2006), however other reports have failed to replicate this association (Barton et al., 2004, Martinez et al., 2005, Caponi et al., 2005, Burr et al., 2010). Polymorphisms in the *PTPN22* gene, which regulates B and T cell activation thresholds, are also associated with RA pathology (Plenge et al., 2005, Hinks et al., 2005), with mutations rendering lymphocytes hyperactive.

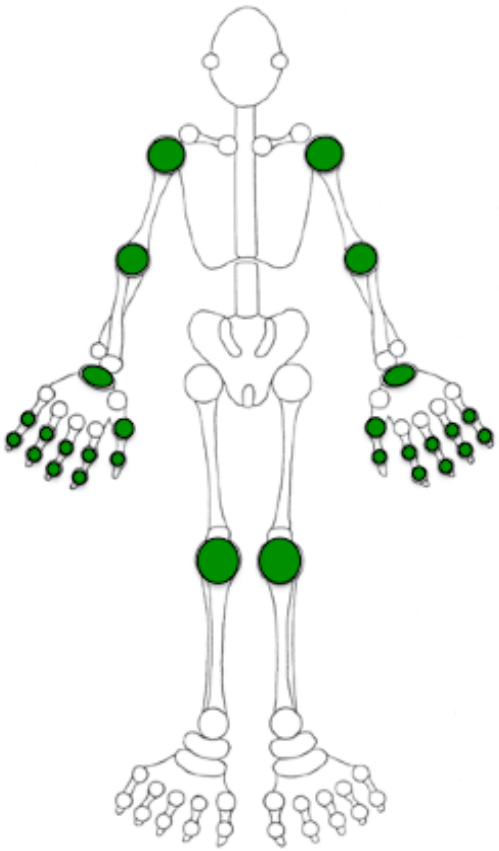
Environmental factors, such as smoking, are also linked to the initiation of RA, as demonstrated by the aforementioned association between the non-shared epitope HLA-DRB1*15 allele and RA smokers. In addition, PAD-2 expression is significantly increased in cells derived from the bronchoalveolar lavage (BAL) of smokers compared to non-smokers (Makrygiannakis et al., 2008), which is thought to increase citrullinated autoantigens within the pulmonary vasculature. Further investigations have implicated mucosal inflammation and the microbiome as extra-articular triggers of RA (Farquharson et al., 2012, Brusca et al., 2014).

To conclude, the initiation and progression of RA is dependent on a complex interplay between genetic predisposition and environmental factors. Growing evidence suggests that mucosal inflammation may contribute to the initiation of RA, however the precise mechanism underlying loss of tolerance remains unclear.

1.5.1.2 Clinical features of rheumatoid arthritis

RA patients typically present with symmetrical peripheral polyarthropathy affecting synovial joints. Synovitis of the hands and feet are common early features of RA. With disease progression, additional joints including the knees, hips, shoulders, elbows and spine are affected. Joint involvement is progressive, with synovial inflammation leading to thinning of cartilage, erosion of bone, and formation of an abnormal layer of fibrovascular tissue called the pannus.

To ensure consistency amongst international groups conducting research into RA, the American College of Rheumatology (ACR) and European League Against Rheumatism (EULAR) devised RA classification criteria. The most recent revision of these criteria was in 2010 (Aletaha et al., 2010). Clinicians often refer to these research criteria to aid diagnosis of RA in clinical practice. These classification criteria can be grouped into 4 main categories: joint involvement, serology, acute-phase reactants and symptom duration. Joint involvement refers to the presence of tender or swollen joints on examination, taking into consideration the size and number of affected joints. Serologic tests determine the presence of RhF and ACPA. C-reactive protein (CRP) levels and erythrocyte sedimentation rate (ESR) are measured to assess levels of inflammation. The duration of symptoms is also considered, with symptoms lasting for more than 6 weeks being required for diagnosis As well as aiding diagnosis, several of these parameters are also included in the disease activity score (DAS)28, which is derived from examination of 28 joints for tenderness and swelling, measurement of ESR and global health score (Figure 1.10). This score is routinely used in clinical practice to evaluate disease activity and guide treatment.



Clinical Variable	Value
Tender Joint Count (0-28)	x
Swollen Joint Count (0-28)	y
ESR (mm/hr) or CRP (mg/L)	ESR or CRP
Global Assessment of Health Score	z

DAS28 Calculations

$$0.56\sqrt{x} + 0.28\sqrt{y} + 0.7\ln(ESR) + 0.014z$$

or

$$0.56\sqrt{x} + 0.28\sqrt{y} + 0.36\ln(CRP + 1) + 0.014z + 0.96$$

Figure 1.10: Formula for calculation of DAS28. Clinical assessment of RA relies on the assessment of tenderness or swollenness of 28 joints. These joints are marked in green in the above diagram. DAS28 is then calculated by considering joint involvement, with ESR or CRP and a global assessment of health. Skeletal image was adapted from the Arthritis Research UK website.

Whilst RA is typically characterised by articular features, extra-articular manifestations are also important. UK cross-sectional studies report an increase in mortality associated with cardiovascular disease, pulmonary complications and solid tumours in RA patients (Young et al., 2007, Olson et al., 2011). RA patients are at increased risk of atherosclerosis and heart disease (Wallberg-Jonsson et al., 1999, Riise et al., 2001, Goodson and Symmons, 2002, Nicola et al., 2006, Cojocaru et al., 2010). Pulmonary manifestations are increasingly recognised, with estimates of both preclinical and clinical RA-associated interstitial lung disease (ILD) ranging between 10-30% (Cortet et al., 1995, Gabbay et al., 1997, Demir et al., 1999, Tanaka et al., 2004, Ayhan-Ardic et al., 2006, Mori et al., 2008, Georgiadis et al., 2009, Kim et al., 2010). A study following 1429 RA patients across England for up to 18 years, reported similar findings to previous population-based studies, with 31% of deaths attributed to cardiovascular disease and 22% of deaths due to respiratory complications (Young et al., 2007). In addition, further analysis of this study found 32% of patients presenting with rheumatoid nodules on the skin, 3% with vasculitis and 1% with neuromyopathy (Young and Koduri, 2007).

1.5.1.3 Immunopathology of rheumatoid arthritis

B and T cell dysregulation, proinflammatory cytokine production and autoantibody generation are key features of RA immunopathology. RA was largely considered a lymphocyte-mediated disorder, therefore extensive work has examined the contribution of adaptive immunity to RA pathology. The contribution of innate immune cells is being increasingly recognised in RA pathogenesis. Given the importance to this thesis, the relevance of neutrophils will be discussed in greater detail in section 1.6.1.

The earliest event in RA pathology is believed to be activation of the innate immune response, in particular DCs (Smolen and Steiner, 2003, Smolen et al., 2007). Studies have found DCs to be elevated in the synovial fluid (SF) of RA patients compared to peripheral blood (Moret et al., 2013), suggesting DCs may migrate to the synovium and facilitate T cell activation.

Whilst substantial evidence supports T cell involvement in RA, direct targeting of T cells has limited efficacy in treatment (Panayi, 2006), suggesting a more complex pathological mechanism. Activated $CD4^+$ T cells are a major cell type within the RA pannus (Panayi et al., 1992). Phenotypic analysis of synovial T cells found a large subpopulation of $CD45RO^+$ T cells, indicative of mature memory T cells, which can stimulate B cells to produce antibodies (Panayi et al., 1992).

Th1 cells have conventionally been considered to drive RA pathology, however there is growing interest in the involvement of Th17 cells. Th17 cells secrete proinflammatory mediators that can suppress Treg generation (Chabaud et al., 1998, Miossec et al., 2009). Elevated Th17 and reduced Treg differentiation have been described in RA patients, which promote inflammatory cell phenotypes (Miao et al., 2014). Inflammatory monocyte-derived DCs enhance Th17 cell generation through the secretion of cytokines such as IL-1β, IL-6 and IL-23 (Estrada-Capetillo et al., 2013). In addition, Tregs isolated from RA patients have limited suppressive activity (Behrens et al., 2007, Cribbs et al., 2014), which is attributed to low expression of cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) (Cribbs et al., 2014). This reduced Treg population, with defective suppressive capability, fails to suppress autoreactive T cells (Rapetti et al., 2015).

Other cells types have also been implicated in RA pathology. NKT cells have been identified in RA patients (Aggarwal et al., 2014), however their contribution to pathology is unclear, as evidence suggests a reduced proliferative capacity (Gutowska-Owsiak et al., 2014). $CD4^+CD8^+$ double positive T cells are more abundant in ACPA positive RA patients, which secrete IL-4, IL-21 and interferon (IFN)-γ in the synovium (Quandt et al., 2014), thus contributing to the proinflammatory cytokine milieu.

B cells are also found in RA synovium, where they form aggregates with T cells. The efficacy of rituximab, which selectively depletes $CD20^+$ B cells, supports B cell involvement in RA (Edwards et al., 2004). B cells contribute to RA pathology not only through antigen presentation, but also by producing autoantibodies (Smolen et al., 2007). Autoantibodies form ICs that stimulate the production of proinflammatory cytokines (Smolen et al., 2007), which

promote T cell, B cell and macrophage activation (Smolen and Steiner, 2003, Smolen et al., 2007). There are reduced numbers of Bregs in RA patients, which inversely correlate with both DAS28 scores and levels of RhF and ACPA (Daien et al., 2014). Moreover, the number of Bregs positively correlates with Treg numbers in new-onset RA patients, leading to the hypothesis that Bregs support Treg differentiation via IL-10 secretion (Ma et al., 2014).

Macrophages also contribute to synovitis through a combination of proinflammatory cytokine production, ROS generation, release of matrix-degrading enzymes, phagocytosis and antigen presentation (Haringman et al., 2005). Macrophages therefore contribute to the proinflammatory synovial environment, whilst supporting the differentiation and activation of B cells and T cells. Macrophage-derived cytokines also drive fibroblast-like synoviocyte (FLS) proliferation and activation, whilst the release of ROS induces chondrocyte apoptosis, both of which facilitate cartilage erosion. Proinflammatory cytokine secretion amplifies osteoclast differentiation and activation (Schett and Teitelbaum, 2009), which promotes bone destruction.

The immunopathology of RA is complex with numerous cellular interactions crossing both innate and adaptive immunity. Abnormal cellular activation and the promotion of proinflammatory cell subsets results in the production and maintenance of a proinflammatory environment that supports the destruction of cartilage and bone.

1.5.1.4 Current treatment for rheumatoid arthritis

Patients are regularly assessed to monitor disease activity and response to therapy. Assessment is comprised of two components: clinical assessment of disease activity and blood tests for inflammatory markers and evidence of toxicity. Clinical assessment of disease activity is made using the DAS28 (Figure 1.10) (Wells et al., 2009), which not only monitors disease activity but also defines eligibility for biological therapy. Laboratory tests normally include inflammatory markers such as CRP and ESR to gauge systemic inflammation, as well as full blood count, renal and liver function to assess for disease complications and toxicity of treatment. DAS28 scores of 2.6 or below indicate disease remission, a score between 2.6-3.2

implies low disease activity, whilst a value of 3.2-5.1 is classified as moderate disease activity and a DAS28 score of 5.1 or higher indicates high disease activity.

RA management is achieved through both medical and non-medical therapy. Prompt diagnosis is essential to reduce permanent joint damage and patients benefit from the specialist input of a team within a rheumatology unit (including doctors, nurses, physiotherapists, podiatrists and psychologists), as well as information from leaflets, websites and local patient groups. Drug therapy includes: analgesics; non-steroidal anti-inflammatory drugs (NSAIDs); glucocorticoids; disease-modifying anti-rheumatic drugs (DMARDs); and biologics. Analgesics do not alter disease progression nor reduce inflammation but are used to ease pain.

NSAIDs are used to relieve inflammation, pain and stiffness but do not slow the progression of RA. This group of drugs can be categorised based upon their ability to inhibit cyclooxygenase (COX)-1 and/or -2 into traditional non-selective NSAIDs, such as ibuprofen, naproxen and diclofenac, and COX-2-selective inhibitors, including celecoxib and etoricoxib. NSAID treatment has been associated with an increased risk of cardiovascular and gastrointestinal complications (Ong et al., 2007), so patients are evaluated to determine the most appropriate NSAID treatment. For additional pain relief, paracetamol and co-codamol are commonly prescribed analgesics.

Glucocorticoids are ideally given on a short-term basis to reduce pain, stiffness and swelling in patients who have yet to start, or are failing to respond to, DMARDs. Prednisolone is a commonly prescribed glucocorticoid. Pain relief varies between patients and often depends on disease severity. The mainstay however, of long-term therapy is the use of multiple DMARDs as combination therapy, started early in the disease, to reduce inflammation and help prevent further joint damage. Methotrexate, hydroxychloroquine (HCQ) and sulfasalazine are commonly prescribed DMARDs, which require regular monitoring for side effects and efficacy.

Biological therapies are routinely used as second line agents to treat RA. In the UK, biologics are given to patients who have failed to respond to at least two different DMARDs, one of which should be methotrexate unless contraindication, and have a DAS28 score of 5.1 or higher on two separate occasions one month apart. Biological treatments are usually

monoclonal antibodies or fusion proteins, which modulate the immune system by targeting the cytokines or cells that contribute to RA pathology. Biologics prescribed in RA can deplete peripheral $CD20^+$ B cells (rituximab), block IL-6 signalling (tocilizumab), suppress T cells activation (abatacept) or inhibit tumour necrosis factor (TNF)-α signalling (infliximab, etanercept, adalimumab, certolizumab pegol and golimumab). First line biological treatment is anti-TNF-α therapy, however if unsuccessful other therapies may be prescribed. Biological therapy is commonly given in combination with DMARDs, such as methotrexate, with the goal to treat-to-target to induce remission and suppress disease activity.

1.5.2 Systemic lupus erythematosus

SLE is an ARD with a wide range of clinical features and a predilection for women and certain ethnic groups (Rahman and Isenberg, 2008). SLE patients commonly have circulating autoantibodies that contribute to SLE pathology, which can affect several organ systems. In addition, multiple immune cells have been shown to be important to the immunopathology underlying SLE.

1.5.2.1 Aetiology of systemic lupus erythematosus

The prevalence of SLE in the UK varies by ethnicity and is estimated to be 134 cases per 100,000 amongst Caucasian women, rising to 516 cases per 100,000 amongst Afro-Caribbean women (Rees et al., 2016). SLE is characterised by the dysregulation of both innate and adaptive immunity, with immune manifestations including defects in apoptotic cell clearance, disturbances in cytokine production, B cell immunity and T cell signalling. SLE is a multifactorial disease, arising from various genetic, environmental and hormonal factors.

The first genetic associations with SLE were made with HLA haplotypes (Grumet et al., 1971). Genetic studies have estimated that the HLA-DRB1*1501 and HLA-DRB1*0301 alleles confer a 2-3 fold increased risk of developing SLE in Caucasian populations (Fernando et al., 2007, Graham et al., 2007). Deficiencies in components of the complement cascade are also associated with SLE (Agnello et al., 1972, Nishino et al., 1981), implicating defects in complement activation and opsonisation. Polymorphisms in FcγRI, FcγRIIA and FcγRIIIB have

been associated with both increased susceptibility to SLE and disease severity (Brown et al., 2007). These polymorphisms alter FcγR affinity, modulating how immune cells engage with ICs (Salmon et al., 1996, Wu et al., 1997, Kyogoku et al., 2002, Karassa et al., 2002, Karassa et al., 2003, Lee et al., 2009), suggesting that defective immune recognition may contribute to pathology. Mutations in *ITGAM*, the gene encoding the α_M integrin subunit, have been strongly associated with SLE. This mutation will be discussed in greater detail in section 1.7.2. Whilst these genes are examples of some genetic factors underlying SLE pathology, many other genes have been implicated, including those involved in lymphocyte signalling (*BANK1, CD80, CSK, IL10, SLK, STAT4*), innate immune signalling (*IRAK1, IRF5, IRF7, IRF8, TLR7, TLR9, TYK2*) and IC clearance (*ATG5, DNASE1, TREX1*) (Ramos et al., 2010, Mohan and Putterman, 2015).

A common environmental factor for SLE is ultraviolet light, which can provoke a photosensitive rash. This effect is dose dependent and is thought to act by inducing apoptosis and cytokine production (Caricchio et al., 2003). Additional environmental factors that are implicated include cigarette smoke, infection, vitamin D deficiency, exogenous oestrogen uptake, conventional drugs, biological agents, and pesticides (Zandman-Goddard et al., 2012).

Given the gender bias towards women, female sex hormones are thought to contribute to SLE aetiology and pathophysiology (Straub et al., 2013). In humans, oestrogen metabolites are thought to enhance humoral immune responses (Cutolo et al., 2010). Early studies found abnormal oestrogen metabolism in SLE patients of both sexes (Lahita et al., 1983, Sequeira et al., 1993, Mok and Lau, 2000). Furthermore, antibodies targeting oestrogen receptor (ER)-α were identified in 45% of an Italian SLE cohort but not in HCs (Colasanti et al., 2012), which induced T cell activation and subsequently apoptosis. In addition, anti-ER-α antibodies were shown to drive proliferation of CD3-stimulated T cells (Colasanti et al., 2012), which was proposed as a mechanism that promotes autoreactive T cell expansion.

1.5.2.2 Clinical features of systemic lupus erythematosus

Given the multi-systemic nature of SLE, patients can present with a broad range of clinical symptoms. The largest prospective lupus cohort, the Euro-Lupus cohort, consists of

1000 patients followed since 1991 (Cervera et al., 2009c) and has documented the following disease manifestations in patients: arthritis in 48.1%; malar rash in 31.1%; nephropathy in 27.9%; photosensitivity in 22.9%; neurologic involvement in 19.4% (including acute confusional state, aseptic meningitis, cerebrovascular disease, cognitive dysfunction, cranial neuropathy, demyelinating syndrome, headache, myelopathy, polyneuropathy and seizure); thrombocytopenia in 13.4%; oral ulcers in 12.5%; discoid lesions in 7.8%; and haemolytic anaemia in 4.8%.

Frequencies of clinical manifestations vary between published cohorts, most likely due to ethnic differences. For example, Asian SLE patients have higher frequencies of skin and renal involvement, with 76.1% of patients having malar rash and 74% diagnosed with nephropathy (Wang et al., 1997). A greater prevalence of arthritis (88.1%), photosensitivity (60.2%) and oral ulcers (52.8%) have also been reported in an American SLE cohort (Alarcon et al., 2002).

Several indices exist to measure disease activity in SLE, each of which assigns scores based on the extent of organ involvement and clinical manifestations, alongside laboratory-based investigations. The disease activity index used will depend on the country in which patients are diagnosed and managed (Nuttall and Isenberg, 2013).

1.5.2.3 Immunopathology of systemic lupus erythematosus

Immune cell dysregulation is central to SLE pathology, affecting both innate and adaptive immune cells, culminating in the activation of polyclonal B cells, increased number of plasma cells, hypergammaglobulinaemia and IC formation. Given the relevance to this thesis, neutrophil involvement in SLE will be discussed in section 1.6.2.

SLE patients have greater populations of activated polyclonal peripheral B cells compared to controls (Klinman et al., 1991). Studies have found a shift towards an immature B cell phenotype that is independent of disease activity (Dorner et al., 2011). Moreover, raised levels of $CD27^{+}IgD^{-}$ peripheral memory B cells have been identified in SLE patients and these cells are less susceptible to immunosuppression (Dorner et al., 2011). This increase in

$CD27^{+}IgD^{-}$ peripheral memory B cells is also associated with higher disease activity and lupus nephritis (Dorner et al., 2011). Reports also suggest that SLE B cells are more sensitive to cytokine stimulation (Linker-Israeli et al., 1991), display abnormal receptor-mediated signalling (Liossis et al., 1996) and have a greater propensity to undergo epitope spreading (Monneaux and Muller, 2002). Defective Breg activity has also been reported in SLE, with a population of IL-10 secreting Bregs identified that lack suppressive functionality within *in vitro* assays (Blair et al., 2010).

Aberrant T cell activation has also been linked to SLE pathology, with reports documenting abnormal TCR signalling and T cell hyper-responsiveness. Decreased expression of the TCRζ chain has been observed in SLE patients (Takeuchi et al., 2012), however studies have found that SLE T cells can replace TCRζ with the more potent FcγR (Kyttaris and Tsokos, 2011). Studies also report impaired CTLA-4 function in SLE patients (Jury et al., 2010). Normally, CTLA-4 activates the tyrosine phosphatase SHP-2, which inactivates TCRζ and disrupts TCR signalling (Lee et al., 1998). Due to the combination of decreased TCRζ expression and impaired CTLA-4 function, this pathway is impaired in SLE patients.

Impaired TCRζ signalling has been suggested to interfere with thymic T cell selection and promote the release of autoreactive T cells into the periphery (Tanaka et al., 2010). Abnormalities in other signalling molecules have been reported, including Ras, Syk and ERK (Cedeno et al., 2003, Krishnan et al., 2008, Tanaka et al., 2010), which contribute to T cell anergy (Yi et al., 2000). SLE $CD4^{+}$ T cells overexpress the α_L integrin subunit, perforin 1 and CD70 (Balada et al., 2014), however the significance of this overexpression is unclear, but may contribute to pathogenesis through increased cellular interactions and co-stimulatory signals. It is important to note that other signalling molecules and pathways, including PKC and mTOR, are abnormal in SLE T cells (Moulton and Tsokos, 2011).

Th17 cells also contribute to SLE pathology, with high levels of Th17 cells and IL-17 found in both mouse models and patients (Wong et al., 2000, Hsu et al., 2008). For example, in the BXD2 mouse, which displays a lupus-like phenotype, Th17 cells contribute to the formation of germinal centres and autoantibody production (Hsu et al., 2008). Elevated Th17 cells and IL-

17 have also been reported in other mouse models, where they promote renal inflammation (Moisan et al., 2007). Expression of signal transducer and activator of transcription 3 (STAT3), a transcription factor mediating Th17 development, is increased in human SLE T cells (Harada et al., 2007). Moreover, STAT3 inhibition in lupus prone mice has been shown to delay disease onset (Edwards et al., 2015), implicating Th17 cells in pathophysiology.

The contribution of Tregs to SLE pathology remains controversial. Some groups report decreased Treg populations in SLE patients (Miyara et al., 2005, Bonelli et al., 2008), whilst others find no difference (Alvarado-Sanchez et al., 2006, Vargas-Rojas et al., 2008). Discrepant results may be due to differences in cell isolation techniques and Treg characterisation. Some studies suggest that Treg-mediated suppression is impaired in SLE patients (Alvarado-Sanchez et al., 2006, Valencia et al., 2007, Bonelli et al., 2008). In contrast, others studies found that SLE Treg function is unaffected but that autoreactive SLE T cells are less susceptible to suppression (Vargas-Rojas et al., 2008). Moreover, HC Tregs have been shown to suppress SLE effector T cells (Valencia et al., 2007), which may suggests that defective SLE Treg-mediated suppression contributes to SLE pathology.

Monocytes and macrophages are also important in SLE pathology. Monocytes isolated from SLE patients have elevated expression of activation markers and adhesion molecules (Funauchi et al., 1993, Nockher et al., 1994, Egerer et al., 2000, Jin et al., 2005). Some groups have documented elevated $CD14^{+}CD11^{+}$ and $CD14^{+}CD16^{+}$ monocytes populations in SLE patients (Figueroa-Vega et al., 2006, Sullivan et al., 2007), which have increased expression of tissue factor (TF) (Nojima et al., 2008). There is also evidence for deregulated cytokine production, with SLE monocytes secreting greater levels of IL-6 and IL-10 compared to control (Linker-Israeli et al., 1991, Llorente et al., 1993, Llorente et al., 1994). Increased IL-6 and IL-10 section promotes the production of anti-dsDNA autoantibodies by B cells (Llorente et al., 1995, Kanda et al., 1999). SLE monocytes differentiating into macrophages also exhibit elevated expression of genes involved in immune processes and signal transduction (Korman et al., 2014). Elevated macrophage activation marker expression has also been identified in renal tissue from lupus nephritis patients (Yang et al., 1998a, Tomasoni et al., 1998, Frosch et al.,

2004, Ikezumi et al., 2005). Macrophage infiltration also correlates with renal disease (Yang et al., 1998a). SLE macrophages display reduced phagocytosis of apoptotic cells and elevated production of proinflammatory cytokines (Lovgren et al., 2006, Sestak et al., 2011). Aberrant autophagy has been reported in splenic and renal macrophages isolated from an activated lymphocytes-derived DNA-induced murine lupus model, which is believed to promote the production of proinflammatory cytokines (Li et al., 2014).

The precise function of NK cells in SLE is unclear. Several groups have reported reduced NK cell populations in SLE patients (Erkeller-Yuksel et al., 1997, Park et al., 2009, Huang et al., 2011a), which are associated with elevated IFN-α secretion (Huang et al., 2011a). Several groups have reported lower levels of NK cell inhibitory receptors in SLE patients (Schepis et al., 2009, Hervier et al., 2011, Puxeddu et al., 2012, Ye et al., 2014). Discrepancies arise when examining activating NK receptors, with some groups finding increased expression compared to controls (Hervier et al., 2011), whilst others report lower expression (Li et al., 2010b, Puxeddu et al., 2012, Ye et al., 2014) and some finding no difference (Schepis et al., 2009). Discrepant results may be attributed to variations in patient demographics, treatment regimens, cell characterisation and the specific receptors studied. A new class of autoantibodies have been identified, which target lectin-like NK cell receptors and interfere with their regulation (Hagberg et al., 2015), further implicating NK cell involvement in SLE.

DCs are important to SLE pathology, with DC depletion in lupus prone mice ameliorating disease, reducing B and T cell expansion and suppressing autoantibody titres (Teichmann et al., 2010, Rowland et al., 2014). Peripheral DCs are reduced in SLE patients (Migita et al., 2005, Fiore et al., 2008, Tucci et al., 2008), however this reduction may be due to greater migration into the tissues. In support of this hypothesis, plasmacytoid DCs (pDC), a DC subset, have been found to be elevated in skin and renal tissues taken from SLE patients (Farkas et al., 2001, Blomberg et al., 2001, Fiore et al., 2008, Tucci et al., 2008). SLE pDCs have a reduced response to *in vitro* TLR-9 stimulation compared to control (Kwok et al., 2008). Studies have found greater expression of CD40 and CD86 in SLE pDCs, which potently induce T cell proliferation (Jin et al., 2010, Nie et al., 2010). Interestingly, whilst promoting effector T

cell expansion, SLE pDCs fail to induce Treg differentiation (Jin et al., 2010). Recent work has found that SLE pDCs also fail to induce Breg differentiation, but promote plasmablast expansion and autoantibody production (Menon et al., 2016). Circulating ICs lead to the chronic activation of pDCs (Barrat et al., 2005), inducing the secretion of type I IFNs (Obermoser and Pascual, 2010). Secreted IFN can subsequently activate other cells and drive SLE pathology.

1.5.2.4 Current treatment for systemic lupus erythematosus

SLE patients are regularly monitored, by clinical assessment of disease activity and measurement of laboratory tests. Laboratory tests normally include inflammatory markers, similar to those assessed for RA, to evaluate levels of systemic inflammation. Current pharmacological treatments for SLE can be divided into 4 broad groups: NSAIDs; glucocorticoids; immunosuppressive drugs; and biologics. Treatment strategies depend upon the degree of disease activity and organ involvement with NSAIDs and HCQ given to treat mild disease with the addition of glucocorticoids and immunosuppressive therapy, such as azathioprine, to treat moderate and severe disease. Increasingly, biologic drugs are considered in patients who fail to respond to these conventional therapies (Lisnevskaia et al., 2014).

Patients prescribed NSAIDs are monitored for adverse effects on bowel, gastrointestinal and renal function (Bertsias and Boumpas, 2008). Glucocorticoids, commonly prednisolone, may be used to treat all features of SLE. HCQ is the most commonly prescribed immunomodulatory drug in SLE patients, particularly to treat arthritis, rash and fatigue. Its precise mechanism of action however, is unclear with HCQ being shown to block inflammatory pathways (Willis et al., 2012), as well as disrupting TLR-3, TLR-7 and TLR-9 signalling (Kuznik et al., 2011).

Those patients who fail to respond to conventional immunosuppressive drugs may be considered for biological therapy. Rituximab is the most common biologic prescribed to SLE patients. It is a chimeric monoclonal antibody originally developed for the treatment of B cell lymphomas (Grillo-Lopez et al., 1999), which targets CD20 and induces apoptosis (Eisenberg, 2005). The National Institute for Health and Care Excellence (NICE) has also recently approved

belimumab, which inhibits B cells survival, for the treatment of SLE. Additional biologics are currently in development and subject to pre-clinical studies. These include drugs that modulate B cell function (epratuzumab), inhibit B cell survival (atacicept) and target plasma cells (bortezomib). In addition, biologic therapy can also target T cells (abatacept, ruplizumab, toralizumab), IFN (rontalizumab, sifalimumab), IL-6 (tocilizumab) and TNF-α (infliximab, etanercept) (Lisnevskaia et al., 2014). These therapies however, require further clinical studies to evaluate any potential benefits to patients.

1.5.3 Antiphospholipid syndrome

The hallmarks of APS are recurrent thrombosis and/or pregnancy morbidity, such as recurrent pregnancy loss (Hughes, 1993, Cervera et al., 2002, Levine et al., 2002). The full clinical description of this unique form of autoantibody-induced thrombophilia was made in the early 1980s, reporting an association between recurrent thrombosis and pregnancy morbidity with the persistent presence of circulating antiphospholipid antibodies (aPL) (Hughes, 1983).

In contrast to their name, aPL do not directly bind phospholipids but target serum cofactors, which in turn bind anionic phospholipids. The most important of these serum cofactors is β_2-glycoprotein I (β_2GPI) (Matsuura et al., 1990, McNeil et al., 1990). Lupus anticoagulants (LA) are a heterogeneous class of Ig that can develop spontaneously or in association with ARDs. LA bind serum cofactor-phospholipid complexes, such that phospholipid-dependent coagulation is prolonged (Arnout, 2001). Anti-cardiolipin antibodies (aCL), which are closely related to LA, have been shown to bind cardiolipin associated with serum cofactors (Galli et al., 1990). Persistent aCL positivity has been associated with an increased risk of arterial or venous thrombosis (Ginsburg et al., 1992). Therefore, the three standard (known as criteria) tests for detection of aPL are LA, aCL and anti-β_2GPI antibodies. The persistent presence of one or more of these criteria aPL tests in the presence of vascular thrombosis and/or pregnancy morbidity defines APS. APS can be further characterised as either primary APS, which occurs in the absence of another ARD, or ARD-associated APS when diagnosed in the presence of other ARD, most commonly being SLE (Miyakis et al., 2006).

1.5.3.1 Aetiology of antiphospholipid syndrome

Early family-based studies reported higher incidences of aCL in first-degree relatives of APS patients (Mackworth-Young et al., 1987, Goldberg et al., 1995), suggesting a genetic component to APS. Several reports have associated HLA alleles with aCL and anti-β_2GPI antibodies. HLA-DR4 and -DR7 were increased in European aCL positive patients (Hartung et al., 1992). HLA-DR7 was also increased in Mexican aCL positive patients (Granados et al., 1997). Studies in European cohorts found positive associations between aCL and HLA-DRB1*04, -DRB1*0402|3, -DRB1*07, -DRB3*0301, -DQA1*0201, -DQA1*0301 and -DQB1*0302 (Galeazzi et al., 2000). The authors also reported positive associations between anti-β_2GPI antibodies and HLA-DRB1*0402|3 and -DQB1*0302. An association between HLA-DQB1*0302 and anti-β_2GPI antibodies was also observed in a Mexican population (Arnett et al., 1999). More recently, positive correlations between HLA-DQB1*0604|5|6|7|9, -DQA1*0102 and -DRB1*1303 with the presence of anti-β_2GPI antibodies were reported, which associated with an increased thrombotic risk (Caliz et al., 2001). These studies demonstrate that contribution of HLA is complex and varies between ethnic groups. The expression of certain HLA haplotypes may increase the presentation of peptides derived from β_2GPI or its associated proteins, making patients prone to generating aPL and drive pathology.

Chromosome mapping has highlighted four major β_2GPI polymorphisms: S85N, L247V, C306G and W316S. The functional effects of these polymorphisms are not fully defined. The L247V polymorphism has been located to domain V, which is thought to be a potential epitope for anti-β_2GP1 antibodies (Ichikawa et al., 1994). The L247V polymorphism was more frequently detected in an Asian APS cohort compared to control, which is associated with the presence of anti-β_2GPI antibodies, but did not associate with increased thrombotic risk (Hirose et al., 1999). A similar distribution of the L247V polymorphism was observed in APS patients and controls in Caucasian populations (Pardos-Gea et al., 2012). This group did report an enrichment of the W316S polymorphism amongst patients, however this finding did not associate with aPL or clinical manifestations. Overall, these results do not provide strong

evidence that β_2GPI polymorphisms contribute to APS pathology, although they may promote the generation of anti-β_2GPI antibodies.

PSGL-1 polymorphisms have been implicated in the predisposition to thrombosis in APS patients. Examination of the variable number of tandem repeats (VNTR) polymorphisms in PSGL-1 highlighted three alleles, encoding 14, 15 or 16 tandem repeats within the mucin-like regions of PSGL-1 (Afshar-Kharghan et al., 2001). Enrichment of the allele encoding 15 VNTR was observed in thrombotic APS patients compared to patients without thrombosis and HCs (Diz-Kucukkaya et al., 2007). This observation suggests that PSGL-1 polymorphisms may predispose patients to thrombotic events, most likely by promoting aberrant leukocyte interactions with ECs and platelets.

TLR-4 polymorphisms D299G and T399I have been implicated in hypo-responsiveness to inhaled LPS in humans (Arbour et al., 2000, Fageras Bottcher et al., 2004). Significantly reduced frequencies of both polymorphisms have been found in APS patients (Pierangeli et al., 2007). The reduced prevalence of hypo-responsive TLR-4 polymorphisms may indicate that APS patients are more susceptible to TLR-4 mediated signalling, which is important in aPL-induced cellular activation (Raschi et al., 2003, Mulla et al., 2009). Other groups however, have been unable to replicate these results in other cohorts (Erridge et al., 2003), which make the significance of these results unclear.

Limited studies examine the environmental factors that trigger clinical events in APS patients, however infection is generally considered to be the most influential environmental factor (Blank et al., 2002, Cervera et al., 2004, Shoenfeld et al., 2006). To conclude, aPL induce a pro-thrombogenic state through cellular activation. Combined with a genetic predisposition arising through polymorphisms in numerous genes, the vasculature of APS patients is primed such that when challenged, thrombosis is rapidly induced.

1.5.3.2 Clinical features of antiphospholipid syndrome

To diagnose patients with APS, physicians often refer to APS classification criteria, which require patients to present with at least one clinical manifestation consistent with APS, as

well as two positive tests for aPL at least 12 weeks apart (Wilson et al., 1999, Miyakis et al., 2006). This timing of aPL tests was chosen to ensure the detection of persistent aPL and avoid misdiagnosis through identification of aPL that are only transiently positive, such as may occur in certain infections. Data obtained from the Euro-Phospholipid project, comprising 1000 APS patients, found that peripheral thrombosis was the most common clinical feature, affecting 63.7% of patients (Cervera et al., 2009a). The most common manifestation was deep vein thrombosis in the leg (38.9%), but venous thrombosis was also observed in the arms (3.4%). Arterial thrombosis in both arms and legs were seen in smaller numbers of patients (2.7% and 4.3% respectively). Obstetric and foetal manifestations were also common in female patients, with 9.5% of the 590 pregnant patients developing pre-eclampsia. Moreover, in the 1580 pregnancies, 52.3% miscarried and 67.7% of these miscarriages occurred within the first 10 weeks of pregnancy (Cervera et al., 2009a).

Neurological manifestations were also common amongst the Euro-Phospholipid cohort, affecting 65.8% of patients. Migraine was the most frequent symptom, which was reported in 20.2% of patients. Thrombosis also contributed to neurological complications, with 19.8% of patients having suffered from a stroke and 11.1% diagnosed as having a transient ischaemic attack (Cervera et al., 2009a). Other clinical features include thrombocytopenia (29.6%), cardiac involvement (26.9%), livedo reticularis (24.1%), pulmonary embolism (14.1%), haemolytic anaemia (9.7%) and retinal thrombosis (2.4%) (Cervera et al., 2009a).

Catastrophic APS (CAPS) is the most severe presentation of APS, but only occurs in 0.8% of patients (Cervera et al., 2009a). CAPS is classified by widespread small-vessel thromboses affecting at least three different organs within one week, micro-thrombosis in at least one organ and persistent aPL positivity (Asherson et al., 2003, Cervera et al., 2005). CAPS is associated with multiple organ failure and a high mortality rate (Asherson et al., 2003).

1.5.3.3 Immunopathology of antiphospholipid syndrome

Studies have shown that aPL targeting β_2GPI can activate ECs and monocytes (Pierangeli et al., 2008). β_2GPI is formed of 5 domains, with domain V mediating phospholipid

binding (Wurm, 1984, Sheng et al., 1996). Multiple groups suggest that pathogenic anti-β_2GPI antibodies target domain I (Iverson et al., 1998, de Laat et al., 2006, Ioannou et al., 2007), although anti-β_2GPI antibodies targeting other domains have also been described (George et al., 1998), which may be clinically relevant (Andreoli et al., 2015).

Multiple studies have shown that aPL activate ECs, monocytes and platelets (Pierangeli et al., 1999, Shoenfeld et al., 2006, de Laat et al., 2008). Anti-β_2GPI antibodies have been shown to activate cells by cross-linking membrane bound β_2GPI with TLR-4 (Raschi et al., 2003, Mulla et al., 2009). Evidence exists implicating other receptors, including apolipoprotein E receptor 2 (Romay-Penabad et al., 2011, Ramesh et al., 2011, Ulrich et al., 2016) and TLR-2 (Satta et al., 2007, Satta et al., 2011) in aPL-mediated cellular activation.

Treatment with aPL upregulates endothelial ICAM-1, VCAM-1, E-selectin and P-selectin, as well as inducing TF expression in ECs and monocytes (Pierangeli et al., 2001, Lopez-Pedrera et al., 2008). ICAM-1 deficient mice, ICAM-1/P-selectin deficient mice and mice injected with VCAM-1 blocking antibodies are all resistant to aPL-induced leukocyte adhesion and thrombosis in a pinch-injury mouse model (Pierangeli et al., 2000), suggesting that cell adhesion molecule upregulation is important to APS pathogenesis. Moreover, thrombus formation is either reduced or completely abrogated in mice lacking ICAM-1, E-selectin or P-selectin (Pierangeli et al., 2001, Espinola et al., 2003). Endothelial activation contributes to thrombosis in mice (Meroni et al., 2004), which is mediated by NF-κB and p38 MAPK (Vega-Ostertag et al., 2005, Montiel-Manzano et al., 2007). These studies demonstrate the importance of EC adhesion molecules, which contribute to pathology through increased interactions with circulating leukocytes and platelets.

Infections, inflammation and other pro-coagulant stimuli can initiate thrombosis (de Groot and Derksen, 2005), leading to the 'two-hit hypothesis' underpinning APS. The basis of this hypothesis stems from the observations that aPL are necessary but not sufficient to trigger the coagulation cascade. According to this hypothesis, circulating aPL provide the first hit by activating the cells within the vasculature, inducing a pro-thrombotic state. A second hit is then required, such as mechanical, physical, inflammatory and/or infectious stimuli (Blank et al.,

2002, Cervera et al., 2004, Fischetti et al., 2005, Shoenfeld et al., 2006), which promotes β_2GPI deposition on ECs and initiates thrombus formation (Meroni and Riboldi, 2001, Agostinis et al., 2011).

Autoantibodies can disrupt mitochondrial function in monocytes and neutrophils, leading to ROS generation and TF expression (Perez-Sanchez et al., 2012). Anti-β_2GPI antibody-induced TF expression has been shown to increase TNF-α secretion in monocytes (Sorice et al., 2007). Studies have shown aPL can directly activate complement and interact with various complement-regulatory proteins including Factor X, plasmin, prothrombin and protein C, all of which inhibit fibrinolysis (de Groot and Derksen, 2005, Pierangeli et al., 2008). In addition, aPL can activate platelets, increasing expression of thromboxane A2 and the platelet integrin $\alpha_{IIb}\beta_3$, enhancing binding to von Willebrand factor and fibrinogen (Robbins et al., 1998). Interactions between activated cells promote thrombus formation. Furthermore, through the inhibition of fibrinolysis, aPL can delay the breakdown of clots and drive thrombosis.

Complement activation has also been implicated in the mechanisms underlying APS immunopathology. Reports have shown that anti-β_2GPI antibody-mediated complement activation contributes to thrombogenesis, as demonstrated by the efficacy of C5 inhibition in preventing blood clots in mice and rats administered intravascular infusions of purified anti-β_2GPI antibodies (Fischetti et al., 2005, Agostinis et al., 2014, Romay-Penabad et al., 2014). Moreover, $C3^{-/-}$ and $C5^{-/-}$ mice subjected to a pinch-injury thrombosis model were found to have reduced thrombus size compared to $C3^{+/+}$ and $C5^{+/+}$ littermate controls (Pierangeli et al., 2005). These findings suggest that complement activation may contribute to the pathology of aPL-induced thrombosis.

Whilst the precise mechanisms underlying obstetric APS are poorly understood, there is evidence implicating complement activation in aPL-induced pregnancy morbidity. Administration of aPL-IgG has been shown to localise to the placenta (Ikematsu et al., 1998), where it can restrict intrauterine growth and induce foetal loss in pregnant mice (Holers et al., 2002). Studies have found that mice deficient in C3, C4 or C5 were protected from aPL-induced pregnancy morbidity (Salmon et al., 2002, Girardi et al., 2003). In addition, administration of

monoclonal antibodies targeting C3 or C5 also prevented foetal loss (Xu et al., 2000, Girardi et al., 2003). These observations indicate that complement activation may be required in the pathogenesis of aPL-induced pregnancy morbidity. Other studies have associated placental thrombosis and abnormalities of annexin V with aPL-mediated pregnancy morbidity in animal models and patients (Rand et al., 1994, Rand et al., 1997). Reports also suggest that aPL reduce trophoblast invasion, induce extravillous and syncytiotrophoblast activation, disrupt syncytium formation and promote thromboembolism within the decidual vessels that lead to pre-eclampsia (Abrahams, 2009, Kwak-Kim et al., 2013).

1.5.3.4 Current treatment for antiphospholipid syndrome

Pharmacological management of thrombotic APS centres on reducing the risk of clot development (Giannakopoulos and Krilis, 2009). Patients with a history of either arterial or venous thrombosis are normally treated with an anticoagulant, most commonly warfarin. Duration and intensity of anticoagulation are dependent on the site (arterial vs. venous) and recurrence of clot. In the event of thrombosis or sudden deterioration, heparin may be administered as it has a faster mode of action. Treatment regimens are complex in ARD-associated APS, where patients are treated for both APS and the associated ARD.

Obstetric APS is treated by a combination of aspirin and heparin, however recommendations vary depending on disease history (Bates et al., 2008). Due to possible beneficial effects during the early stages of implantation, aspirin is recommended prior to conception (Carmona et al., 2001). APS patients without previous thrombosis but recurrent early miscarriage during the pre-embryonic or embryonic stages are typically prescribed low-dose aspirin with low molecular weight heparin (LMWH) (de Jesus et al., 2014). If patients have had previous foetal death after 10 weeks gestation or early delivery due to severe pre-eclampsia, low-dose aspirin is prescribed combined with LMWH. In both cases, women are treated with aspirin or LMWH for up to 6 weeks postpartum.

Given the rarity of CAPS, randomised controlled trials evaluating the efficacy of drugs do not exist. There is however a CAPS registry, which documents 280 reports of CAPS patients,

so treatment recommendations are based on these case studies (Bucciarelli et al., 2009, Cervera et al., 2009b). Initial treatment involves intravenous heparin and oral anticoagulants administration to prevent further thrombosis. Corticosteroids are given to suppress inflammation. Plasma exchange and intravenous Ig may also be administered. If patients continue to deteriorate then cyclophosphamide or rituximab may also be prescribed (Asherson et al., 2003).

1.6 Neutrophil involvement in autoimmune rheumatic diseases

Neutrophils are emerging as important regulators of both innate and adaptive immune responses. Abnormalities in neutrophil phenotype and function have been described in various ARDs and suggest that neutrophils may contribute to immunopathology. This section evaluates the current evidence implicating neutrophil involvement in the pathology of RA, SLE and APS.

1.6.1 Neutrophils in rheumatoid arthritis

RA is a complex disorder affecting both cellular and humoral immunity. A key feature of RA is the erosion of cartilage and destruction of the underlying bone. Hyaluronic acid, a macromolecule endowing the SF with lubricant properties (Ogston and Stanier, 1953), protects the joint from erosion. ROS and granular enzymes secreted by neutrophils have been shown to depolymerise long chain hyaluronic acid *in vitro* (Grootveld et al., 1991, Parkes et al., 1991). Therefore, activated neutrophils within the synovium may reduce the lubricant properties of the SF and promote joint destruction. Exposure to ICs, Ig and cytokines within the SF activate neutrophils and contributed to cartilage destruction via the externalisation of neutrophil granule contents (Emery et al., 1988, Chatham et al., 1990).

Early studies identified neutrophils in the SF of RA patients, implicating neutrophils in joint destruction (Dularay et al., 1988, Dularay et al., 1990, den Broeder et al., 2003). Activated neutrophils have been reported in the SF and synovial tissue (Barnhart et al., 1967, Hughes et al., 1995, Belcher et al., 2002). Neutrophils have also been shown to contribute to pathology in three RA mouse models: the collagen-induced arthritis (CIA) model (Griffiths et al., 1995), K/BxN serum transfer model (Wipke and Allen, 2001) and proteoglycan-induced arthritis

model (Gal et al., 2005). Interestingly, disease remission in RA patients has been linked with reduced neutrophil synovial migration (Dominical et al., 2011).

Neutrophils migrate into the RA joint, attracted by IL-8 (Brennan et al., 1990), TNF-α (den Broeder et al., 2003) and granulocyte colony-stimulating factor (G-CSF) (Eyles et al., 2008). Evidence suggests that neutrophils can orchestrate their own recruitment via C5a receptor and FcγRIIIB signaling (Sadik et al., 2012). Neutrophils can also contribute to the proinflammatory environment within the synovium. Following activation by GM-CSF, IL-1, LPS or TNF-α, neutrophils can initiate *de novo* cytokine synthesis and secretion, including IFN-α (Shirafuji et al., 1990), IL-1β (Marucha et al., 1990, Lord et al., 1991, Malyak et al., 1994, Quayle et al., 1995), IL-6 (Cicco et al., 1990, Palma et al., 1992, Melani et al., 1993, Zimmermann et al., 2016), IL-8 (Strieter et al., 1992, Takahashi et al., 1993, Fujishima et al., 1993), transforming growth factor (TGF)-β (Grotendorst et al., 1989) and TNF-α (Dubravec et al., 1990, Djeu et al., 1990).

Neutrophils have also been implicated in bone resorption. Studies examining receptor activator of NF-κB (RANK) and RANK ligand (RANKL) expression reported higher levels in RA SF-derived neutrophils compared to osteoarthritis (OA) controls (Poubelle et al., 2007). LPS can induce neutrophil RANKL expression, which activates osteoclasts and stimulates bone resorption (Chakravarti et al., 2009). Moreover, neutrophils cultured in cell-free RA SF, but not OA SF, have been shown to induce RANKL-dependent osteoclastogenesis and bone resorption.

Neutrophils engulf opsonised pathogens via phagocytosis, which are then destroyed by the granular proteases and ROS released into the phagosome. When neutrophils encounter Ig-opsonised cartilage, they are unable to phagocytose the cartilage and underlying bone, therefore undergo 'frustrated phagocytosis'. As a result of frustrated phagocytosis, neutrophils release their granular contents into the extracellular space, a process with parallels to NETosis (Dularay et al., 1988, Edwards et al., 1988, Nurcombe et al., 1991a, Nurcombe et al., 1991b, Robinson et al., 1992, Robinson et al., 1993), which contributes to joint destruction.

Peripheral blood- and SF-derived RA neutrophils are both prone to spontaneous NETosis. Elevated NETs have been reported in RA serum and SF compared to OA and HCs,

with levels of NETs correlating with ACPA titres and markers of inflammation (Sur Chowdhury et al., 2014). Serum, purified IgG and SF of ACPA high patients enhance NETosis of control neutrophils (Khandpur et al., 2013), which expose citrullinated autoantigens that are targeted by autoantibodies (Khandpur et al., 2013, Pratesi et al., 2013). NETs can activate synovial fibroblasts, inducing the synthesis of proinflammatory cytokines, chemokines and adhesion molecules (Khandpur et al., 2013). The importance of NETs has also been demonstrated in mice, with PAD-4 inhibition preventing NETosis and reducing disease severity in the CIA mouse model (Willis et al., 2011).

Analysis of mRNA and protein expression in models of acute and chronic arthritis, using the streptococcal cell wall-induced arthritis and CIA models respectively, found PAD-4 mRNA and protein were only detected in neutrophils of arthritic mice (Vossenaar et al., 2003). Neutrophils have also been shown to release functionally active PAD-2 and PAD-4 during NETosis, which can be detected in RA SF, but not in OA or psoriatic arthritis controls (Spengler et al., 2015). Therefore, synovial neutrophils may contribute to RA pathology, not only through the generation of NETs, but also by releasing active PAD isoforms that can citrullinate extracellular synovial autoantigens.

To conclude, there is increasing evidence that neutrophils contribute to RA pathology. Through the secretion of proinflammatory and chemotactic cytokines, ROS and NETs, neutrophils can facilitate cartilage erosion and bone destruction.

1.6.2 Neutrophils in systemic lupus erythematosus

Adaptive immunity has an established role in SLE pathology, however in more recent years, neutrophils are being increasingly studied. Early studies noted that SLE sera induced neutrophil aggregation and interfered with phagocytosis and degranulation (Abramson et al., 1983). Defective phagocytosis, reduced responsiveness to cytokines and increased senescence are reported in SLE neutrophils (Brandt and Hedberg, 1969, Wu et al., 2007, Hsieh et al., 2008). Uptake of circulating nucleosomes activates neutrophils (Ronnefarth et al., 2006), which leads to the section of antibacterial proteins. Elevated bactericidal proteins have been documented in

SLE sera, which correlate with autoantibody titres (Bakkaloglu et al., 1998) and disease activity (Sthoeger et al., 2009, Vordenbaumen et al., 2010, Ma et al., 2012).

Increased neutrophil apoptosis has also been reported in SLE, correlating with disease activity and anti-dsDNA antibody titres. Anti-dsDNA and anti-La antibodies are thought to modulate neutrophil cell death and function respectively (Armstrong et al., 2006). In SLE, apoptotic neutrophils are not recognized by C1q-calreticulin and CD91 mediated clearance pathways (Donnelly et al., 2006). In addition, scavenger receptors display reduced binding to NETs (Schorn et al., 2012), which prevents their clearance.

Aberrant NETosis and impaired clearance contribute to SLE pathology (Hakkim et al., 2010, Villanueva et al., 2011, Garcia-Romo et al., 2011, Lande et al., 2011). NETosis releases dsDNA and inflammatory cytokines, which correlates with anti-dsDNA antibodies titres (Villanueva et al., 2011). ICs bind NETs and prevent their degradation and promote uptake by pDCs, which stimulates IFN-α secretion (Lande et al., 2011), and primes neutrophils for further NETosis (Garcia-Romo et al., 2011). Impaired DNase I-mediated NET degradation promotes complement activation and exacerbates disease (Hakkim et al., 2010, Leffler et al., 2012). NETs have been shown to stimulate the inflammasome in macrophages, which also drives proinflammatory responses (Kahlenberg et al., 2013).

Animal models also implicate NETosis in SLE pathology. Antibodies targeting NETs have been identified in NZM2328 lupus prone mice, which induce NETosis *in vitro* (Knight et al., 2013). Neutrophils infiltrate the kidneys of NZM2328 mice, where NETs directly impair endothelial function. Administration of Cl-amidine, an irreversible pan-PAD inhibitor, significantly reduced NETosis, complement activation and renal IC deposition in NZM2328 mice. This group noted that whilst serum autoantibodies increased, most likely due to reduced renal deposition, endothelial function improved (Knight et al., 2013).

When examining SLE peripheral blood mononuclear cells (PBMCs), Bennett et al. found a significant upregulation of granulocyte-specific gene expression (Bennett et al., 2003). Several studies have described an abnormal neutrophil subset in SLE patients (Hacbarth and Kajdacsy-Balla, 1986, Bennett et al., 2003, Denny et al., 2010, Leffler et al., 2012). Lower in

density than 'conventional' neutrophils, the subset was called low-density granulocytes (LDGs). LDGs express the same cell surface markers as mature neutrophils, however their nuclear morphology is more consistent with immature neutrophils. LDGs are therefore thought to have an immature neutrophil phenotype, instead of being activated neutrophils. This hypothesis is supported by increased expression of early granulopoiesis genes (Nakou et al., 2008). LDGs may represent an aberrant immature neutrophil subset that persists within the vasculature and tissues of SLE patients (Carmona-Rivera and Kaplan, 2013).

LDGs are highly proinflammatory and secrete greater levels of TNF-α, type I and type II IFNs than conventional neutrophils (Denny et al., 2010, Carmona-Rivera and Kaplan, 2013). LDGs more readily undergo spontaneous NETosis (Denny et al., 2010) and express greater levels of various bactericidal proteins (Villanueva et al., 2011). Furthermore, LDGs have an increased capacity to kill ECs on contact and produce NETs on stimulation (Denny et al., 2010, Villanueva et al., 2011). NETosis in both SLE neutrophils and LDGs can be induced by SLE-IgG (Carmona-Rivera et al., 2015). The combination of increased cytokine production, elevated NETosis and induction of EC death is central to the pathogenic contribution of LDGs.

To conclude, there is a growing understanding of how neutrophils contribute to the pathogenesis of SLE. Through the aberrant activation of neutrophils, NETs are produced and cause endothelial damage. LDGs also contribute to the level of NETosis, whilst inducing EC death and dysfunction. Defective clearance of NETs facilitates immune activation, deposition of ICs and endothelial damage, all of which enhance pathology.

1.6.3 Neutrophils in antiphospholipid syndrome

Relatively less work has explored neutrophils in APS pathology. A C5a receptor-TF crosstalk has been described in neutrophils, whereby the *in vitro* aPL-mediated complement activation led to neutrophil C5a receptor engagement. This interaction subsequently induced TF expression and enhanced procoagulant activity (Ritis et al., 2006).

This observation was replicated *in vivo*, with C5a generated by aPL inducing neutrophil TF expression in an aPL-induced pregnancy loss mouse model (Redecha et al., 2007).

Inflammation and pregnancy loss was reduced in TF knockout mice and wild-type mice administered TF blockade. Further immunohistochemical analysis found that TF expressed in neutrophils, but not trophoblasts, associated with foetal injury. Generation of myeloid-specific TF knockout mice found a significant reduction in ROS production following aPL treatment. Redecha et al. therefore proposed that aPL activated trophoblasts and initiated the complement cascade. C5a then bound neutrophils, which induced TF expression and oxidative burst, causing trophoblast injury, inflammation and foetal death (Redecha et al., 2007).

Redecha and colleagues subsequently explored the mechanisms by which TF contributed to neutrophil activation and trophoblast injury. Greater expression of protease-activated receptor (PAR)-2 was observed in neutrophils of aPL-treated mice (Redecha et al., 2008). ROS generation, phagocytosis and foetal loss were reduced in aPL-treated PAR-2 knockout mice. Wild-type and *Par2*$^{-/-}$ neutrophils had similar respiratory burst and phagocytic responses following PMA stimulation *in vitro* (Redecha et al., 2008), suggesting that PAR-2 signalling may modulate aPL-induced neutrophil activation. The authors also found that aPL-induced activation was dependent on TF/Factor VIIa interactions (Redecha et al., 2008). These results could not be replicated by PAR-1 blockade, suggesting that TF/Factor VIIa/PAR-2 signalling mediate neutrophil activation and foetal death in the aPL-induced pregnancy loss mouse model.

A caveat of this model is the amount of purified IgG administered to mice. 10mg of human whole IgG was injected intraperitoneally at days 8 and 12 of pregnancy. For experimental procedures, 6-8 week old female C57BL/6 mice were used, weighing 15-20g. Given that C57BL/6 mice have approximately 58.5ml/kg of blood, 10mg of human IgG was injected into 0.87-1.17ml of blood at each administration. Whilst 10mg/ml roughly represents normal serum IgG levels in humans (Gonzalez-Quintela et al., 2008), serum IgG levels are 10-fold lower in mice at 1.22mg/ml (Klein-Schneegans et al., 1989). As mice were administered at least 20mg of IgG over a 5-day period, it is possible that neutrophils are responding to the vast amount of human IgG. Experiments using physiologically representative IgG levels for mice would be interesting, but may give rise to more subtle differences that are harder to interpret.

Recent work has explored the effects of APS sera and IgG upon NETosis (Yalavarthi et al., 2015). APS-IgG induced significantly higher levels of NETs compared to HC-IgG, which was abrogated following depletion of β_2GPI-specific IgG (Yalavarthi et al., 2015). In addition, *ex vivo* APS neutrophils were predisposed to produce NETs spontaneously. This group also found elevated NETs in the sera and plasma of primary APS patients compared to healthy volunteers. NETs have been shown to induce thrombosis in the absence of aPL both *in vitro* (Fuchs et al., 2010), and also promote deep vein thrombosis in mice (Brill et al., 2012), which may contribute to APS pathology. Therefore, APS-IgG, in particular anti-β_2GPI antibodies, may prime neutrophils such that they more readily activate and release NETs to promote thrombosis.

In summary, there is increasing interest in the contribution of neutrophils in APS pathology. Due to their ability to produce large quantities of ROS and NETs, both of which are known to cause cellular damage and are associated with increased thrombosis, neutrophils may promote APS pathogenesis. Table 1.3 summarises key publications that support the role of neutrophils in RA, SLE and APS.

1.7 Integrins in autoimmune rheumatic disease

Defects in integrin regulation have been implicated in the immunopathology of several ARDs, with aberrant cellular interactions being reported in RA, SLE and APS. This section will explore the literature to evaluate the evidence for integrin involvement in RA, SLE and APS.

1.7.1 Integrins in rheumatoid arthritis

The β_1 integrins can regulate T cell cytokine production. ECM components have been shown to modulate IL-2 production in 2B4 T cells following stimulation with suboptimal doses of an anti-CD3 antibody (Takahashi et al., 1991). Cells stimulated in fibrinogen-, laminin- or collagen-coated or uncoated wells produced low levels of IL-2. In contrast, cells stimulated in fibronectin- or vitronectin-coated wells produced significantly higher IL-2 levels, which was reduced by β_1 integrin blockade (Takahashi et al., 1991), implicating a β_1 integrin-dependent mechanism of IL-2 production in 2B4 T cells.

Autoimmune Rheumatic Disease	Summary of Major Findings Implicating Neutrophil Involvement	Key Evidence Demonstrating the Contribution of NETs to Pathology
Rheumatoid Arthritis	• Neutrophils important in mouse models of RA (Griffiths et al., 1995, Wipke and Allen, 2001, Gal et al., 2005) • Disease remission associated with reduced synovial neutrophils (Dominical et al., 2011) • Neutrophils can stimulate bone resorption via RANKL (Poubelle et al., 2007) • RA neutrophils prone to spontaneous NETosis (Sur Chowdhury et al., 2014) • RA-IgG enhances NETosis of control neutrophils (Khandpur et al., 2013)	• NETs are a source of autoantigens (Pratesi et al., 2013) • Elevated NETs in RA serum and synovial fluid (Sur Chowdhury et al., 2014)
Systemic Lupus Erythematosus	• Neutrophil activation correlates with disease activity (Vordendaumen et al., 2010, Ma et al., 2012) • Neutrophils are important to renal disease in mouse models of lupus (Knight et al., 2013) • LDGs contribute to SLE pathology (Carmona-Rivera and Kaplan, 2013) • LDGs are more prone to NETosis (Denny et al., 2010) • SLE-IgG induces NETosis of neutrophils and LDGs (Carmona-Rivera et al., 2013)	• DNase I degradation of NETs is defective in patients (Hakkim et al., 2010) • NETs activate pDCs to secrete IFN-α (Lande et al., 2011) • NETs activate macrophages to secrete proinflammatory cytokines (Kahlenberg et al. 2013) • Inhibition of NETs reduces renal disease in mouse models of lupus (Knight et al., 2013)
Antiphospholipid Syndrome	• Neutrophils mediate aPL-mediated foetal loss in mice (Redecha et al., 2007) • APS neutrophils more prone to NETosis (Yalavarthi et al., 2015) • APS-IgG induces NETosis of control neutrophils (Yalavarthi et al., 2015)	• NETs promote thrombosis in vitro (Fuchs et al., 2010) • NETs promote deep vein thrombosis in mice (Brill et al., 2012) • Elevated NETs in APS serum and plasma (Yalavarthi et al., 2015)

Table 1.3: Evidence for neutrophil involvement in autoimmune rheumatic disease. Listed above are key publications that document the contributory role of neutrophils and NETs to RA, SLE and APS.

Early studies found that SF T cell proliferation and IFN-γ production could be modulated through interactions with both native and denatured collagen (Ofosu-Appiah et al., 1989a, Ofosu-Appiah et al., 1989b). Early work also reported elevated VLA-1 (CD49a/CD29, $\alpha_1\beta_1$) expression in RA SF lymphocytes compared to peripheral blood lymphocytes (Hemler et al., 1986). The authors suggested that following recruitment to the synovium, T cells upregulate VLA-1 expression, which may contribute to disease. A limitation to this study is the lack of healthy or disease controls, meaning that whether this upregulation is RA-specific or a result of transmigration is unclear. Similar observations were made with VLA-4 expression on $CD3^+$ T cells. Whilst there were no significant differences between RA and control peripheral blood T cells, matched peripheral blood and SF RA T cells highlighted that SF-derived T cells expressed significantly higher levels of VLA-4 (Laffon et al., 1991).

Additional work observed upregulation of IL-1α, IL-1β, IFN-γ and TNF-α mRNA when RA PBMCs were cultured on fibronectin, laminin or collagen, but not bovine serum albumin (BSA), which was mitigated by β_1 but not β_2 integrin blockade (Miyake et al., 1993). The use of only 3 patients is a limitation to this study, however the data supports previous work by Ofosu-Appiah and colleagues showing modulation of cytokine production by the presence of β_1 integrin ligands. Characterisation of larger RA and control cohorts are required, however these observations suggest that synovial T cells upregulate β_1 integrins, which contributes to RA pathology through increased proinflammatory cytokine production.

VLA-2 has also been implicated in both the antigen-induced arthritis (AIA) and human TNF-α transgenic mouse models (Peters et al., 2012). *Itga2*$^{-/-}$ mice do not express the α_2 integrin subunit, therefore lack VLA-2 ($\alpha_2\beta_1$), but still express the other β_1 integrins. In both models, *Itga2*$^{-/-}$ mice had significantly reduced pannus formation, cartilage erosion and joint inflammation. VLA-2 deficient mice also had reduced MMP-3 expression and suppressed FLS proliferation and attachment to cartilage (Peters et al., 2012). This group therefore concluded that VLA-2 contributed to RA pathology by inducing MMP-3 expression and promoting FLS proliferation and attachment to ECM. Early work in keratinocytes found that VLA-2 ligation

upregulated MMP-1 expression (Pilcher et al., 1997), which may suggest that in RA, VLA-2 is able to upregulate expression of several MMPs.

Integrin expression between RA FLS and control FLS has also been compared. Significantly higher expression of the $\alpha_{1\text{-}6}$, β_1 and β_4 integrin subunits was reported in RA FLS compared to controls (Rinaldi et al., 1997a), suggesting an upregulation of VLA-1 to -6 and $\alpha_6\beta_4$. RA FLS also displayed increased adhesion to collagen, fibronectin, laminin and tenascin, all of which were inhibited by integrin-specific blockade. Further work found differential $\alpha_V\beta_3$ regulation in FLS derived from OA patients and RA patients (Rinaldi et al., 1997b). Whilst similar basal $\alpha_V\beta_3$ expression was observed in RA and OA FLS, stimulation with IL-1β or TNF-α increased expression in OA FLS but decreased expression in RA FLS, however the basis of this differential regulation remains unclear.

RA synovial cells have been shown to express significantly higher levels of VLA-2, VLA-5 and $\alpha_V\beta_3$ compared to OA controls (Nakayamada et al., 2003). Moreover, crosslinking of β_1 integrins induced ICAM-1 and Fas expression in RA synoviocytes but not in OA cells. Given the wealth of evidence, obtained from independent groups using various methodologies, the β_1 integrins appear to contribute to the pathology of RA, by promoting cell adhesion, MMP production and proinflammatory cytokine secretion.

Early work found that β_2 integrin blockade reduced acute arthritis and promoted the amelioration of chronic inflammation in an AIA rabbit model (Jasin et al., 1992). Early signs of inflammation were reduced in rabbits treated with an anti-β_2 integrin antibody compared to saline control. The authors also noted a striking decease in infiltrating PMNs, suggesting that β_2 integrin blockade reduced the number of infiltrating neutrophils in the acute inflammatory phase of arthritis (Jasin et al., 1992).

Immunohistochemical analysis of human RA synovial tissue sections found prominent ICAM-1 expression in the cells lining the synovium, believed to macrophages and fibroblasts (Lindsley et al., 1993). Given that ICAM-1 is a ligand for LFA-1 and the beneficial effects of β_2 integrin blockade, the rabbit AIA model was proposed to be LFA-1-dependent. This hypothesis was supported by studies using LFA-1 blockade and LFA-1 knockout mice, which highlighted

the importance of LFA-1 in the migration and recruitment of T cells, monocytes and neutrophils to sites of inflammation (Berlin-Rufenach et al., 1999, Ding et al., 1999, Mine et al., 2002). Moreover, LFA-1 blockade was also shown to reduce disease severity in the CIA and K/BxN serum transfer mouse models of arthritis (Kakimoto et al., 1992, Watts et al., 2005). Interestingly, β_2 integrin deficient mice have been found to be resistant to the development of arthritis (Watts et al., 2005).

These observations led to the development of a LFA-1 small molecule antagonist, BMS-587101, which was efficacious in *in vitro* and *in vivo* preclinical disease models (Suchard et al., 2010). BMS-587101 treatment *in vitro* inhibited human T cell proliferation, cytokine production and adhesion to ECs. *In vivo* administration of BMS-587101, in both AIA and CIA mouse models, significantly reduced disease severity either comparable to, or better than, treatment with an anti-LFA-1 antibody. AIA mice treated with BMS-587101 had lower levels of cytokine mRNA within the joints, whilst BMS-587101 treatment in the CIA model conferred a marked protection against inflammation and bone destruction (Suchard et al., 2010). LFA-1 blockade in humans was trialled, using the monoclonal antibody efalizumab, but was withdrawn after some patients developed central nervous system infections including progressive multifocal leukoencephalopathy, caused by reactivation of latent JC virus (Major, 2010).

Several reasons could account for the differences observed between mice and humans. A key difference is that experimental animals are immunologically naïve, whilst patients have been exposed to numerous pathogens. Therefore, it would have been impossible for mice to present with reactivation of JC virus, as reported in clinical studies. It is also worth consideration that LFA-1 also binds ICAM-3, an important interaction during antigen presentation (de Fougerolles and Springer, 1992). Efalizumab would therefore prevent LFA-1 interactions with not only ICAM-1, but also ICAM-3, which may suppress the early stages of antigen presentation. Reports have found that efalizumab also inhibits VLA-4 (Guttman-Yassky et al., 2008). This integrin crosstalk may contribute to the T cell hypo-responsiveness observed in clinical trials (Major, 2010). Mac-1 has also been implicated in animal models. Interestingly,

administration of the small molecule Mac-1 agonist leukadherin-1 (LA-1) reduced inflammation and leukocyte extravasation in animal models, despite Mac-1 activation (Maiguel et al., 2011).

In conclusion, increased β_1 integrin expression in T cells and FLS enhances proinflammatory cytokines production within the RA joint. LFA-1 has also been implicated, however blockade was not beneficial in patients. Due to the importance of LFA-1 in immune homeostasis and the documented integrin crosstalk, LFA-1 may not be a suitable target in RA. Further investigation of other integrins and signalling pathways may highlight novel therapeutic targets.

1.7.2 Integrins in systemic lupus erythematosus

One of the strongest susceptibility risk loci for SLE is *ITGAM*, the gene encoding the α_M subunit of Mac-1 (Nath et al., 2008, Hom et al., 2008, Harley et al., 2008, Yang et al., 2009, Han et al., 2009, Kim-Howard et al., 2010, Warchol et al., 2011). Multiple *ITGAM* gene variants have been identified, encoding point mutations in various domains of the α_M subunit, the most studied being the *rs1143679* variant. Examination of the *rs1143679*, *rs1143638* and *rs1143678* Mac-1 variants, encoding the amino acid changes R77H, A858V and P1146S respectively, found that Mac-1 function was altered (Zhou et al., 2013). Whilst there was no difference in surface expression between mutated and wild-type Mac-1, R77H neutrophils had reduced phagocytosis of IgG-opsonised sheep erythrocytes and adhesion to ICAM-1, P-selectin and TNF-α stimulated ECs (Zhou et al., 2013). These results support earlier work that found that the R77H mutation compromised phagocytosis of iC3b-opsonised sheep erythrocytes and adhesion to iC3b and ICAM-1 (MacPherson et al., 2011).

Similar observations were made in monocytes and macrophages derived from SLE patients with the R77H mutation (Rhodes et al., 2012). In agreement with previous work, there was no difference in Mac-1 expression between wild-type and R77H cells. R77H variant monocytes and macrophages displayed reduced adhesion to immobilised fibrinogen, human serum albumin, ICAM-1, iC3b and an anti-Mac-1 antibody, as well as displaying defective phagocytosis of IgM-opsonised sheep erythrocytes (Rhodes et al., 2012). The R77H Mac-1

mutation has also been studied in SLE DCs, monocytes, macrophages and neutrophils. In this study, the group only found phagocytosis of iC3b-opsonised guinea pig erythrocytes to be defective in R77H cells, whilst Mac-1 expression, adhesion and transmigration were all unaffected (Fossati-Jimack et al., 2013).

All groups agree that the R77H Mac-1 variant does not affect Mac-1 expression but leads to defective phagocytosis. There is disagreement over the effects on adhesion and transmigration. As integrin engagement of opsonised pathogens precedes phagocytosis, it is reasonable to suggest that adhesion and transmigration may also be defective in R77H cells.

To better understand these observations, Rosetti and colleagues conducted various assays to interrogate Mac-1 function, including evaluating the kinetics of ligand binding, adhesion assays and neutrophil spreading and crawling experiments (Rosetti et al., 2015). The authors reported that the R77H Mac-1 variant had impaired affinity for ligands. This impairment was not due to a reduced ability to engage with ligands, but due to reduced bond stability. The group found that the wild-type variant allows the 'force-induced' allosteric bond stabilisation required for integrin binding, which was lost in the R77H Mac-1 variant.

Taken together, substantial evidence indicates that point mutations in the α_M chain contribute to the susceptibility of developing SLE. Several studies demonstrate an association between the *rs1143679* Mac-1 variant and SLE in several populations (Nath et al., 2008, Hom et al., 2008, Harley et al., 2008, Yang et al., 2009, Han et al., 2009, Kim-Howard et al., 2010, Warchol et al., 2011). Recent work found that the R77H Mac-1 variant affects ligand affinity by reducing bond stability, thus conferring defective Mac-1 function to leukocytes.

The $\alpha_E\beta_7$ integrin has also been implicated in SLE pathology. Expression of $\alpha_E\beta_7$ is largely restricted to mucosal $CD8^+$ T cells (Cerf-Bensussan et al., 1987). Less than 2% of peripheral T cells express $\alpha_E\beta_7$ (Cerf-Bensussan et al., 1987), however expression can be induced following stimulation with antigens, cytokines, mitogens or phorbol esters (Schieferdecker et al., 1990, Parker et al., 1992). This observation suggests that the $\alpha_E\beta_7$ integrin may act as an activation marker. Examination of $\alpha_E\beta_7$ expression in healthy volunteers, SLE patients, Sjögren's syndrome patients and polymyositis/dermatomyositis patients found similar

basal expression. Following phytohaemagglutinin (PHA) stimulation, $\alpha_E\beta_7$ expression was significantly higher in SLE lymphocytes compared to healthy and disease controls (Pang et al., 1998). Within the SLE cohort, $\alpha_E\beta_7$ expression associated with oral ulcers and serositis, implicating $\alpha_E\beta_7$ in epithelial inflammation.

Stimulation of β_1 integrins induces ICAM-1 expression and Fas-mediated apoptosis in RA synovial cells (Nakayamada et al., 2003), as well as promoting T cell proliferation and IL-2 production (Ennis et al., 1993, Kamiguchi et al., 1999). Given these observations, Nakayamada et al. explored β_1 integrins in SLE. Peripheral $CD3^+$ T cells from SLE patients had significantly higher levels of the common β_1 integrin chain (CD29) compared to RA patients and healthy volunteers (Nakayamada et al., 2007). CD29 stimulation induced proliferation and CD40L expression in SLE T cells but not controls, which required crosslinking of CD29 and CD3. These results suggest that β_1 integrin stimulation is sufficient to induce SLE T cell activation and proliferation, which may promote autoreactive T cell expansion.

To conclude, both meta-analyses and experimental evidence associate defective Mac-1 activity with SLE. There is also evidence for increased β_1 integrin and $\alpha_E\beta_7$ expression and activity on T cells from patients with SLE, albeit in smaller studies that would require further work to validate findings.

1.7.3 Integrins in antiphospholipid syndrome

Increased arterial thrombotic risk in a cohort of Spanish APS patients was found to be associated with heterozygous polymorphisms in both platelet VLA-2 (glycoprotein Ia/IIa) and $\alpha_{IIb}\beta_3$ (glycoprotein IIb/IIIa) (Jimenez et al., 2008). The functional implications of these polymorphisms were not explored, however alterations in platelet integrin activity may contribution to thrombotic risk.

Integrin expression was also examined in the cardiac endothelium of heart valves obtained from APS patients or controls (Afek et al., 1999). Immunohistochemical staining found VLA-3 expression in diseased APS valves, but not in the unaffected APS or control valves. Greater expression of collagen, fibronectin and laminin was observed in the endothelial

basement membrane of diseased APS valves. These observations suggest that altered integrin and ECM expression may contribute to cardiac disease in APS.

Adhesion molecules are important in regulating trophoblast migration and invasion. Fibronectin, a constituent of the endometrial stroma, increases in concentration with decidualization during pregnancy (Aplin et al., 1988, Aplin et al., 1999). Attachment to fibronectin has been shown to induce differentiation of trophoblasts *in vitro* (Kao et al., 1988), with some evidence suggesting that adhesion to fibronectin inhibits trophoblast invasion (Damsky et al., 1994, Bischof et al., 1995). VLA-5 is an important trophoblast integrin, which is thought to be the main integrin mediating *in vitro* adhesion to fibronectin (Burrows et al., 1995) and regulating *in vivo* trophoblast invasion (Damsky et al., 1994, Bischof et al., 1995, Zeng et al., 2007). In normal placental development, trophoblasts orchestrate integrin expression to ensure successful invasion, by first upregulating VLA-5 (Coutifaris et al., 2005) but then switching to VLA-1 (Damsky et al., 1994, Zhao et al., 2012).

APS-IgG has been shown to significantly reduce both gonadotropin secretion and trophoblast invasion compared to HC-IgG (Di Simone et al., 2000). Further investigation found that APS-IgG significantly increased mRNA and protein of the α_5 subunit, whilst decreasing the α_1 subunit compared to HC-IgG (Di Simone et al., 2002). These results suggest that APS-IgG upregulates VLA-5 and supresses VLA-1, such that trophoblast invasion is impaired. Integrin dysregulation may contribute to the pathogenesis underpinning pregnancy loss in APS patients, which is supported a study demonstrating that APS-IgG reduces trophoblast invasion compared to HC-IgG (Poulton et al., 2015).

ICAM-1 expression is required for aPL-induced pregnancy loss in mice (Mo and Salmon, 2001). In this report, the authors examined foetal loss in a mouse model of pregnancy morbidity, in both wild-type and ICAM-1 knockout mice. Foetal loss was only observed in wild-type mice, suggesting that pregnancy morbidity is ICAM-1-dependent. Additional work is required to explore these observations, but given the restricted binding of ICAM-1, these results implicate β_2 integrins in APS pathology.

In conclusion, there is evidence to suggest that dysregulated integrin expression, in particular the β_1 integrins, contribute to APS pathology. Polymorphisms in VLA-2 have been reported in platelets, dysregulated expression of endothelial VLA-3 was observed in APS patients with cardiac valvulopathy, and there is differential regulation of VLA-1 and VLA-5 in trophoblasts exposed to APS-IgG. These observations suggest that β_1 integrins may contribute to APS pathophysiology.

1.8 Hypoxia and neutrophil biology

Neutrophils circulate within the vasculature before migrating into infected and inflamed tissue, so are exposed to a wide range of oxygen levels. Atmospheric air contains 20.3% oxygen (154 mmHg), which is similar to the oxygen concentrations within the pulmonary vasculature, normally at 19.7% oxygen (150 mmHg) (Volkholz et al., 1984, Caldwell et al., 2001). Within the circulation, normal oxygen levels can range between 5.0-13.2% (38-100 mmHg) (Vaupel et al., 1973, Caldwell et al., 2001). Normal oxygen levels within tissues are even lower, ranging from 0.5-2.7% oxygen (4-20 mmHg) (Braun et al., 2001). Given that normal oxygen levels within circulation and tissues can be described as hypoxic, the effects of hypoxia are important and relevant to neutrophil function under physiological conditions and in disease states.

1.8.1 Hypoxia and hypoxia-inducible factors

Hypoxia can be defined as a state where the oxygen supply to a tissue does not meet its demand. Hypoxia modulates gene expression in both unicellular and multicellular organisms (Bunn and Poyton, 1996). Studies of the haematopoietic growth factor, erythropoietin, highlighted the existence of a hypoxia-regulated transcription factor that upregulated erythropoietin expression (Semenza and Wang, 1992). Further analysis found that the oxygen-sensing system regulating erythropoietin was widespread in mammalian cells and mediated other adaptive responses to hypoxia (Maxwell et al., 1993). This hypoxia-regulated transcription factor was demonstrated to be HIF-1 (Wang et al., 1995), founding the HIF family of transcription factors. To date, 3 HIF family members have been described, composed of one of the three α subunits (HIF-1α, HIF-2α or HIF-3α) and the common HIF-1β subunit, sometimes

referred to as aryl hydrocarbon receptor nuclear translocator protein (ARNT). HIF transcription factors bind conserved 5'-[A/G]CGTG-3' DNA sequences, referred to as hypoxia responsive elements (HREs) within the genome to modulate gene expression (Mole et al., 2009).

HIF-1α was first described in 1995 as a transcription factor expressed in Hep3B human hepatoma cells when cultured at 1% oxygen, but not 20% oxygen, or by treatment with hypoxia mimetics (Wang et al., 1995). These observations implicated HIF-1α in the regulation of gene expression under hypoxia. Characterisation of HIF-1α structure highlighted four key regions: period circadian protein (PER), ARNT, and single-minded protein (SIM), which form a PER-ARNT-SIM (PAS) domain, and a basic helix-loop-helix (bHLH) domain (Wang et al., 1995) (Figure 1.11). The bHLH-PAS motif is functionally important as it enables α subunit dimerisation with HIF-1β, which allows for transcriptional regulation (Kallio et al., 1997).

Endothelial PAS domain protein-1 (EPAS-1) was described shortly after the discovery of HIF-1 (Tian et al., 1997). EPAS-1 was expressed by cells cultured under hypoxia or treated with the hypoxia mimetics cobalt chloride or desferrioxamine (Tian et al., 1997, Wiesener et al., 1998). Due to high expression in ECs, EPAS-1 was hypothesised to regulate vascularisation by modulating endothelial gene expression under hypoxia. EPAS-1 exhibited high degrees of homology to HIF-1α, containing the same bHLH and PAS domains (Figure 1.11). Moreover, HIF-1α and EPAS-1 also displayed similar patterns of mRNA and protein expression in response to hypoxia and both facilitate transactivation of erythropoietin and vascular endothelial growth factor (VEGF) (Tian et al., 1997, Wiesener et al., 1998). EPAS-1 was independently identified by three other groups, reported as HIF-like factor (Ema et al., 1997), member of PAS superfamily 2 (Hogenesch et al., 1997) and HIF-related factor (Flamme et al., 1997). EPAS-1 was therefore considered to belong to the HIF transcription factor family and was subsequently referred to as HIF-2α.

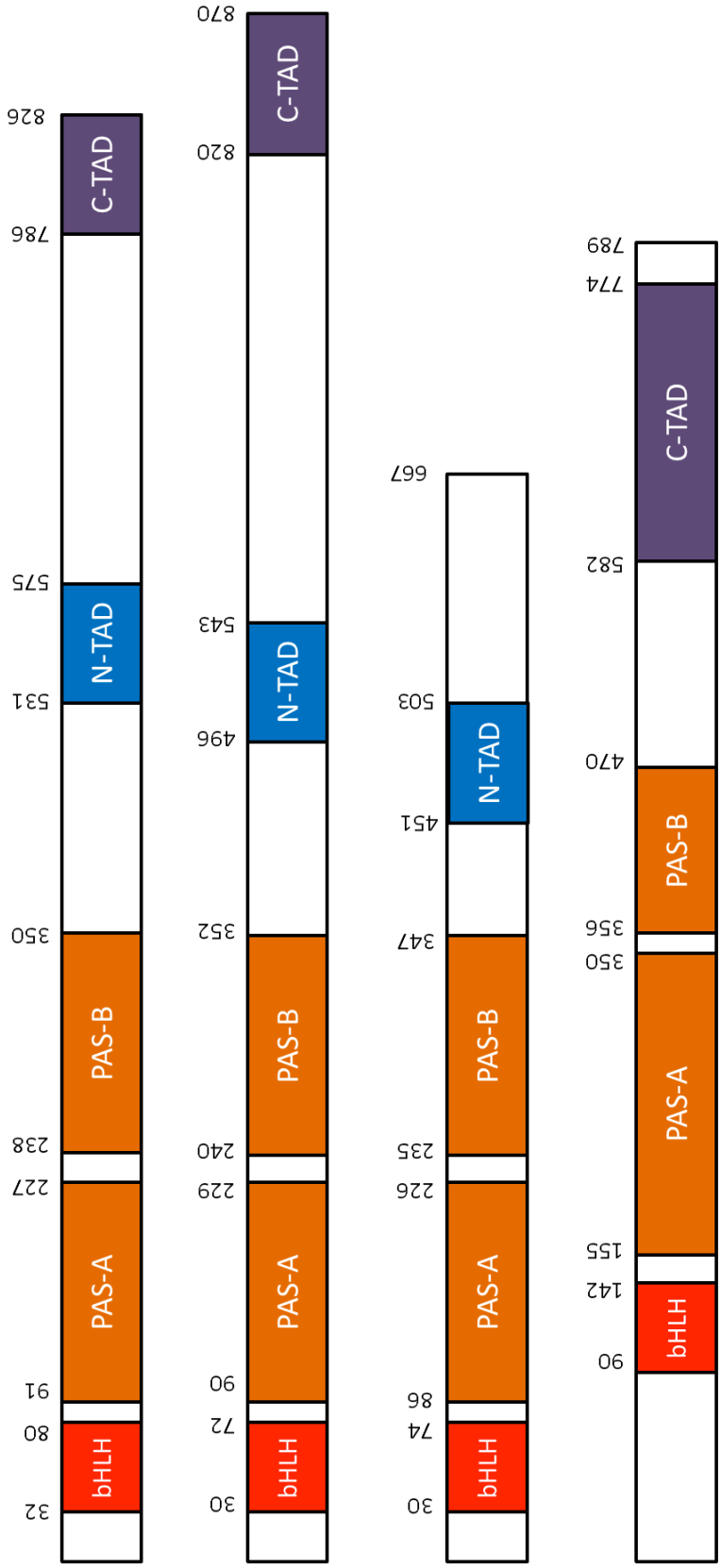


Figure 1.11: Schematic of the primary structures of the HIF transcription factors. Depicted above are the primary structures of HIF-1α, HIF-2α, HIF-3α2 (the first HIF-3 isomer described) and HIF-1β. Functionally important domains for HIF-mediated transcriptional activity have also been shown. Abbreviations: bHLH, basic helix-loop-helix domain; C-TAD, C-terminal transactivation domain; N-TAD, N-terminal transactivation domain; PAS, period circadian protein (PER), aryl hydrocarbon receptor nuclear translocator protein (ARNT), single-minded protein (SIM) domain.

HIF-2α was initially believed to modulate EC function in response to hypoxia, due to a perceived restricted expression to blood vessels (Tian et al., 1997, Wiesener et al., 1998), however HIF-2α was subsequently found to be expressed by multiple cell types (Wiesener et al., 2003). There is a general consensus that HIF-1α mediates responses to acute hypoxia (under 24 hours) and HIF-2α regulates expression under chronic hypoxic exposure (beyond 24 hours).

The final member of the HIF superfamily, HIF-3α, was characterised in 1998 (Gu et al., 1998). The precise function of HIF-3α is unclear and remains controversial. HIF-3α expresses both bHLH and PAS domains and can dimerise with HIF-1β (Gu et al., 1998). Whilst HIF-1α and HIF-2α both contain N-terminal and C-terminal transactivation domains (N-TAD and C-TAD respectively), HIF-3α only possesses a N-TAD. HIF-3α has also contains evolutionary conserved motifs and domains not found in either HIF-1α or HIF-2α (Zhang et al., 2012). Due to alternative mRNA splicing, ten different human HIF-3α isoforms have been identified to date: HIF-3α1-10 (Maynard et al., 2003, Pasanen et al., 2010).

Murine HIF-3α, inhibitory PAS domain protein (IPAS), enhances HRE-driven transcription in COS-7 cells co-transfected with both IPAS and HIF-1β (Gu et al., 1998). Studies in transfected COS-7 cells found that when HIF-3α and HIF-1β were co-transfected, HRE-driven transcription was enhanced (Hara et al., 2001). Interestingly when HIF-3α was co-transfected with HIF-1α or HIF-2α, transactivation was suppressed (Hara et al., 2001). Similar observations were made in the hepatoma cell line, Hepa 1c1c7, whereby HIF-1α and HIF-2α driven transcription was suppressed when co-transfected with IPAS (Makino et al., 2001). IPAS also suppresses hypoxia-induced VEGF expression in primary mouse corneal epithelial cells (Makino et al., 2001). Mice implanted with IPAS-expressing hepatoma cells had significantly reduced tumour growth compared to control, suggesting IPAS could suppress hypoxia-driven tumour growth (Makino et al., 2001). An additional murine HIF-3α variant, neonatal and embryonic PAS protein (NEPAS) has been implicated in embryonic growth. Abnormal heart development and impaired lung remodelling was observed in NEPAS knockout mice, as well as increased expression of endothelin-1 and platelet-derived growth factor (PDGF)-β in pulmonary endothelium (Yamashita et al., 2008).

Similar observations have been reported in human cells, with human HIF-3α4 shown to be a dominant negative regulator of HIF-1 signalling (Maynard et al., 2005). HIF-3α4 prevents HIF-1 binding to HREs, thus preventing HRE-driven transcription. Interestingly, hypoxia suppresses HIF-3α4 expression in both HepG2 hepatoma and HEK293A cells, suggesting that HIF-3α4 itself may be under hypoxic regulation (Maynard et al., 2005). Other groups also provide evidence that HIF-3α splice variants are regulated by HIF-1α (Pasanen et al., 2010). HIF-3α4 has also been shown to impair angiogenesis and proliferation in human hypervascular meningioma cell lines (Ando et al., 2013). Recent reports found that HIF-3α9 activates gene expression distinct from HIF-1α in zebrafish embryos, which was replicated *in vitro* using human U2 osteosarcoma and HEK293T cells transfected with HIF-3α9 (Zhang et al., 2014).

HIF activity is regulated by the post-translational modification of the α subunit. Under normoxia the α subunit is targeted for degradation, whilst in hypoxia there is rapid cytosolic stabilisation of the α subunit (Kaelin and Ratcliffe, 2008, Majmundar et al., 2010). This regulation is achieved by the combined activity of prolyl-hydroxylases (PHDs) and factor inhibiting HIF-1α (FIH1). Under normoxia, PHDs hydroxylate conserved proline residues, Pro402 and Pro564, within the α subunit N-TAD (Ivan et al., 2001, Jaakkola et al., 2001, Masson et al., 2001, Yu et al., 2001). PHD-mediated hydroxylation is achieved via the coupling with the oxidative carboxylation of the tricarboxylic acid (TCA) cycle intermediate, 2-oxoglutarate, into succinate and carbon dioxide in the presence of oxygen. The modified subunit is then recognised by the E3 ubiquitin ligase, von-Hippel-Lindau tumour suppressor, which is ubiquitinated and targeted for proteasomal degradation (Figure 1.12).

In hypoxic environments, low levels of oxygen result in the accumulation of TCA cycle intermediates, other than 2-oxoglutarate, which inhibit the catalytic activity of PHDs (Selak et al., 2005). Studies suggest that when subjected to 1.5% oxygen (11.4 mmHg) or lower, mitochondria produce ROS that further suppress PHD activity (Klimova and Chandel, 2008). PHD inactivation prevents the ubiquitination and degradation of the HIF α subunit, enabling its stabilisation within the cytosol. The HIF α subunits are then trafficked to the nucleus and dimerise with HIF-1β. HIF transcription factors can then bind HREs and recruit transcriptional

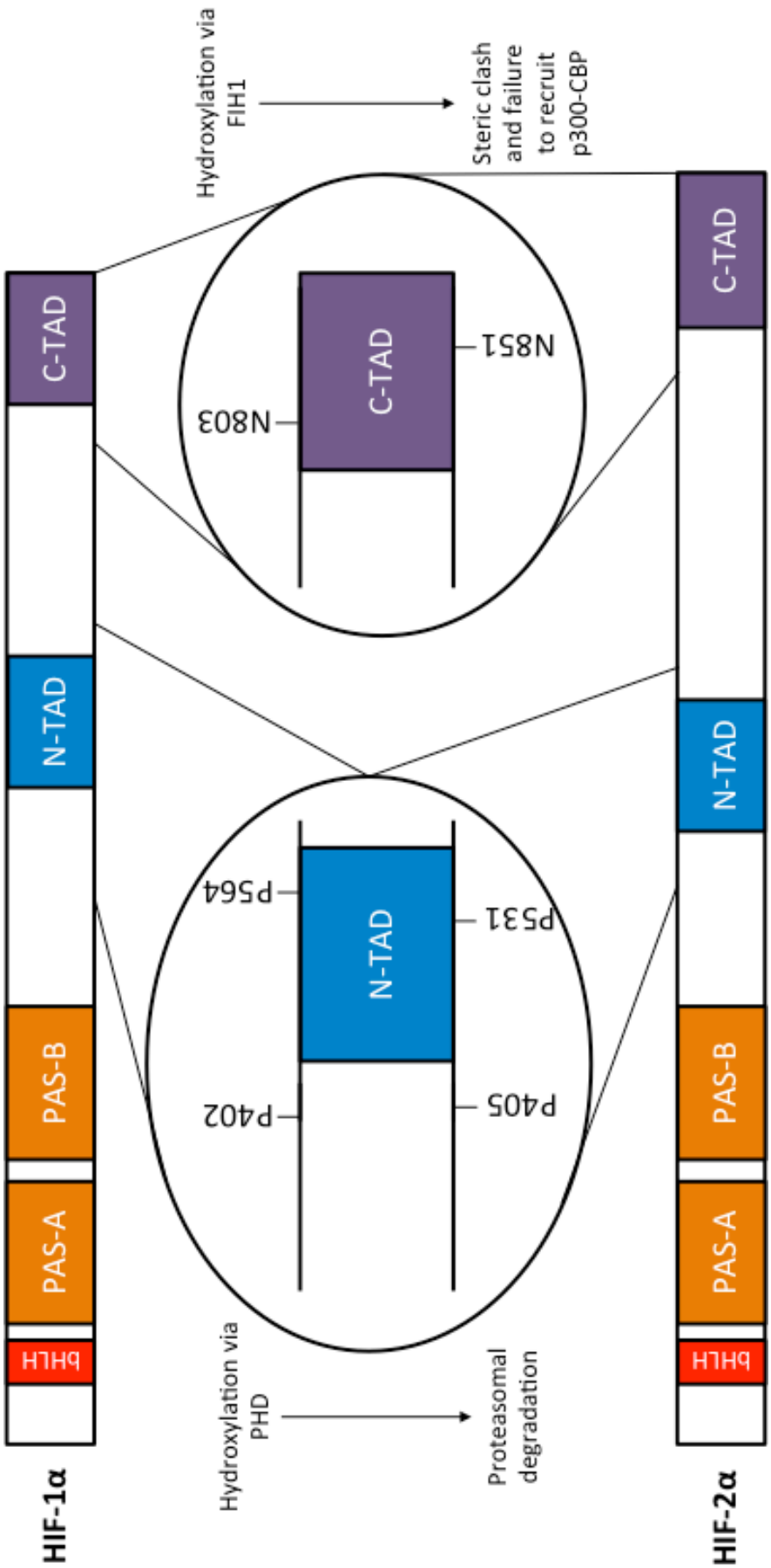


Figure 1.12: Oxygen-dependent hydroxylation of key residues regulates HIF-1α and HIF-2α activity. Both HIF-1α and HIF-2α are regulated by oxygen-dependent enzymes that prevent their activity under normal oxygen levels. PHDs hydroxylate proline residues located localised to N-TAD region, which targets the protein for ubiquitation and proteasomal degradation. In contrast, FIH1 hydroxylates asparagine residues in the C-TAD, which induces a steric clash that prevents the recruitment of p300-CBP, ultimately inhibiting HIF transcriptional activity.

co-activators, including p300-CREB-binding protein (CBP) to modulate gene expression.

FIH1 is also dependent on 2-oxoglutarate and hydroxylates Asn803 on HIF-1α or Asn851 on HIF-2α, located within their C-TADs (Lando et al., 2002, McNeill et al., 2002) (Figure 1.12). Asparagine hydroxylation introduces a steric clash that prevents p300-CBP recruitment and suppresses transactivation (Hewitson et al., 2002). Reports found FIH1 remains active at higher oxygen levels than PHD, so may prevent HIF activity during milder levels of hypoxia (Dayan et al., 2006). HIF-1α is readily suppressed by FIH1, however HIF-2α displays a degree of resistance to FIH1-mediated inactivation (Bracken et al., 2006).

It is important to consider the physiological oxygen gradient from the lungs to tissues. Jiang et al. examined HIF-1α and HIF-1β in nuclear extracts, as well as HIF-1 DNA-binding activity in HeLa S3 cells exposed to 0-20% oxygen (0-152 mmHg) (Jiang et al., 1996). Significant increases in HIF-1α protein and DNA-binding activity, thus HRE-driven transcription, in nuclear extracts were only observed when oxygen levels reached 5% (38 mmHg) or lower. As oxygen levels can fall to 5% within the circulation and even lower in tissues, HIF signalling is an important factor in many cellular interactions. Furthermore, as hypoxia is a feature of several inflammatory diseases, the effects of hypoxia upon cell functions may contribute to pathophysiology.

1.8.2 Neutrophil function and hypoxia

A fine balance exists in the regulation of neutrophil survival. If neutrophil survival is prolonged, resolution of inflammation is delayed and there can be damage to surrounding cells and tissues, whilst if neutrophils undergo apoptosis too soon, they cannot mediate their antimicrobial functions (Savill, 1997, Rossi et al., 2006). As neutrophils migrate into tissues with hypoxic environments, it is important to consider the effects of hypoxia on neutrophil function. Hypoxia inhibits neutrophil apoptosis and promotes survival via HIF-1α-dependent NF-κB activation (Walmsley et al., 2005, Cross et al., 2006). As hypoxia drives neutrophil survival, the modulatory role of hypoxia upon other neutrophil functions is physiologically relevant and important in understanding disease pathology.

The precise effect of hypoxia upon neutrophil function is not fully understood. Comparisons between published reports is made difficult by the fact these studies have employed different oxygen levels, experimental conditions and methods of neutrophil isolation. In neutrophil isolation, choice of anticoagulant affects both neutrophil yields and *ex vivo* function. For example, the highest yields of neutrophil isolation are achieved with EDTA-treated blood, however responses to PMA are reduced in neutrophils isolated from EDTA-treated blood compared to citrate- and heparin-treated blood (Haslett et al., 1985, Freitas et al., 2008). Therefore, variations in the anticoagulant used between studies may influence functional readouts.

Overall, reports suggest neutrophil function is generally enhanced under hypoxia. A study subjecting healthy volunteers to systemic hypoxaemia, defined by an arterial oxygen saturation of 68%, found that neutrophils from hypoxaemic volunteers had enhanced NE release on fMLP stimulation compared to normoxic volunteers (Tamura et al., 2002). In a similar study, healthy volunteers were exposed to oxygen concentrations ranging from 12-20% oxygen (91.2-152 mmHg) for 2 hours and neutrophil function examined. Neutrophils isolated from the volunteers subjected to 12% oxygen had significantly enhanced chemotaxis, respiratory burst and phagocytosis compared to volunteers exposed to 20% oxygen (Wang and Liu, 2009).

Evidence suggests that phagocytosis is elevated in hypoxia. Neutrophils isolated from hypoxaemic blood (5.0% oxygen, 38 mmHg) has significantly higher levels of phagocytosis compared to normoxic controls (Simms and D'Amico, 1994). Moreover, isolated neutrophils cultured under hypoxia (2.9% oxygen, 22 mmHg) display enhanced phagocytosis compared to normoxic controls (18.8% oxygen, 142.5 mmHg) (Walmsley et al., 2006).

Early reports found neutrophil migration was inhibited under hypoxia (3.9% oxygen, 30 mmHg) compared to normoxia (20.0% oxygen, 152 mmHg) (Rotstein et al., 1988). More recently, Wang and Lui provided evidence that chemotaxis was enhanced by hypoxia (12.0% oxygen, 91.2 mmHg) (Wang and Liu, 2009). McGovern et al. however, found hypoxia (3.0% oxygen, 22.5 mmHg) had no effect on neutrophil transmigration (McGovern et al., 2011). These

discrepancies are likely to be due to a combination of differing isolation techniques, experimental oxygen levels and chemoattractants used.

Other studies have shown that hypoxia causes defective respiratory burst in neutrophils. McGovern et al. found that superoxide generation by both fMLP- and PMA-stimulated neutrophils was reduced under hypoxia (3% oxygen, 22.5 mmHg) compared to normoxia (20% oxygen, 152 mmHg), whilst NADPH oxidase expression, a key enzyme for respiratory burst, was not affected (McGovern et al., 2011). This observation may indicate that whilst expression of NADPH may not be oxygen sensitive, its activity may be regulated by hypoxia.

Conflicting evidence may arise due to the broad use of the term 'hypoxia' to describe oxygen levels below 20% oxygen. There are differences between cells cultured in 1% oxygen and those cultured in 12% oxygen, emphasised by work demonstrating that HRE-mediated binding is only detected at 5% oxygen and lower (Jiang et al., 1996). Variations in anticoagulation and isolation techniques also contribute to differences. Careful consideration should be taken when drawing conclusions of the effects of hypoxia upon neutrophil function.

1.8.3 Hypoxia and neutrophil extracellular trap formation

Relatively little work has examined the effects of hypoxia upon NETosis. McInturff et al. implicate HIF-1α as a regulator of NETosis (McInturff et al., 2012). This group found that both LPS and platelet-activating factor (PAF) activated mTOR, which induced HIF-1α stabilisation under normoxia and mediated NETosis. Inhibition of either HIF-1α, with 2-methoxyestradiol, or mTORC1, with either rapamycin or the mTORC1 selective inhibitor torin-1, reduced NETosis. These findings suggest that HIF-1α regulates NET production.

Increased bactericidal capacity was also reported in phagocytes treated with the HIF-1α agonist mimosine (Zinkernagel et al., 2008). This group measured killing of *Staphylococcus aureus* by all phagocytes, isolated neutrophils and the human monocytic U937 cell line in the presence of varying concentrations of mimosine. Greater mimosine concentrations induced higher levels of HIF-1α and fewer surviving *S. aureus*, suggesting HIF-1 signalling may influence bacterial killing. Whilst the authors were unable to demonstrate differences in the

levels of NETs between untreated and mimosine-treated neutrophils, increased bactericidal activity was mitigated by DNase I treatment, implicating a NET-dependent mechanism.

1.9 Hypoxia in autoimmune rheumatic disease

Hypoxia is a potent cell stimulus that induces transcriptional change and modulates protein expression. Hypoxia has been implicated to varying degrees in RA, SLE and APS pathogenesis. This section will evaluate the evidence relating to hypoxia in each disease.

1.9.1 Hypoxia and rheumatoid arthritis

Various methodologies have independently demonstrated that the RA synovial joint is hypoxic. Early investigations using microelectrodes found lower oxygen tensions in RA SF (3.5 ±2.5% oxygen, 26.5 ±19.3 mmHg) compared to control (8.3 ±2.5% oxygen, 63.0 ±19.2 mmHg) (Lund-Olesen, 1970). Additional groups have independently published results demonstrating the hypoxic nature of the RA synovial joint, finding increased carbon dioxide tensions (Richman et al., 1981, James et al., 1990), raised lactate (Goetzl et al., 1971, James et al., 1990), reduced glucose values (Richman et al., 1981) and acidosis (Geborek et al., 1989, James et al., 1990) in RA joints compared to control.

More recently, Sivakumar et al. replicated these findings by directly measuring the partial pressure of oxygen using microelectrodes in the joints of RA patients and non-RA controls undergoing hand surgery (Sivakumar et al., 2008). Intra-operative matched *in vivo* synovial measurements were made for both affected and unaffected joints. RA joints were significantly more hypoxic than controls, with an average reading of 26 mmHg (3.4% oxygen) and 74 mmHg (9.7% oxygen) respectively (Sivakumar et al., 2008). This group also suggested that disease activity affects oxygen tension. The mean oxygen tension for unaffected RA joint synovia was 46 mmHg (6.1% oxygen), however the oxygen tension of joints with evidence of synovitis was significantly lower, with a mean of 40 mmHg (5.3% oxygen) (Sivakumar et al., 2008). Interestingly RA joints with severe synovitis, as determined by direct visualisation at surgery, were even more hypoxic, with a mean oxygen tension of 26 mmHg (3.4% oxygen)

(Sivakumar et al., 2008). Synovial hypoxia has been shown to promote VEGF expression, angiogenesis and cell migration (Sivakumar et al., 2008, Akhavani et al., 2009).

Under the hypoxic environment of the synovial joint, mitochondria generate ROS (Klimova and Chandel, 2008). Early studies found that ROS activated osteoclasts and stimulated bone resorption both *in vitro* and *in vivo* (Garrett et al., 1990). Hydroxyl radicals are able to target and degrade hyaluronic acid (Grootveld et al., 1991), which decreases synovial lubrication and contributes to synovitis. Therefore, ROS generated under hypoxia drive hyaluronic acid degradation, which is supported by early reports demonstrating lower hyaluronic acid content in RA SF (1.15 ±0.37mg/ml) compared to controls (3.21 ±0.38mg/ml) (Decker et al., 1959). Hydroxyl radicals also modify amino acids, so may facilitate generation of autoantibodies targeting modified proteins (Bodamyali et al., 1998, Chandel et al., 2000).

T cell function is affected by oxidative stress. Early work found that antioxidant depletion suppresses T cell proliferation, IL-2 secretion and IL-2 receptor expression (Chaudhri et al., 1988). Hypoxia also upregulates IL-1 and TNF-α, whilst suppressing IL-2 expression (Ghezzi et al., 1991), suggesting that hypoxia may modulate T cell function.

Hypoxia and cellular stress have been shown to increase expression and activity of PAD-2 in astrocytes (Sambandam et al., 2004, Algeciras et al., 2008). A significant increase in PAD-2 mRNA was seen within 2 hours of hypoxic exposure (2% oxygen, 15.2 mmHg), translating to elevated PAD-2 protein by 8 hours of hypoxia (Sambandam et al., 2004). Increased glial fibrillary acidic protein citrullination was also reported, demonstrating increased PAD-2 activity (Sambandam et al., 2004). Given the importance of PAD-2 and PAD-4 in generating citrullinated proteins, pattern of expression and hypoxic regulation of PAD-2 in astrocytes, the hypoxic RA joint may favour the generation of citrullinated autoantigens through the activation of PAD-4.

Hypoxia has also been found to increase the production of various inflammatory cytokines, MMPs and VEGF by synovial fibroblasts, which promoted recruitment of monocytes, B cells and T cells to the RA synovium (Hu et al., 2014). Interestingly, HIF-1α stabilisation was found to enhance synovial fibroblast-mediated expansion of Th1 and Th17

cells that lead to elevated levels of both IFN-γ and IL-17 (Hu et al., 2014), which contribute to the proinflammatory environment of the RA synovium.

Taken together, hypoxia may contribute to RA pathology in a number of ways. Low oxygen levels promote the recruitment of inflammatory cells, skew T cell polarisation to favour TNF-α secretion, promote ROS generation that can induce post-translational modifications, which may facilitate the generation autoantigens.

1.9.2 Hypoxia and systemic lupus erythematosus

The role of hypoxia in SLE pathophysiology is not as well studied as in RA. HIF-1α signalling has been implicated in mesangial cell proliferation in patients with lupus nephritis. Using a renal pathology activity index defined to help refine prognosis for SLE patients with diffuse proliferative or membranoproliferative glomerulonephritis (Austin et al., 1984), greater levels of HIF-1α were found to associate with increased mesangial cell proliferation, more severe degrees of renal pathology and clinical manifestations (Deng et al., 2014). The enhanced mesangial cell proliferation observed in patient samples could be replicated in murine mesangial cells by treatment by the PHD inhibitor dimethyloxaloylglycine (DMOG) (Deng et al., 2014), implicating HIF-1α signalling in the pathology of lupus nephritis.

1.9.3 Hypoxia and antiphospholipid syndrome

APS is characterised by the persistent presence of aPL that induce a pro-thrombogenic state. These aPL exist within the circulation at 5.0-13.2% oxygen (38-100 mmHg) (Vaupel et al., 1973, Caldwell et al., 2001), without inducing any clinical thrombotic events, but require a second hit to elicit an effect. Therefore hypoxia in venous stasis or tissue surrounding an unstable plaque may enhance aPL-mediated effects.

Hypoxia and HIF-mediated signalling regulate placental morphogenesis, angiogenesis and cell fate decisions (Adelman et al., 2000). Placental development is initiated when the blastocyst makes contact with the epithelial lining of the uterus following implantation. Placental villi subsequently develop, which consist of a mesenchymal core surrounded by a mononuclear villous cytotrophoblast stem cell monolayer. These stem cells either fuse to form

the overlying multinucleated syncytiotrophoblast or differentiate into extravillous trophoblasts, which grow out from the villous and spread laterally around the placenta (Irving et al., 1995). As extravillous trophoblasts migrate away from the placenta, they differentiate into an invasive phenotype in a process that is essential for successful placental development and pregnancy.

Extravillous trophoblast invasion into the walls of the uterine spiral arteries is essential in adapting these vessels to become capable of delivering the increased blood supply required during the second and third trimesters of pregnancy (Brosens et al., 1967, Zhou et al., 1997). As these extravillous trophoblasts invade the spiral arteries in early pregnancy, they occlude the arteries and prevent maternal blood from entering the intervillious space, thereby creating an environment of physiological hypoxia (Jaffe et al., 1997, Burton et al., 1999). Direct measurements of oxygen tension also demonstrate the hypoxic nature of the early placenta. From 8-10 weeks of gestation, the partial pressure of oxygen within the placenta (17.9 mmHg, 2.4% oxygen) was significantly lower than that of the endometrium (39.6 mmHg, 5.2% oxygen) (Rodesch et al., 1992).

Studies examining murine placental cell fates found that HIF-1α and HIF-2α regulate trophoblast differentiation (Cowden Dahl et al., 2005). In this study, placentas from mice deficient of HIF-1α and HIF-2α or HIF-1β exhibited defective placental vascularisation and aberrant cell fate adaption. This group also showed that in the absence of either HIF-1α or HIF-1β, trophoblast stem cell differentiation is also disrupted. Therefore, as trophoblast invasion and differentiation within the first trimester of pregnancy occur under physiological hypoxia, HIF signalling may contribute to obstetric complications associated with APS in early pregnancy. Clinical observations also implicate hypoxia in the pathogenesis of certain obstetric manifestations of APS (Branch, 1994, Galli and Barbui, 2003).

Hypoxia is a feature in animal models of APS, in particular *in vivo* ischemia/reperfusion (I/R) experiments to model myocardial infarction or stroke. In a mouse model of mesenteric I/R-induced injury, CR2 deficiency conferred resistance to tissue damage, as reported in *Cr2*$^{-/-}$ mice (Fleming et al., 2004). In this experiment infusion of purified mouse or human aPL-IgG before I/R injury was administered, reconstituted I/R-induced intestinal and lung tissue damage

in *Cr2*$^{-/-}$ mice compared with control IgG, which lacked this effect. Similar observations were made in a rat model of I/R stroke injury, in which aPL-IgG exacerbated stroke severity post I/R injury and caused larger infarct compared to control IgG (Pericleous et al., 2014). These results demonstrate that circulating aPL may enhance cellular and tissue damage following the hypoxic insult caused by I/R injury.

1.10 Introduction summary

There is a growing awareness of the importance of neutrophils in ARD pathology, including RA, SLE and APS. Neutrophil integrin activation initiates numerous signalling cascades and mediates cell functions including transmigration, cytokine production, ROS generation and NETosis. Hypoxia is known to modulate several aspects of neutrophil biology and may also contribute to the pathology of RA, SLE and APS. The effects however, of hypoxia on neutrophil integrin activation and NETosis in these ARDs are not fully understood. In particular, although purified IgG from RA, SLE and APS patients have been shown to modulate aspects of neutrophil activation, their effects upon integrin activation, ROS generation and NETosis in hypoxia remains poorly characterised.

1.11 Hypothesis and aims of this thesis

My hypothesis is that hypoxia and purified IgG, from patients with ARDs, promote neutrophil integrin activation, ROS generation and NETosis. I have tested this hypothesis, with the following aims to examine:

- The effects of hypoxia on integrin activation and neutrophil adhesion
- The effects of hypoxia on ROS generation and NETosis
- The effects of purified IgG upon neutrophil adhesion and activation

Chapter Two:
Materials and Methods

2.1 Materials

2.1.1 General materials, equipment and buffers

The general equipment and materials used for experimental procedures throughout this thesis are listed in appendix I. In addition, the formations of all experimental buffers are detailed in appendix II.

2.1.2 Antibodies for ELISAs

Optimised enzyme-linked immunosorbent assay (ELISA) antibodies dilutions can be found in Table 2.1. Capture antibodies were diluted in phosphate-buffered saline (PBS) and coated overnight at 4^{o}C.

Capture Antibody	Host	Concentration	Company
Human IgG Fc	Goat	400ng/ml	Millipore
MPO	Mouse	1µg/ml	Abcam
Detection Antibody	**Host**	**Concentration**	**Company**
Citrullinated histone H3	Rabbit	1µg/ml	Abcam
HRP-conjugated Secondary Antibody	**Host**	**Dilution**	**Company**
Human Ig	Goat	1:2000	Sigma
Rabbit Ig	Goat	1:500	Dako

Table 2.1: Optimised antibody concentrations used for ELISA experiments. Detailed above are the immunogenic targets, host species and optimised working concentrations of all antibodies used for ELISA experiments throughout this thesis.

2.1.3 Antibodies for flow cytometry

Fluorochrome-conjugated antibodies were used at optimal dilutions in sodium HEPES buffer. Antibodies used for all flow cytometry experiments can be found in Table 2.2. Appropriate isotype controls were used from the same company for each antibody to determine background fluorescence.

Immunogen	Isotype	Clone	Fluorochrome	Company
CD11a (α_L)	IgG2a	38	AlexaFluor-647	AbD Serotec
CD11b (α_M)	IgG2b	M1/70.15.11.5	Vio-Blue	Miltenyi Biotec
CD11c (α_X)	IgG1	B-ly6	FITC	BD Biosciences
CD15	IgG1	W6D3	PE	BD Biosciences
CD18 (β_2)	IgG1	TS1/18	FITC	Miltenyi Biotec
CD29 (β_1)	IgG2a	HUTS-21	PE	BD Biosciences
CD49a (α_1)	IgG1	SR84	PE	BD Biosciences
CD49d (α_4)	IgG1	9F10	PE-Cy™5	BD Biosciences
CD49e (α_5)	IgG1	IIA1	PE	BD Biosciences
CD54 (ICAM-1)	IgG1	HCD54	AlexaFluor-647	Biolegend
CD62E (E-selectin)	IgG1	68-5H11	PE	BD Biosciences
CD102 (ICAM-2)	IgG2a	CBR-IC2/2	FITC	Biolegend
CD106 (VCAM-1)	IgG1	51-10C9	PE	BD Biosciences

Table 2.2: Fluorochrome-conjugated antibodies used for flow cytometry. Detailed above are the immunogenic targets, antibody isotypes, clones and conjugated fluorochromes of all antibodies used for flow cytometry throughout this thesis.

2.1.4 Antibodies for functional blocking

Details of the integrin-specific functional blocking antibodies used in this thesis can be found in Table 2.3. Antibodies were titrated and used at optimal concentrations.

Immunogen	Integrin Blocked	Isotype	Clone	Company
CD11a (α_L)	LFA-1	IgG2a	38	Gift from Nancy Hogg (Crick Institute, Lincoln Inns Field, London)
CD11b (α_M)	Mac-1	IgG1	2LPM19c	Santa Cruz Biotechnology
CD18 (β_2)	LFA-1 and Mac-1	IgG1	TS1/18	Gift from Nancy Hogg (Crick Institute, Lincoln Inns Field, London
CD29 (β_1)	VLA-1,-2,-3,-4 and -5	IgG1	P5D2	Santa Cruz Biotechnology
CD51/CD61 ($\alpha_V\beta_3$)	$\alpha_V\beta_3$	IgG1	LM609	Millipore

Table 2.3: Functional blocking antibodies. Detailed above are the immunogenic targets, antibody isotypes and clones of all functional blocking antibodies used throughout this thesis.

2.1.5 Antibodies for immunoblot

All antibodies were diluted in 5% w/v skim milk/tris-buffered saline (TBS)/0.1% Tween-20 for immunoblot, using the dilutions stated in Table 2.4. Horseradish peroxidase (HRP)-conjugated secondary antibodies were also diluted in 5% w/v skim milk/TBS/0.1% Tween-20 using matched dilutions to respective primary antibodies.

Immunogen	Host	Concentration	Company
Citrullinated histone H3	Rabbit	1µg/ml	Abcam
GAPDH	Mouse	5ng/ml	Millipore
HIF-1α	Mouse	250ng/ml	BD Biosciences
HIF-2α	Rabbit	Concentration not provided. Used in a 1:1000 dilution	Cell Signalling
MPO	Rabbit	Concentration not provided. Used in a 1:1000 dilution	Cell Signalling
PAD-4	Goat	500ng/ml	Abcam
β-tubulin	Mouse	50ng/ml	Millipore
HRP-conjugated secondary antibodies			
Goat Ig	Rabbit	Primary antibody dependent	Dako
Rabbit Ig	Goat	Primary antibody dependent	Dako
Mouse Ig	Rabbit	Primary antibody dependent	Dako

Table 2.4: Optimised immunoblot antibody concentrations. Detailed above are the immunogenic targets, host species and optimised concentrations or dilutions of all antibodies used for immunoblot throughout this thesis.

2.1.6 Primary human cells

Human umbilical cord endothelial cells (HUVEC) from pooled donors were purchased from Lonza (Lonza Group Ltd., Switzerland). Cells were purchased at passage 2 (P-2) and

cultured in endothelial basal media (EBM)-2 supplemented with 10% foetal calf serum (FCS) (Gibco, UK), 2mM L-glutamine (Gibco, UK) and growth factors provided in the endothelial growth media (EGM)-2 SingleQuot kit (Lonza Group Ltd., Switzerland). Cells were cultured in plastic 80cm^2 nunclon-treated flasks (VWR International, UK) at a cell density of 2500-5000 cells/cm^2 and allowed to grow to confluency at 37^{o}C in a humidified atmosphere of 5% CO_2 in air. Once 70-80% confluent, the media was aspirated and the cells were washed twice with PBS (Thermo Scientific, UK). Warmed 0.05% trypsin/EDTA (Life Technologies, UK) was added to flasks and incubated for 3 minutes at 37^{o}C, before addition of 10ml EGM-2 growth media to inactivate trypsin. A cell pellet was obtained by centrifugation at 170g for 5 minutes, which was resuspended in 10ml EGM-2 growth media. HUVEC were then seeded into either 80cm^2 tissue culture flasks for further subculture or multi-dishes for experimentation. All HUVEC were used by P-5 for all experimental procedures.

Human neutrophils were isolated from citrated venous blood obtained by informed consent from patients or healthy volunteers (UCL project ID: 13/LO/0900). Isolated neutrophils were resuspended in phenol-free Roswell Park Memorial Institute (RPMI) 1640 supplemented with 10% FCS (heat inactivated to 80^{o}C) and 2mM L-glutamine and cultured at 37^{o}C in a humidified atmosphere of 5% CO_2 in air. All neutrophils were used within 24 hours of isolation.

Human T cells were isolated from citrated venous blood obtained by informed consent from patients or healthy volunteers. T cells were resuspended in RPMI 1640 supplemented with 10% FCS, 2mM L-glutamine and 20ng/ml IL-2 (Gibco, UK), which were cultured at 37^{o}C in a humidified atmosphere of 5% CO_2 in air.

For all experiments exploring the effects of hypoxia, normoxia was defined as 21% oxygen (equivalent to 159.6 mmHg) and hypoxia as 1% oxygen (equivalent to 7.6 mmHg).

2.1.7 Recombinant proteins

Fibrinogen (1mg/ml; Sigma, UK), IL-2 (20µg/ml, Gibco, UK) LPS (1mg/ml; Sigma, UK) and TNF-α (10µg/ml; R&D Systems, UK) were purchased as lyophilised powders and reconstituted as suggested by the manufacturer to the concentrations stated. Proteins were

aliquoted and stored at -20°C until required. ICAM-1-Fc (3mg/ml) was given as a generous gift from Nancy Hogg (The Francis Crick Institute, London). Fibronectin (1mg/ml; Sigma, UK) was purchased as a 0.1% solution and stored at 4°C until required.

2.1.8 Inhibitors, stimuli and other reagents

2', 7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein acetoxymethyl ester (BCECF-AM) (1mM; Life Technologies, UK), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (5mg/ml; Sigma, UK) and PMA (2mM; Sigma, UK) were purchased as lyophilised powders and reconstituted to the concentrations stated. BCECF-AM and PMA were reconstituted using DMSO, whilst MTT was dissolved in distilled water and filter-sterilised. All compounds were aliquoted and stored at -20°C until required. Dextran (Sigma, UK) was purchased and dissolved as a 6% dextran solution in 0.9% sterile saline, which was filter-sterilised and stored at 4°C.

2.2 Methods

2.2.1 Isolation of human peripheral blood cells

Peripheral blood neutrophils and T cells were isolated from venous blood via density centrifugation. 50ml conical centrifuge tubes were layered with 10ml of 6% dextran/0.9% saline solution (at room temperature), to which 20ml PBS and 20ml citrated whole blood were then layered on top and the RBCs allowed to sediment for 45 minutes. The cell-rich plasma layer was transferred to a fresh centrifuge tube and PBS added to a total volume of 50ml. Following centrifugation at 300g for 5 minutes, supernatants were discarded and cells isolated by Percoll density separation, which separates neutrophils from PBMCs based upon their cellular density. To achieve the required Percoll gradient, 27ml of Percoll PLUS (Sigma, UK) was mixed with 3ml of 10xPBS to obtain a 100% Percoll PLUS stock solution. This stock solution was used to prepare 55%, 67% and 81% Percoll PLUS in PBS solutions.

A 4ml aliquot of the 67% Percoll PLUS solution was then carefully layered on top of 4ml 81% Percoll PLUS solution in a 15ml conical centrifuge tube. The previous cell pellet was

resuspended in 4ml of the 55% Percoll PLUS solution. This cell suspension was then carefully layered on top of the Percoll gradient and allowed to separate by centrifugation at 700g for 30 minutes without the break (Figure 2.1). Cells were then isolated as described below.

2.2.1.1 Neutrophils

After separation, the PBMC layer between the 55% Percoll PLUS/67% Percoll PLUS interface was transferred to a fresh 50ml centrifuge tube and processed as described in 2.2.1.2. The neutrophil-enriched layer between the 67% Percoll/81% Percoll PLUS interface was transferred to a fresh 50ml centrifuge tube. The isolated cells were then washed with PBS. If there was a visible RBC contamination, the pellet was resuspended in water for 30 seconds to allow for isotonic RBC lysis. Neutrophils were subjected to centrifugation at 300g for 5 minutes and resuspended for experimental procedures. All cells were used within 24 hours of isolation.

2.2.1.2 T cells

Once transferred into a fresh 50ml centrifuge tube, PBMCs were washed twice with RPMI. PBMCs were resuspended in RPMI/10% FCS at $2.5x10^5$ cells/ml. T cells were expanded by addition of PHA (Gibco, UK) to 1% v/v in RMPI/10% FCS for 72 hours. T cells were then washed and resuspended in RPMI/10% FCS supplemented with 20ng/ml IL-2 at $2.5x10^5$ cells/ml. Cells were allowed to grow until $1x10^6$ cells/ml, at which point they were considered confluent and expanded. T cells were maintained for 1-2 weeks and used between days 10-14.

2.2.2 Determination of cell viability – MTT assay

Cell viability was assessed via the colorimetric reaction of MTT, which is cleaved by mitochondrial succinate dehydrogenase to give a water-insoluble blue precipitate. The colour produced is dependent on cell number and metabolic activity, so only accounts for viable cells.

For HUVEC, $1.5x10^5$ cells were seeded into 96-well tissue culture plates. Stock MTT reagent was diluted to 1mg/ml in culture media and filter-sterilised. Following incubation, media was removed and 50µl of the 1mg/ml MTT working solution was added to wells. Following incubation for 1 hour at 37^{o}C, MTT was removed and 100µl isopropanol was added

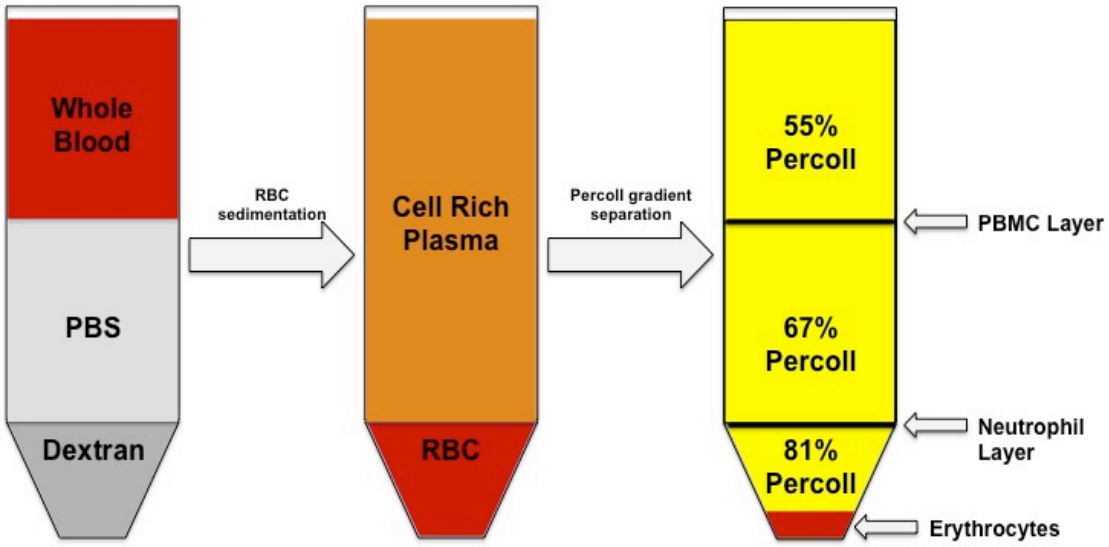


Figure 2.1: Percoll density separation of whole blood. Citrated whole blood was layered upon PBS and 6% dextran/0.9% saline, with red blood cells allowed to sediment for 45 minutes. Cells were obtained by centrifugation of the cell-rich plasma and resuspended in 55% Percoll. This cell suspension was layered upon a Percoll gradient and subjected to centrifugation to allow for density separation. Cells were isolated and processed further. Abbreviations: PBMC, peripheral blood mononuclear cell; RBC, red blood cells.

to wells. The plate was incubated for 10 minutes at room temperature with gentle shaking to solubilise the blue precipitate. Absorbance was measured at 560nm using a Tecan GENios Spectra FLUOR plate reader (Tecan UK Ltd., UK).

For neutrophils, $3.5x10^5$ cells were added to round-bottomed 96-well tissue culture plates. Following incubation, cells were subjected to centrifugation at 350g for 5 minutes. Media was removed and 50µl of the 1mg/ml MTT working solution added and incubated for a further hour at 37°C. After incubation, neutrophils were subjected to centrifugation at 350g for 5 minutes, MTT was removed and 100µl isopropanol added to each well. Precipitates were allowed to solubilise for 10 minutes with gentle shaking and absorbance was read at 560nm.

2.2.3 Neutrophil static adhesion assay

Neutrophil adhesion to immobilised ligands and endothelial monolayers was assessed. To evaluate adhesion to immobilised integrin ligands, 96-well black MaxiSorp microplates (Thermo Scientific, UK) were coated overnight with either 4.5µg/ml ICAM-1-Fc, 200ng/ml fibrinogen or 20µg/ml fibronectin diluted in PBS. After coating, plates were washed three times with TBS/0.1% Tween-20 and blocked with 2% fish skin gelatin/TBS/0.1% Tween-20 for 1 hour at 37°C. For adhesion to endothelial monolayers, HUVEC were grown to confluence in 96-well black polystyrene TC-treated microplates (Corning, UK) that had been coated with attachment factor (Gibco, UK). To evaluate the effects of endothelial hypoxia on adhesion, HUVEC monolayers were incubated overnight in either normoxia or hypoxia.

Neutrophils were isolated from whole blood, washed with a sodium HEPES buffer and resuspended at $1x10^6$ neutrophils/ml. Cells were fluorescently labelled with 2.5µM BCECF-AM (Life Technologies, UK) for 30 minutes at 37°C in the dark, then washed with Hank's balanced salt solution (HBSS) and resuspended to a cell density of $1x10^7$ cells/ml.

Assay plates were washed twice with HBSS and 50µl HBSS containing 100ng/ml LPS, 10ng/ml TNF-α or 20nM PMA in the absence or presence of 200µg/ml purified IgG or 10µg/ml blocking antibodies was added to relevant wells. 50µl of the BCECF-AM labelled neutrophils were added, giving a final concentration of $5x10^5$ neutrophils/well. Plates were centrifuged at

170g for 2 minutes and incubated at 37°C for 30 minutes. Following incubation, fluorescence was measured using a Tecan GENios Spectra FLUOR plate reader (Tecan UK Ltd., UK) with an emission wavelength of 535nm and an excitation wavelength of 490nm to determine total cell fluorescence. Plates were washed three times with HBSS and the fluorescence read again. The percentage of adherent neutrophils was calculated by comparing the fluorescence of washed wells to the initial total fluorescence.

2.2.4 Neutrophil trans-endothelial migration assay

Neutrophil trans-endothelial migration was also measured. HUVEC were grown to confluence on 12mm trans-well inserts with 5µm pores (Millipore, UK). Once confluent, EC monolayers were cultured overnight under normoxia or hypoxia in the absence or presence of 10ng/ml TNF-α. Neutrophils were isolated and resuspended to $1x10^6$ cells/ml in serum-free medium-199 (M-199) and stained with 0.5µM CellTracker (Invitrogen, UK) for 20 minutes on ice. An unstained aliquot of neutrophils was kept aside. Following incubation, neutrophils were resuspended in M-199 supplemented with 1% FCS to a cell density of $5.5x10^6$ neutrophils/ml.

Wells were washed and 900µl of untreated M-199 or 150ng/ml IL-8 diluted in M-199 was added to the lower chamber and 200µl of the neutrophil suspension added to the upper chamber and incubated for 90 minutes at 37°C. After incubation, the media from each of the lower chambers was collected and subjected to centrifugation at 300g for 5 minutes, which was then resuspended in 400µl 1% paraformaldehyde (PFA)/PBS. To this cell suspension, 50µl of CountBright™ absolute counting beads (Invitrogen, UK) was added and samples assessed on a FACSVerse (BD Biosciences, UK). Unlabelled cells were used to define experimental settings and cell gating strategies, based on the forward and side scatter properties of neutrophils. The CountBright™ absolute counting beads enabled the calculation of cell numbers in samples. Data was analysed using FlowJo (TreeStar Inc., UK).

2.2.5 Neutrophil integrin expression analysis

Flow cytometry was used to quantify the expression of neutrophil β_1 and β_2 integrins. Neutrophils were isolated and incubated under normoxia or hypoxia. Cells were resuspended in

sodium HEPES buffer and $2x10^5$ neutrophils incubated with fluorochrome-conjugated integrin-specific or isotype control antibodies for 30 minutes at room temperature in the dark. A cell pellet was obtained by centrifugation at 350g for 5 minutes and washed twice in sodium HEPES buffer. After the final wash, cells were fixed with 200µl 1% PFA/PBS. Unstained cells were used to define gating strategies to analyse neutrophils. Stained cells were analysed, with 20,000 gated events being assessed using a FACSVerse (BD Biosciences, UK). Data was analysed using FlowJo (TreeStar Inc., UK).

2.2.6 Neutrophil nitrite generation analysis

To measure nitrite generation the Griess test was performed on neutrophil supernatants. This assay depends on the production of diazonium salts by organic nitrites, facilitated by the sulphanilamide in the Griess reagent. The subsequent diazonium salt then interacts with the azo-dye N-(1-napthyl)ethylenediamine, also in the Griess reagent, to form a pink aqueous solution that can be measured by absorbance of light.

Isolated neutrophils were stimulated for set time points, after which cell supernatants were obtained by centrifugation at 300g for 5 minutes. 100µl of either a nitrite standard (Sigma, UK) or cell supernatant was added in triplicate to a 96-well flat-bottomed plate. Following addition of 100µl Griess reagent (1% sulphanilamide, 0.1% N-(1-napthyl)ethylenediamine, 2.1% phosphoric acid, made in ddH_2O), plates were incubated for 10 minutes at room temperature and absorbance read at 560nm using a Tecan GENios Spectra FLUOR plate reader (Tecan UK Ltd., UK). Nitrite concentrations were calculated using the standard curve generated by the nitrite standards.

2.2.7 Neutrophil hydrogen peroxide generation analysis

The effects of hypoxia and purified IgG upon hydrogen peroxide generation were evaluated using the Amplex UltraRed® assay. The assay reagent (10-acetyle-3,7-dihydroxyphenoxazine) is a colourless HRP substrate, which in the presence of hydrogen peroxide, is rapidly converted into the fluorescent Amplex UltraRed® compound in a 1:1 stoichiometry, thus allowing for the measurement of hydrogen peroxide production.

Neutrophils were isolated and resuspended to $2x10^6$ cells/ml. Neutrophil FcγRs were blocked by incubation with FcR block (Miltenyi Biotec, UK) for 20 minutes to exclude a non-specific FcγR-mediated effects. Cells were preconditioned with either purified IgG or inhibitors for one hour at 37°C. Next, HRP (0.5U/ml) (Sigma, UK) was added to cell suspensions and then 60nM Amplex® UltraRed (Invitrogen, UK). 200µl per well of the cell suspension was added in triplicate to a 96-well black microplate (Thermo Scientific, UK) and placed into a FLUOstar Omega microplate reader (BMG Labetech, Germany). Fluorescence was measured using an excitation wavelength of 544nm and emission wavelength of 590nm, with a gain of 1004. Basal levels of hydrogen peroxide generation were assessed between the 1st and 3rd cycles. 50nM PMA was added on the 4th cycle and fluorescence followed over time. Rates were determined using the Omega Mars Analysis software (BMG Labtech, Germany).

2.2.8 Immunofluorescence visualisation of neutrophil extracellular traps

NETs were qualitatively evaluated by immunofluorescence microscopy. Assessment of co-localisation between DNA and proteins associated with NET structure was possible by 4'-6-diamidino-2-phenylindole (DAPI) and indirect staining of histone H3.

13mm glass coverslips (Fischer, UK) were sterilised with ethanol and air-dried in a 24-well tissue culture plate. Once dry, coverslips were coated with 200µg/ml fibrinogen (Sigma, UK) in PBS overnight (400µl/well) at 4°C. Coverslips were washed for 5 minutes first with PBS and then RPMI. $5x10^5$ neutrophils were added to coverslips and allowed to settle for 30 minutes. Cells were then stimulated for 4 hours. Following incubation, coverslips were washed with ice-cold HBSS (Life Technologies, UK) and fixed for 15 minutes with 4% PFA/PBS at room temperature.

Coverslips were blocked in 10% goat serum/1% BSA/2mM EDTA/HBSS/0.1% Tween-2 (blocking buffer) overnight at 4°C with gentle agitation on an orbital shaker. Coverslips were washed once with HBSS and incubated with an anti-histone H3 antibody (Abcam, UK) diluted in blocking buffer for one hour at room temperature on an orbital shaker. Coverslips were washed twice with HBSS and incubated with an AlexaFluor® 488-conjugated secondary

antibody (Life Technologies, UK) diluted 1:1000 in blocking buffer for a further hour at room temperature on an orbital shaker. After incubation, coverslips were washed twice for 5 minutes with HBSS and once with ddH_2O before being mounted and sealed on microscope slides with a DAPI mounting medium. Slides were subsequently visualised using a Zeiss Axio Imager.A1 inverted fluorescence microscope (Zeiss, Germany) and images analysed using Image J.

2.2.9 Neutrophil extracellular trap quantification – extracellular DNA quantification

Measurement of cell-free dsDNA was conducted to provide a quantitative readout of NETosis. The Quanti-iT™ PicoGreen® dsDNA kit is widely used for this purpose and works by the PicoGreen® reagent integrating into extracellular dsDNA, but not single stranded DNA and extracellular RNA, allowing for specific and sensitive measurement of cell-free dsDNA. For assessment of NETosis, neutrophil supernatants were obtained by centrifugation at 350g for 5 minutes following stimulation. Supernatants were stained using the Quanti-iT™ PicoGreen® dsDNA kit (Invitrogen, UK), and measured using a fluorescence plate reader.

500µl of cell supernatants were added to microcentrifuge tubes and 500µl of Quanti-iT™ PicoGreen® dsDNA reagent added (diluted 1:200 in 1xDNase-free TE buffer), mixed and incubated at room temperature for 3 minutes. To quantify NETs, a standard curve was generated using known concentrations of λ dsDNA ranging from 1µg/ml to 1ng/ml. Following incubation, 300µl of dsDNA standard or sample was added in triplicate to the wells of a 96-well polystyrene TC-treated microplate. Fluorescence was read with an excitation wavelength of 490nm and an emission wavelength of 535nm. Cell-free dsDNA concentrations were calculated using the dsDNA standard curve.

2.2.10 Neutrophil extracellular trap quantification – capture ELISA

A capture ELISA was developed to quantify NETs, rather than extracellular dsDNA, by targeting two components of NET structure. Streptavidin-coated plates (Fisher Scientific, UK) were coated with 1µg/ml of a biotin-conjugated anti-MPO capture antibody (Abcam, UK) overnight at 4^{o}C. Plates were washed three times with PBS/0.1% Tween-20 and blocked with 0.5% BSA/PBS/0.1% Tween-20 for 1 hour at 37^{o}C. Plates were then washed and incubated with

100µl of neutrophil supernatants for 2 hours at 37°C. Following incubation, wells were washed and 100µl of an anti-citrullinated histone H3 detection antibody (Abcam, UK), diluted to 1µg/ml in 0.5% BSA/PBS/0.1% Tween-20, added and incubated for 1 hour at 37°C. Plates were washed and incubated with an HRP-conjugated secondary antibody (Dako, UK) for 1 hour at 37°C. 100µl/well of room temperature SureBlue TMB Microwell Peroxidase Substrate (KPL, UK) was added and incubated in the dark at 37°C for 20 minutes. The reaction was then stopped by the addition of 100µl/well of TMB stop solution (KPL, UK). Absorbance was read at 450nm using a Tecan GENios Spectra FLUOR plate reader (Tecan UK Ltd., UK).

2.2.11 Endothelial adhesion molecule expression analysis

Endothelial adhesion molecules expression was evaluated, as they represent physiological integrin ligands. Levels of E-selectin, ICAM-1, ICAM-2 and VCAM-1 expression were assessed on HUVEC by flow cytometry. $5x10^5$ HUVEC were seeded in 6-well tissue culture plates and allowed to grow to confluence. Cells were treated with 100ng/ml LPS, 10ng/ml TNF-α or media alone under normoxia or hypoxia. After incubation, media was aspirated and cells washed twice with PBS. 0.5ml warmed cell dissociation buffer was added and incubated for 5 minutes at 37°C. HUVEC were transferred to polystyrene round-bottomed tubes (BD Falcon, UK) and subjected to centrifugation at 350g for 5 minutes. Cells were washed twice with sodium HEPES buffer and incubated with fluorophore-conjugated antibodies targeting endothelial adhesion molecules for 30 minutes at room temperature in the dark. Following incubation, cells were centrifuged at 350g for 5 minutes, washed twice with sodium HEPES buffer and fixed with 200µl 1% PFA/PBS. HUVEC were selected based on their forward and side scatter properties, with a gate being placed around cells of the correct size and granular properties. Stained HUVEC were analysed, with 20,000 gated events being assessed using a FACSVerse (BD Biosciences, UK). Data was analysed using FlowJo (TreeStar Inc.)

2.2.12 Whole IgG purification

Whole IgG fractions were purified from human sera using protein G agarose spin columns (Thermo Scientific, USA). Protein G selectively binds the Fc portion of IgG, thus

allowing the separation from other Ig isotypes (IgA, IgM, IgD and IgE). Patient or healthy volunteer serum was selected and IgG purified by passing through a protein G column. Separate protein G spin columns were allocated for each group to prevent cross-contamination between IgG populations. Columns were allowed to reach room temperature before removal of the base and lid and allowing the storage solution (0.01% sodium azide/PBS) to run through. The column was then washed with 5ml binding buffer (0.1M phosphate buffer, pH 7.2) by gravity flow. Serum was diluted 1:1 with binding buffer and allowed to run through the protein G column. IgG-depleted serum was collected in conical centrifuge tubes and stored at -20^{o}C. The column was washed three times with 5ml binding buffer. Bound IgG was eluted by 5ml elution buffer (0.1M glycine, pH 3.0) into conical centrifuge tubes containing 500µl of neutralisation buffer (1M Tris, pH 9.0).

2.2.13 IgG concentration

Eluted IgG was concentrated into PBS using 100kDa centrifugal filter units (Merck Millipore, Ireland). A 4ml aliquot of purified IgG was added to the filter unit and filtered by centrifugation at 2050g for 20 minutes. Then 2ml of the filtration waste was discarded and the remaining 1ml of the eluted purified IgG sample added to the filtration unit before repeating the centrifugation step. The sample was washed twice and dialysed by adding 2ml endotoxin-free PBS (Thermo, Scientific, USA) and subjecting to centrifugation for a further 20 minutes at 2050g. Endotoxin-free PBS was used to make up the final concentrated sample to 1ml, which was then transferred to a sterile microcentrifuge tube. Protein concentration was then determined using a NanoDrop 1000 Spectrophotometer (Thermo Scientific, USA).

2.2.14 Endotoxin removal

Endotoxin was removed from purified and concentrated IgG by passage through a Detoxi-Gel endotoxin removal column (Thermo Scientific, USA) under laminar airflow. The column matrix contains immobilised polymyxin B beads that binds and removes LPS from samples. Columns were equilibrated at room temperature and the storage solution (25% ethanol) was removed by gravity flow. Columns were then washed sequentially with: 5ml of

1% sodium deoxycholate/endotoxin-free water (0.22µm filter-sterilised); 4ml endotoxin-free water; and 4ml sterile PBS. Purified IgG was then added and allowed to enter the column matrix. IgG was eluted by addition of 1.2ml sterile PBS, which was collected in a microcentrifuge tube. The protein concentration was re-tested using a NanoDrop 1000 Spectrophotometer (Thermo Scientific, USA) to confirm the presence of purified IgG. The concentration of purified IgG was then determined using the IgG ELISA detailed in section 2.2.16. All further use of purified IgG was always under laminar airflow to prevent endotoxin contamination.

2.2.15 Endotoxin quantification– Limulus Amoebocyte Lysate assay

Once purified, IgG endotoxin content was determined using the Limulus Amoebocyte Lysate (LAL) test. For this assay, aqueous extracts from the horseshoe crab (*Limulus polyphemus*) was mixed with the purified IgG. When endotoxin is present, the LAL reacts to form a solid gel, which can be quantified by comparison with endotoxin standards run in tandem with the unknown samples.

Endotoxin standards were made using LPS of known endotoxin units (EU). Stock LPS (Sigma, UK) containing 3,000,000 EU/ml of endotoxin was diluted to prepare 4, 2, 1, 0.5, 0.25, 0.125, 0.06 and 0.03 EU/ml standards. All samples were prepared under laminar airflow, using sterile capped polystyrene 14ml sterile cell culture tubes and endotoxin-free water. Endotoxin standards were mixed by vortex (Scientific Industries Inc., USA) and used at the time of preparation. E-toxate test vial (Sigma, UK) was reconstituted in sterile endotoxin-free water, gently mixed and allowed to chill at 4^{o}C immediately until required to test samples. Remaining E-toxate reagent was aliquoted and kept at -20^{o}C.

200µg IgG was diluted in endotoxin-free water to a final volume of 100µl in 10x75mm glass tubes. Endotoxin standards were added to separate 10x75mm glass tubes, with 100µl of endotoxin-free water as a negative control. Next, 100µl E-toxate was added to each tube and gently mixed. Tube mouths were covered with parafilm and then allowed to incubate undisturbed for 1 hour at 37^{o}C. Samples positive for endotoxin formed a solid gel, whilst those

negative remain in the liquid phase. Samples testing positive at more than 0.25 EU/ml were re-passed through the Detoxi-Gel endotoxin removal column as detailed in the previous section until sufficient endotoxin had been removed.

2.2.16 Purified IgG quantification

Following endotoxin removal, concentrations of purified IgG were determined using an IgG capture ELISA. The test half of a MaxiSorp plate was coated with 400ng/ml of goat anti-human IgG Fc antibody (I8885; Sigma, UK) in PBS overnight at 4^{o}C, whilst the control half was coated with PBS alone to determine the relative backgrounds for each sample. Coated plates were washed twice with PBS/0.1% Tween-20 and blocked with 100µl of 2% BSA/PBS for 1 hour at 37^{o}C. After blocking, plates were washed three times with PBS/0.1% Tween-20 and serially diluted purified samples loaded (50µl/well) onto the plate alongside prepared IgG standards using IgG from human serum of known concentrations (I2511; Sigma, UK), which were allowed to incubate for 1 hour at room temperature. After incubation, plates were washed three times and a HRP-conjugated anti-human IgG (A6029; Sigma, UK) was added and incubated for a further hour at room temperature. Following three washes, 100µl of a HRP substrate was added and incubated for 15 minutes in the dark at room temperature before the reaction was stopped by addition of 100µl of stop solution. Absorbance was then read at 450nm using a Tecan GENios Spectra FLUOR plate reader (Tecan, UK Ltd., UK). The concentration of purified IgG samples was determined by comparison with the standard curve generated by the known concentrations of human IgG.

2.2.17 Anti-citrullinated protein antibody ELISA

To quantify the ACPA activity of purified IgG, samples were tested using the EDIA™ anti-CCP-2 kit (Euro Diagnostica, Sweden) as per instruction. In brief, 100µl of anti-CCP calibrators (human plasma of known CCP-2 activity) ranging from 0 U/ml to 300 U/ml and purified IgG diluted 1:101 in sample diluent (phosphate buffer/0.5% sodium azide) were added to 96-well plate strips in duplicate and incubated for 1 hour at room temperature. Wells were washed three times with diluted wash buffer (borate buffer/0.4% sodium azide) and 100µl of an

alkaline phosphatase-conjugated goat polyclonal anti-human IgG antibody was added to each well and then incubated for a further 30 minutes at room temperature. After washing, 100µl substrate solution (phenolphthalein monophosphate, Mg^{2+} buffer solution) was added to each well for 30 minutes at room temperature and the reaction was then stopped with 100µl of stop solution (sodium hydroxide in an EDTA/carbonate buffer, pH>10). Absorbance was read at 560nm using a Tecan GENios Spectra FLUOR plate reader (Tecan, UK Ltd., UK) and the activity determined using a standard curve derived from the calibrators.

2.2.18 Anti-neutrophil cytoplasmic antibody ELISA

To determine the ANCA positivity of purified IgG, samples were tested for PR3- and MPO-ANCA using the WIESLAB® ANCA screen kit (Euro Diagnostica, Sweden) as per instruction. In brief, purified IgG samples and human serum known to be positive or negative for PR3- and MPO-ANCA were diluted 1:4 in a dilution buffer and 100µl pipetted into either PR3- or MPO-coated wells. Samples were incubated at room temperature for 10 minutes on an orbital shaker. Wells were washed 4 times with diluted wash buffer then incubated with 100µl of an alkaline phosphatase-conjugated goat anti-human IgG antibody for a further 10 minutes at room temperature. Following incubation, wells were washed 4 times and 100µl of alkaline phosphatase substrate (para-Nitrophenylphophate) was added and allowed to incubate for 20 minutes on an orbital shaker at room temperature. Absorbance was subsequently read at 405nm using a Tecan GENios Spectra FLUOR plate reader (Tecan, UK Ltd., UK) and ANCA positivity determined as an absorbance OD ratio relative to negative control.

2.2.19 Protein extraction

Both endothelial and neutrophil proteins were extracted using a T-SDS lysis buffer (0.66mM Tris-HCl pH 7.4, 2% SDS and complete mini protease and phosphatase inhibitor cocktail tablets [Roche]). HUVEC were grown in 6-well tissue culture plates (Thermo Fisher, UK) and treated following experimental protocol. Wells were washed with 1ml ice cold PBS and 50µl of T-SDS lysis buffer was added. Cells were immediately removed by scraping and transferred to microcentrifuge tubes by passage through a 25G needle. Neutrophils were

transferred to ice cold 1.5ml microcentrifuge tubes and centrifuged at 350g for 5 minutes at 4°C. Neutrophils were washed with 1ml ice cold PBS and then resuspended in 50µl of T-SDS lysis buffer, transferred to a fresh microcentrifuge tube by passage through a 25G needle and heated for 5 minutes at 95°C. Lysates were centrifuged at 16,100g for 5 minutes at 4°C to pellet cell debris. For some studies, fractional lysis was performed to obtain subcellular cell fractions using the ProteoExtract® subcellular proteome extraction kit (Millipore, Ireland). Gently and sequential cell lysis steps gave cytosolic, membrane and organelle, nuclear and cytoskeletal fractions. Lysate supernatants were then transferred to fresh microcentrifuge tubes and stored at -20°C for future analysis.

2.2.20 Estimation of protein concentration

Protein concentrations of cell lysates were determined by bicinchoninic acid (BCA) protein assay (Thermo Scientific, UK). This assay relies on the reduction of copper ions by the peptide bonds in the lysates and the subsequent chelation of reduced copper ions by bicinchoninic acid, which induces a colorimetric change. Protein standards were made from dilutions of a 2mg/ml albumin solution to concentrations ranging from 2mg/ml to 25µg/ml. A working reagent was then prepared by mixing 50 parts of BCA reagent A (sodium carbonate, sodium bicarbonate, bicinchoninic acid and sodium tartrate in 0.1M sodium hydroxide) with 1 part BCA reagent B (4% cupric acid). To each well the following agents were added in duplicate: 10µl of the albumin standard; 10µl of cell lysate supernatants; 10µl of T-SDS lysis buffer; and 10µl of PBS. Next 200µl of working reagent was added to each well and gently mixed. The plate was covered and incubated for 30 minutes at 37°C. Following incubation, absorbance was read at 560nm on a Tecan GENios microplate reader. Protein concentrations were then determined using the standard curve obtained by the albumin standards.

2.2.21 Protein detection via immunoblot

Cell lysates were diluted in lithium dodecyl sulphate (LDS) sample buffer (Life Technologies, UK) and heated to 70°C for 10 minutes, before being loaded into pre-cast bis-tris gels (Life Technologies, UK). MES [2-(N-morpholino)-ethanesulfonic acid] running buffer

(Life Technologies, UK) was added to the electrophoresis unit to cover the gel. A pre-stained full range protein marker ladder (New England BioLabs, UK) was added to the first lane and the prepared samples added to subsequent lanes. Proteins were resolved for 45 minutes at 165 volts.

Resolved proteins were transferred to a polyvinylidene fluoride (PVDF) membrane (GE Healthcare, UK), by placing the gel against the methanol-activated PVDF membrane between filter paper and sponges pre-soaked in transfer buffer. Gels were allowed to transfer for 1 hour at 10 volts in an electrophoresis rig containing transfer buffer (Life Technologies, UK).

Membranes were then blocked for 1 hour in 5% skim milk/TBS/0.1% Tween-20 on an orbital shaker before overnight incubation with primary antibodies at 4^{o}C. After overnight incubation, membranes were washed twice with TBS/0.1% Tween-20 for 15 minutes following which membranes were incubated with HRP-conjugated secondary antibodies for 1 hour at room temperature in blocking buffer. Membranes were then washed twice for 15 minutes and protein bands visualised using the Luminata Western HRP substrate system (Millipore, Ireland), allowing for the chemiluminescent detection of the HRP-conjugated secondary antibodies.

2.2.22 Statistical analysis

Data were evaluated using GraphPad Prism software. Experimental data was tested for normality using the Kolmogorov-Smirnov test. Data identified as normally distributed was assessed for statistical significance by means of a one-way analysis of variance (ANOVA) with a Bonferroni multiple comparison post-test analysis, or an unpaired t test depending on the nature of the experiment. If data was non-parametrically distributed, statistical significance was either tested using a Kruskal-Wallis test with a Dunn's comparison post-test analysis or a Mann-Whitney t test. For data comparing multiple parameters, a two-way ANOVA with a Bonferroni post-test analysis was conducted. If data represented matched observations, the appropriate paired statistical test was performed depending on the nature of data distribution. Experiments were conducted on three separate occasions, unless otherwise stated, with data being presented as the mean ±standard deviation (SD) of these independent experiments.

Chapter Three:
The Effects of Hypoxia on Neutrophil Integrin Activation

3.1 Introduction and aims

Early studies demonstrated enhanced neutrophil adhesion to ECs under hypoxia (Palluy et al., 1992, Rainger et al., 1995). Hypoxia has also been shown to increase neutrophil adhesion to epithelial cells (Beck-Schimmer et al., 2001), and promote trans-epithelial migration (Colgan et al., 1996). Increased mRNA and protein of the β_2 integrin subunit (CD18) has been observed in murine and human leukocytes subjected to hypoxia, which promoted adhesion to ECs (Kong et al., 2004). Upregulation of β_1 integrins has also been described under hypoxia (Blaschke et al., 2002, Keely et al., 2009, Lee et al., 2011). There are no reports however, on the effects of hypoxia on β_1 integrins in neutrophils. Therefore, in this chapter, I determined the effects of hypoxia upon neutrophil β_1 and β_2 integrin expression, adhesion to immobilised integrin ligands and ECs, and trans-endothelial migration.

3.2 Neutrophils express both β_1 and β_2 integrins

Prior to examining the effects of hypoxia upon neutrophil integrins, I measured baseline integrin expression in unstimulated neutrophils from 5 healthy donors. All isolated neutrophils stained positive for CD15, which is expressed by mature neutrophils (Figure 3.1A).

Integrin subunits α_1 (CD49a), α_4 (CD49d), α_5 (CD49e), α_L, (CD11a), α_M (CD11b), α_X (CD11c), β_1 (CD29), and β_2 (CD18) were assessed by flow cytometry. Neutrophils expressed varying levels of each of these integrin subunits (Figure 3.1B). Mean fluorescence intensity (MFI) data showed variable expression of all integrin subunits between donors.

In addition, the number of neutrophils that expressed each integrin subunit was also examined (Table 3.1). Expression of α_L, α_M, α_X and β_2 was seen in 94%, 80%, 26% and 95% of neutrophils respectively. In contrast, α_1, α_4, α_5 and, β_1 expression was only observed on 1%, 5%, 9% and 8% of neutrophils respectively. These results show that unstimulated neutrophils have high expression of β_2 integrins and low levels of β_1 integrins.

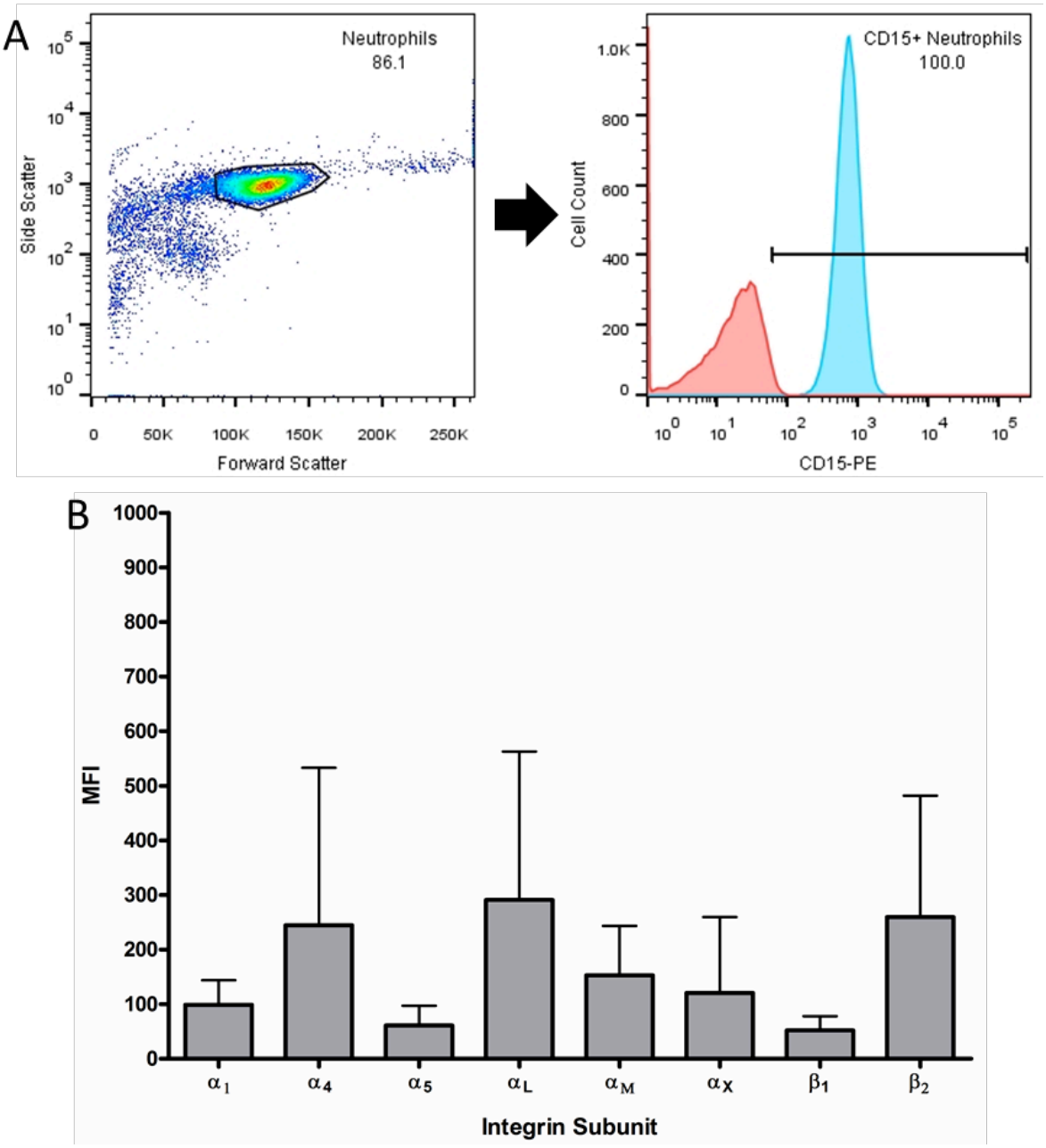


Figure 3.1: Freshly isolated neutrophils express varying levels of β_1 and β_2 integrins. Neutrophils were isolated and fluorescently stained for CD15 and various integrin subunits. **(A)** A representative flow cytometry plot of isolated neutrophils with the cell gate applied for further neutrophil analysis, based upon neutrophil side and forward scatter properties. Isolated cells were stained for CD15 (blue) or an isotype control (red). Gated cells were all found to be $CD15^+$ neutrophils. **(B)** Baseline neutrophil integrin expression was assessed in 5 healthy donors. Neutrophils were found to express varying levels of all integrin subunits analysed. Data is presented as mean fluorescence intensity (MFI) ±SD of data obtained from all 5 donors.

	M/F	Number of Neutrophils (%)							
		α_1 (CD49a)	α_4 (CD49d)	α_5 (CD49e)	α_L (CD11a)	α_M (CD11b)	α_X (CD11c)	β_1 (CD29)	β_2 (CD18)
Donor 1	M	0.1	13.5	21.2	96.4	68.0	40.6	18.1	99.6
Donor 2	M	0.2	0.2	3.1	84.2	64.4	11.3	4.6	86.6
Donor 3	F	1.0	4.0	7.5	94.8	80.4	12.7	4.4	90.0
Donor 4	F	1.1	0.9	2.0	95.3	89.6	35.8	2.0	98.0
Donor 5	F	0.9	4.2	10.6	99.0	98.0	29.4	10.2	99.7
Average	2M/3F	0.7 ±0.4	4.6 ±4.7	8.9 ±6.9	93.9 ±5.1	80.1 ±12.7	26.0 ±12.0	7.8 ±5.8	94.8 ±5.4

Table 3.1: Neutrophils predominately express β_2 integrins. As well as analysing mean fluorescence intensity, the number of neutrophils staining positive for integrin subunits was analysed. Relatively low numbers of neutrophils were positive for α_1 (CD49a), α_4 (CD49d), α_5 (CD49e) and β_1 (CD29) integrin subunits. In contrast, almost all neutrophils expressed α_L (CD11a), α_M (CD11b) and β_2 (CD18) integrin subunits. A quarter of resting neutrophils expressed the α_X (CD11c) integrin unit.

3.3 Expression of neutrophil β_2 integrins, but not β_1 integrins, is enhanced by hypoxia

Having shown that neutrophils express β_1 and β_2 integrins, I then studied the effects of hypoxia upon integrin expression. To begin with, cell viability in response to hypoxia was assessed with MTT. Isolated neutrophils were cultured under hypoxia, which was confirmed by HIF-1α and HIF-2α expression by immunoblot (Figure 3.2A). Neutrophil viability was assessed at 4, 8 and 24 hours of culture under normoxia and hypoxia. Hypoxia did not significantly affect cell viability at 4 and 8 hours culture (Figure 3.2B). Optical density (OD) values decreased following 24 hours culture, indicating neutrophils were undergoing cell death, although these values were not significantly different between normoxia and hypoxia.

To determine the optimal time point to analyse integrin expression, a preliminary time course experiment was conducted with neutrophils isolated from one donor. Surface α_L, α_M and β_2 expression was evaluated after 4, 6, 8 and 24 hours of culture under normoxia or hypoxia. Expression of α_L did not vary between cells cultured up to 8 hours (Figure 3.3A). In contrast, culture under hypoxia for up to 8 hours enhanced α_M expression, which decreased under normoxia (Figure 3.3B). Expression levels of β_2 were also higher in neutrophils cultured under hypoxia compared to normoxia (Figure 3.3C). Integrin expression decreased following 24 hours culture under normoxia and hypoxia, probably as a result of cell death. For subsequent experiments, an 8-hour time point was chosen as neutrophil viability was not significantly affected and differences in α_M and β_2 expression were seen.

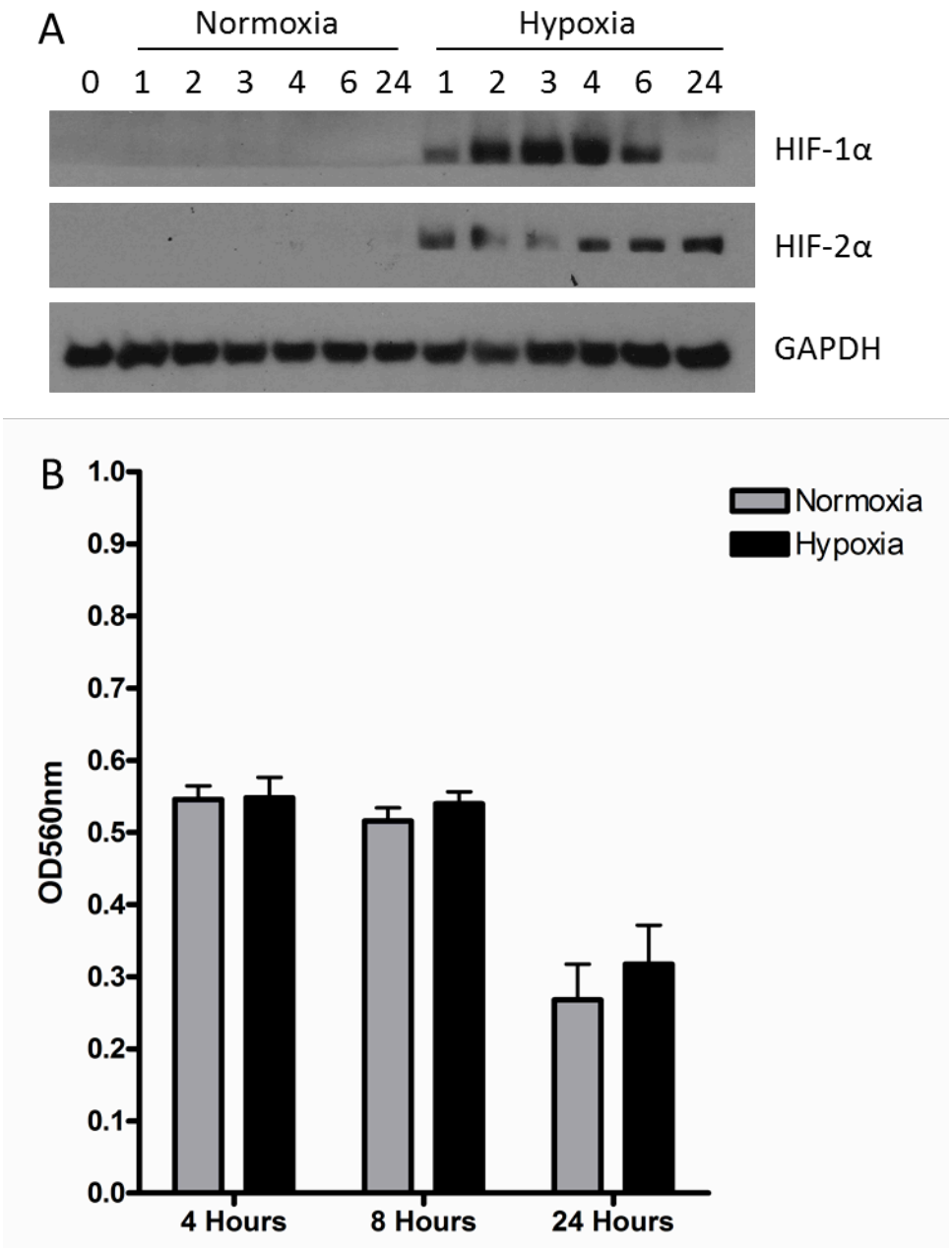


Figure 3.2: Hypoxia does not affect neutrophil viability. MTT assays were performed to assess neutrophil viability in response to hypoxia. **(A)** Hypoxia was confirmed by expression by HIF-1α and HIF-2α by immunoblot. Neutrophils were cultured for 1, 2, 3, 4, 6 or 24 hours under normoxia or hypoxia before lysis with a T-SDS lysis buffer. Resolved cell lysates were transferred to a PVDF membrane and probed for HIF-1α, HIF-2α and GAPDH. **(B)** $3.5x10^5$ neutrophils were cultured for 4, 8 or 24 hours under normoxia or hypoxia before cell viability was assessed. Hypoxia did not significantly affect neutrophil viability at 4 and 8 hours culture. Following 24 hours culture, OD values were 50-60% of earlier time points, indicative of cell death. Statistical analysis was tested by means of a two-way ANOVA with a Bonferroni's post-test. Data is presented as mean OD ±SD of two independent experiments.

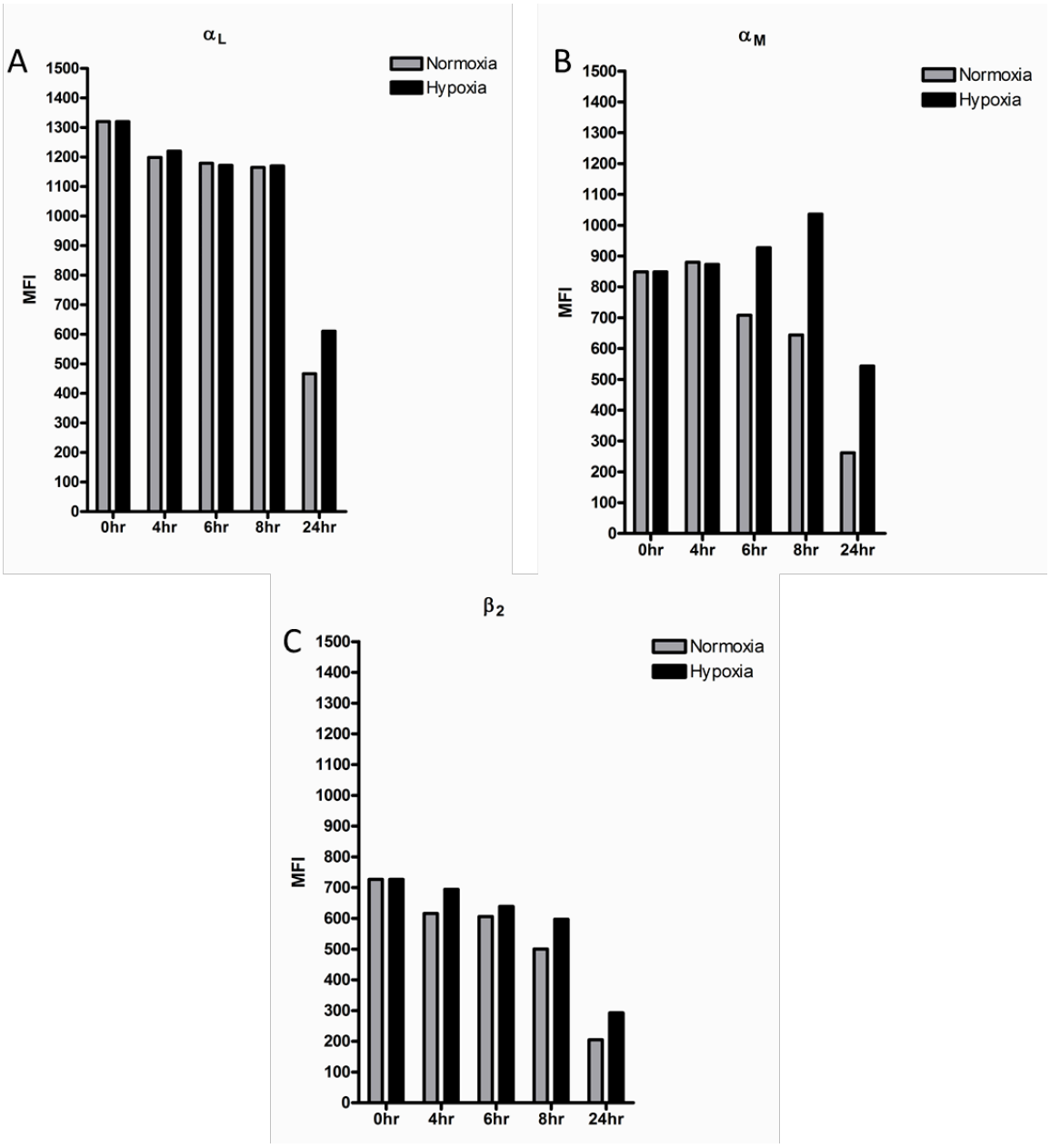


Figure 3.3: Surface expression of α_M increases with culture under hypoxia. A preliminary time course experiment was conducted to evaluate surface integrin expression. Neutrophils were isolated from one donor and cultured under normoxia or hypoxia for 4, 6, 8 or 24 hours and stained for α_L (CD11a), α_M (CD11b) and β_2 (CD18). **(A)** Expression of α_L did not vary between cells cultured under normoxia or hypoxia up until 8 hours culture. **(B)** Hypoxia increased α_M expression up to 8 hours culture. **(C)** Surface β_2 expression was higher in cells cultured under hypoxia. Following 24 hours culture under normoxia and hypoxia, decreased expression of all three integrin subunits was observed, most likely due to cell death. Data is presented as the mean fluorescence intensity (MFI) of 20,000 neutrophils

Neutrophils were isolated from 7 healthy donors and cultured for 8 hours under normoxia or hypoxia. Surface expression of the integrin subunits α_1, α_4, α_5, α_L, α_M, α_X, β_1 and β_2 were assessed by flow cytometry.

Surface expression of β_1 integrins was not modulated by hypoxia (Figure 3.4A-D). Paired statistical tests did not find any differences in MFI of α_1 ($p=0.8750$), α_4 ($p=0.2188$), α_5 ($p=0.1489$) or β_1 ($p=0.1546$). In addition, the number of neutrophils expressing each integrin subunit was not affected by hypoxia (Figure 3.4E-H). These results show that surface expression of VLA-1 ($\alpha_1\beta_1$), VLA-4 ($\alpha_4\beta_1$) and VLA-5 ($\alpha_5\beta_1$) is not modulated by 8 hours hypoxic exposure.

In contrast, β_2 integrin expression was enhanced by hypoxia. Whilst there were no significant differences in MFI of α_L ($p=0.7645$) and β_2 ($p=0.4688$) between cells cultured under normoxia or hypoxia (Figure 3.5A, D), MFI for α_M and α_X significantly increased under hypoxia ($p<0.001$ and $p=0.0038$ respectively) (Figure 3.5B, C). Further analysis found that hypoxia significantly increased the number of neutrophils expressing α_L ($p=0.0156$), α_M ($p=0.0001$), α_X ($p=0.0179$) and β_2 ($p=0.0313$) (Figure 3.5E-H). Taken together, these results indicate that hypoxia induces greater numbers of neutrophils to express the β_2 integrins LFA-1 ($\alpha_L\beta_2$), Mac-1 ($\alpha_M\beta_2$) and p150,95 ($\alpha_X\beta_2$) following 8 hours hypoxic exposure.

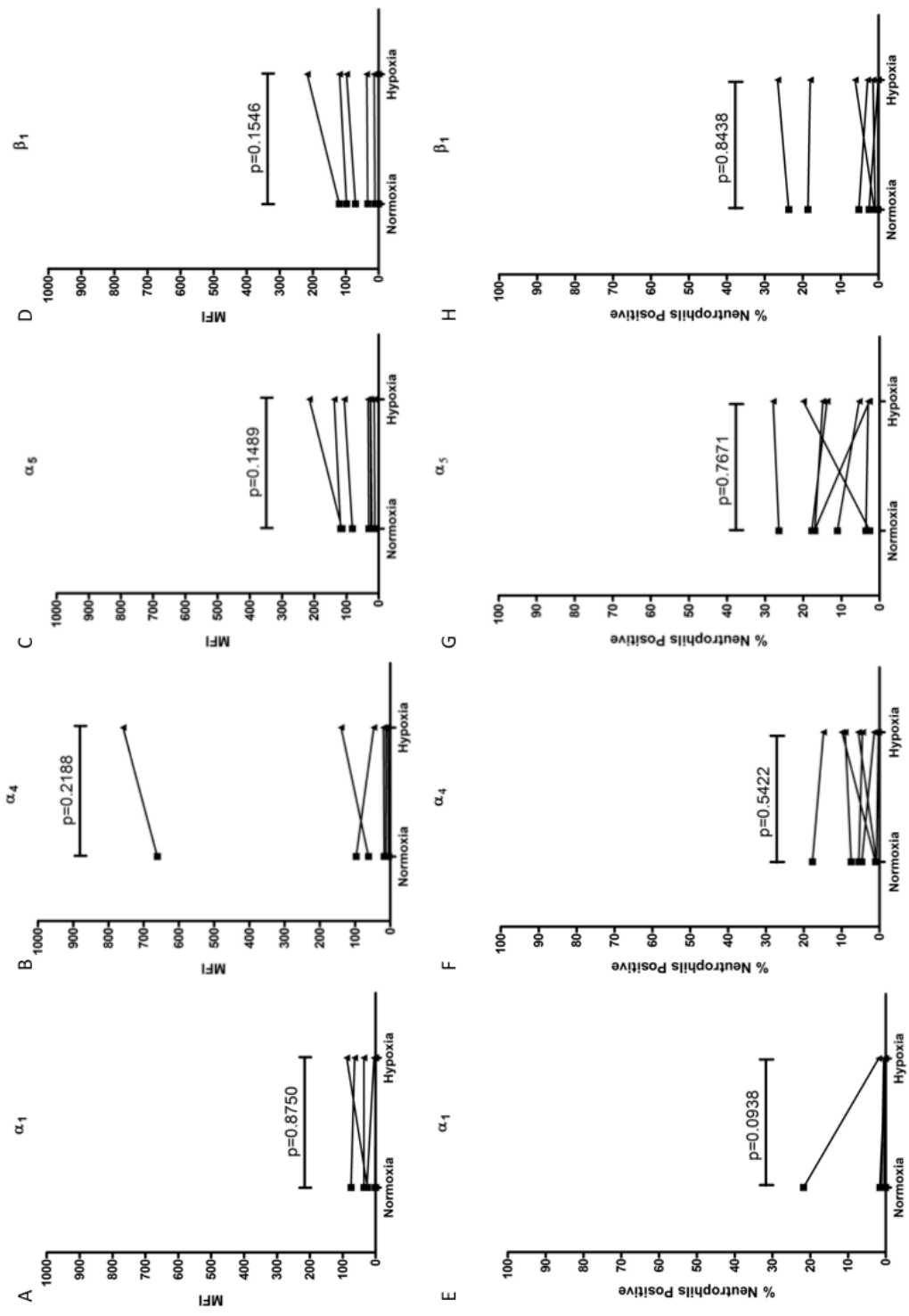


Figure 3.4: β_1 integrin expression is not modulated by hypoxia. Neutrophils were isolated from 7 healthy donors and cultured for 8 hours under normoxia or hypoxia. Neutrophils were stained for α_1 (CD49a), α_4 (CD49d), α_5 (CD49e) and β_1 (CD29) and assessed by flow cytometry. Hypoxia did not significantly affect MFI for **(A)** α_1 (Wilcoxon matched pairs test), **(B)** α_4 (Wilcoxon matched pairs test), **(C)** α_5 (paired t test) nor **(D)** β_1 (paired t test). In addition, culture under hypoxia for 8 hours did not affect the number of neutrophils that stained positive for **(E)** α_1 (Wilcoxon matched pairs test), **(F)** α_4 (paired t test), **(G)** α_5 (paired t test) and **(H)** β_1 (Wilcoxon matched pairs test). Data is presented as matched observations of all 7 donors.

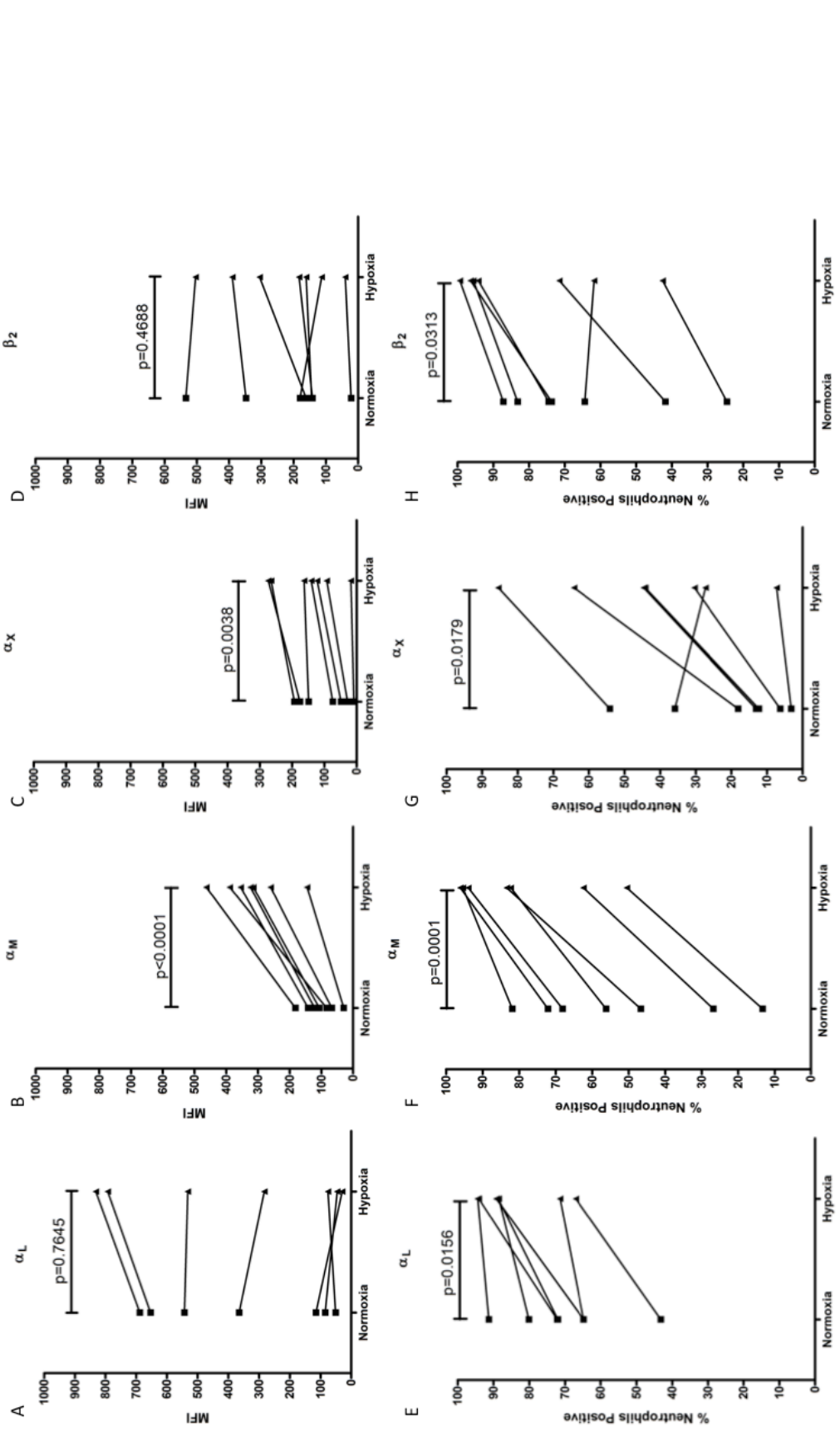


Figure 3.5: Hypoxia enhances expression of β_2 integrins. Neutrophils were isolated from 7 healthy donors and cultured for 8 hours under normoxia or hypoxia. Cells were stained for α_L (CD11a), α_M (CD11b), α_X (CD11c) and β_2 (CD18) and analysed by flow cytometry. **(A)** Surface expression of α_L was not affected by hypoxia (paired t test). **(B)** Hypoxia significantly increased surface α_M (paired t test). **(C)** Surface α_X expression was enhanced by hypoxia (paired t test). **(D)** Surface β_2 expression was not affected by cultured under hypoxia (Wilcoxon matched pairs test). **(E)** The number of neutrophils expressing α_L was significantly higher under hypoxia (Wilcoxon matched pairs test). **(F)** Neutrophils positive for α_M expression were significantly higher under hypoxia (paired t test). **(G)** The number of neutrophils positive for α_X expression was significantly higher in cells cultured under hypoxia (paired t test). **(H)** Neutrophils positive for β_2 expression were significantly higher under hypoxia (Wilcoxon matched pairs test). Data is presented as matched observations of all 7 donors.

3.4 Optimisation of neutrophil static adhesion assays

Having found that hypoxia modulates neutrophil integrin expression, I then evaluated the effects of hypoxia upon neutrophil binding to immobilised integrin ligands. In this thesis, I assessed adhesion to three ligands: fibrinogen, fibronectin and ICAM-1.

3.4.1 Immobilised fibrinogen adhesion assay optimisation

To examine Mac-1 activation, I optimised fibrinogen adhesion assays. Three fibrinogen concentrations were tested for optimal coating concentrations. Adhesion of PMA-stimulated neutrophils was comparable across all fibrinogen concentrations (Figure 3.6A). Given these results, 200μg/ml fibrinogen was chosen as the coating concentration for all further fibrinogen adhesion assays.

Titration of the Mac-1-specific antibody, 2LPM19c, was performed (Figure 3.6B). Inhibition of neutrophil binding to immobilised fibrinogen was observed from 2.5μg/ml. To ensure saturation of the blocking antibody, 10μg/ml was selected for further experiments. Blockade using mAb44, an alternative Mac-1 blocking antibody, was also examined but mAb44 was not found to block neutrophil adhesion to fibrinogen (data not shown). Adhesion to uncoated plastic wells was not blocked by treatment with 10μg/ml 2LPM19c (Figure 3.6C).

Fibrinogen immobilisation was also tested using 50mM and 100mM bicarbonate/carbonate buffers, however no significant differences were observed between buffers (data not shown). Therefore, fibrinogen was diluted in PBS for all further experiments.

3.4.2 Immobilised fibronectin adhesion assay optimisation

To study the effects of hypoxia upon β_1 integrin activation, I evaluated neutrophil adhesion to immobilised fibronectin. To optimise the coating concentration of fibronectin, T cells were used to remove potential Mac-1-mediated interactions. T cells were isolated from healthy donors and phenotypically characterised by flow cytometry to confirmed the isolation of $CD3^+$ T cells (Figure 3.7A). T cells also expressed α_4, α_L, β_1 and β_2, but not α_M, indicating the expression of LFA-1($\alpha_L\beta_2$) and VLA-4 ($\alpha_4\beta_1$), but not Mac-1 ($\alpha_M\beta_2$) (Figure 3.7B).

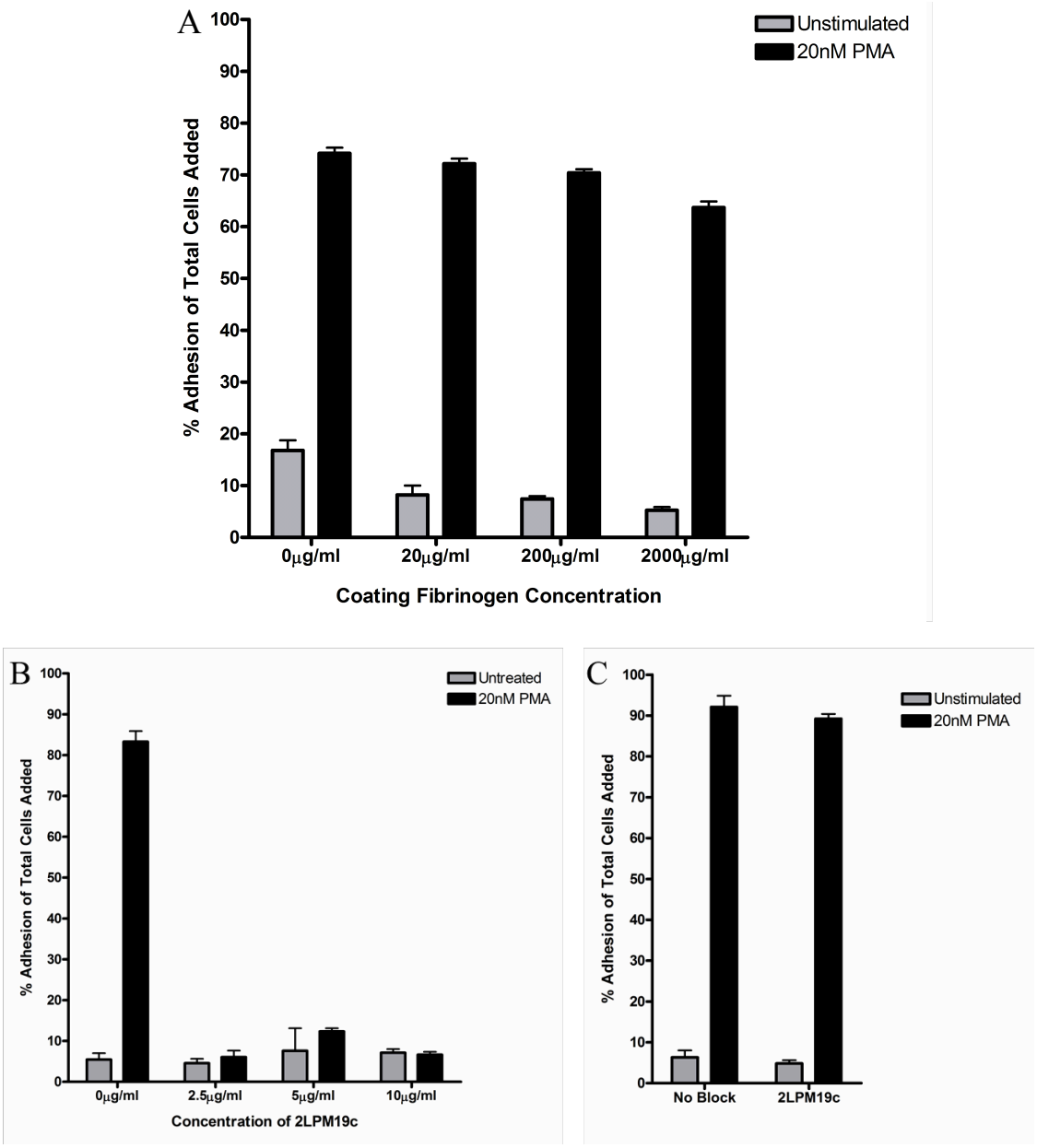


Figure 3.6: Optimisation of fibrinogen adhesion assay. Adhesion to immobilised fibrinogen was used to assess Mac-1 ($\alpha_M\beta_2$) activation. $5x10^5$ fluorescently labelled neutrophils were added to fibrinogen-coated wells in the absence or presence of 20nM PMA for 30 minutes. **(A)** Fibrinogen was immobilised at concentrations of 20µg/ml, 200µg/ml and 2mg/ml. PMA-stimulated neutrophil adhesion did not significantly vary between concentrations. **(B)** Neutrophil adhesion to fibrinogen was evaluated in the presence of the Mac-1 ($\alpha_M\beta_2$)-specific blocking antibody 2LPM19c. Adhesion was blocked from 2.5µg/ml 2LPM19c. **(C)** Neutrophil binding to uncoated plastic well was assessed in the absence and presence of 10µg/ml 2LPM19c. PMA-stimulated neutrophil adhesion was not blocked by Mac-1 ($\alpha_M\beta_2$) blockade by 2LPM19c. Data presented as mean adhesion ±SD of three independent experiments.

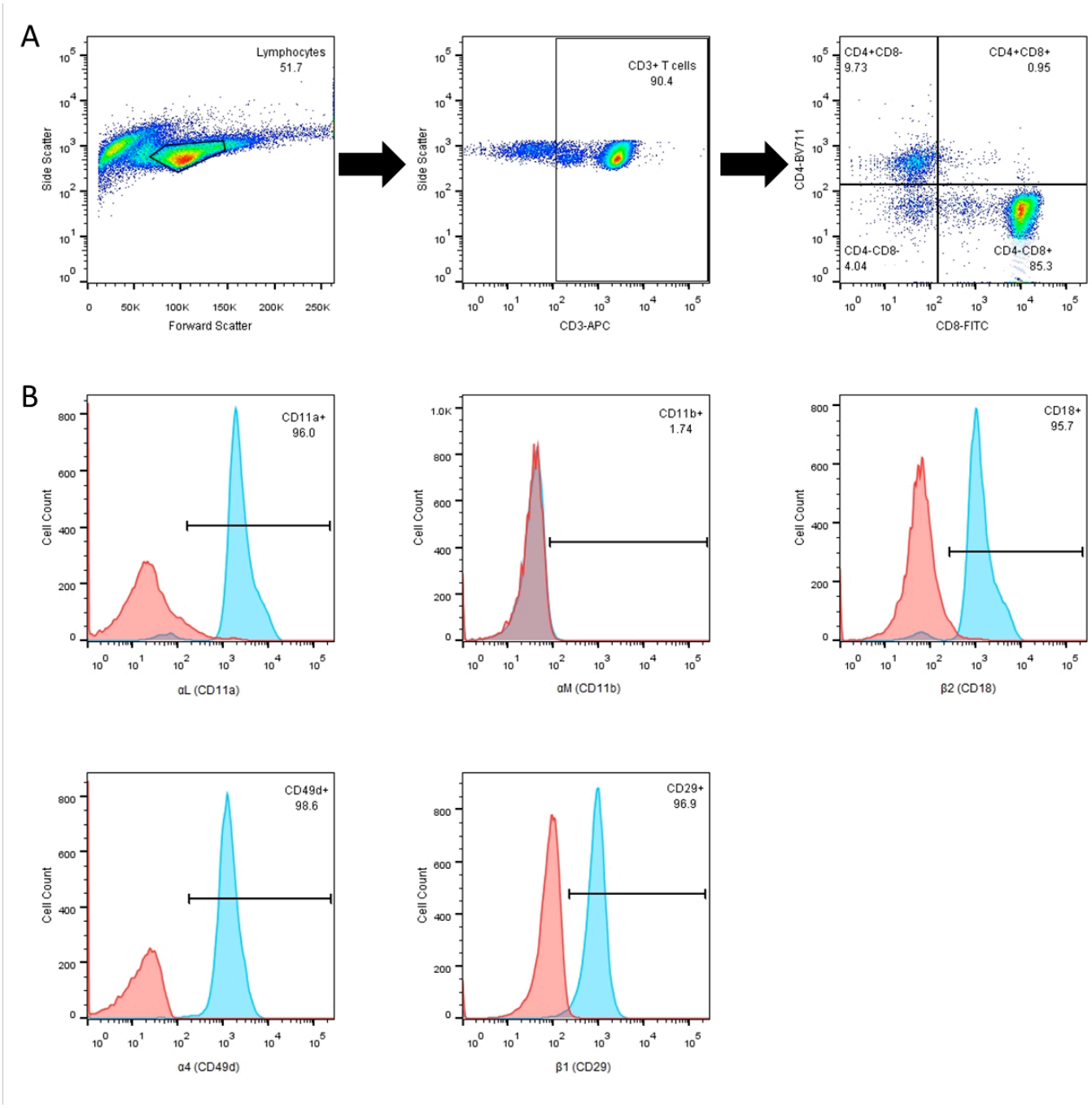


Figure 3.7: CD3$^+$ T cells express LFA-1 and VLA-4, but do not express Mac-1. Isolated T cells were phenotypically characterised by flow cytometry. **(A)** Cultured lymphocytes were stained with the T cell markers CD3, CD4 and CD8. 90.4% of cells stained positive for CD3, which represents 20,000 cells. Further characterisation of these gated CD3$^+$ T cells found that the most were CD8$^+$ T cells. **(B)** CD3$^+$ T cells were found to express α_4 (CD49d), α_L (CD11a), β_1 (CD29) and β_2 (CD18), but not α_M (CD11b). T cell characterisation was performed on three independent occasions, with representative plots and histograms shown above.

Fibronectin was immobilised at concentrations ranging between 10-100μg/ml and T cell adhesion evaluated. $5x10^5$ fluorescently labelled T cells were added to wells in the absence or presence of PMA. PMA-stimulated T cell adhesion was observed with all coating concentrations of fibronectin (Figure 3.8A). For further experiments, 20μg/ml fibronectin was used to coat plates. Immobilisation using 50mM and 100mM bicarbonate/carbonate buffers was also tested, however no significant differences were observed (data not shown), and therefore PBS was used for subsequent experiments to dilute fibronectin.

Neutrophil adhesion to immobilised fibronectin was assessed in the presence of 1, 2, 5 or 10μg/ml of the blocking antibody P5D2, which inhibits CD29 and therefore blocks adhesion mediated by all β_1 integrins. A dose response was observed, with maximal inhibition of PMA-induced neutrophil adhesion achieved with 10μg/ml P5D2 (Figure 3.8B).

3.4.3 Immobilised ICAM-1 adhesion assay optimisation

Finally, I optimised the coating concentration of Fc-conjugated recombinant ICAM-1 (ICAM-1-Fc). T cells were used to remove potential Mac-1-mediated interactions. ICAM-1-Fc was titrated at concentrations ranging from 0.1-5μg/ml and T cell adhesion assessed in the absence or presence of PMA (Figure 3.9A). Increasing adhesion was observed between 3-4μg/ml, which reached a plateau by 5μg/ml. Therefore, 4.5μg/ml ICAM-1-Fc was used to coat wells for further ICAM-1 adhesion assays.

PMA-stimulated T cell adhesion to ICAM-1-Fc was shown to be LFA-1-dependent using: TS1/18, an anti-β_2 antibody that blocks all β_2 integrin adhesion (Figure 3.9B); and mAb38, an anti-α_L antibody that specifically blocks LFA-1 (Figure 3.9C). A dose-dependent inhibitory effect was observed with TS1/18, whilst mAb38 potently inhibited PMA-stimulated T cell adhesion to ICAM-1-Fc.

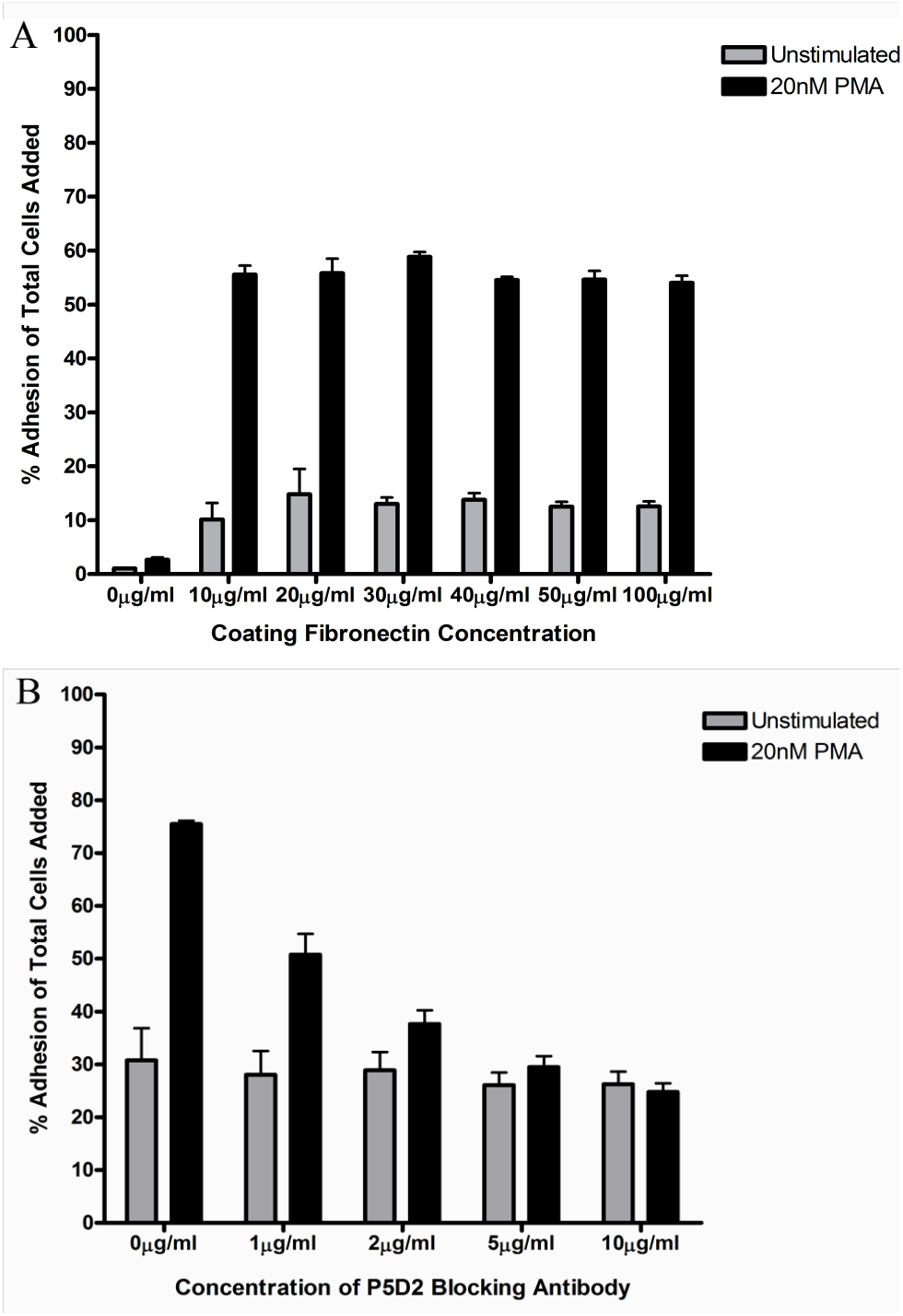


Figure 3.8: Optimisation of fibronectin adhesion assay. Adhesion to immobilised fibronectin was used to evaluate β_1 integrin activation. **(A)** $5x10^5$ fluorescently labelled T cells were cultured in the absence or presence of 20nM PMA for 30 minutes. Adhesion was observed at all coating concentrations tested. 20μg/ml fibronectin was used for further experiments. **(B)** The pan-β_1 integrin blocking antibody, P5D2, was titrated to determine β_1 integrin specific adhesion. $5x10^5$ fluorescently labelled neutrophils were added to fibronectin-coated wells in the absence or presence of 20nM PMA and 1, 2, 5 or 10μg/ml P5D2. A dose-dependent inhibition of neutrophil adhesion was observed. Data is shown as mean adhesion ±SD of three independent experiments.

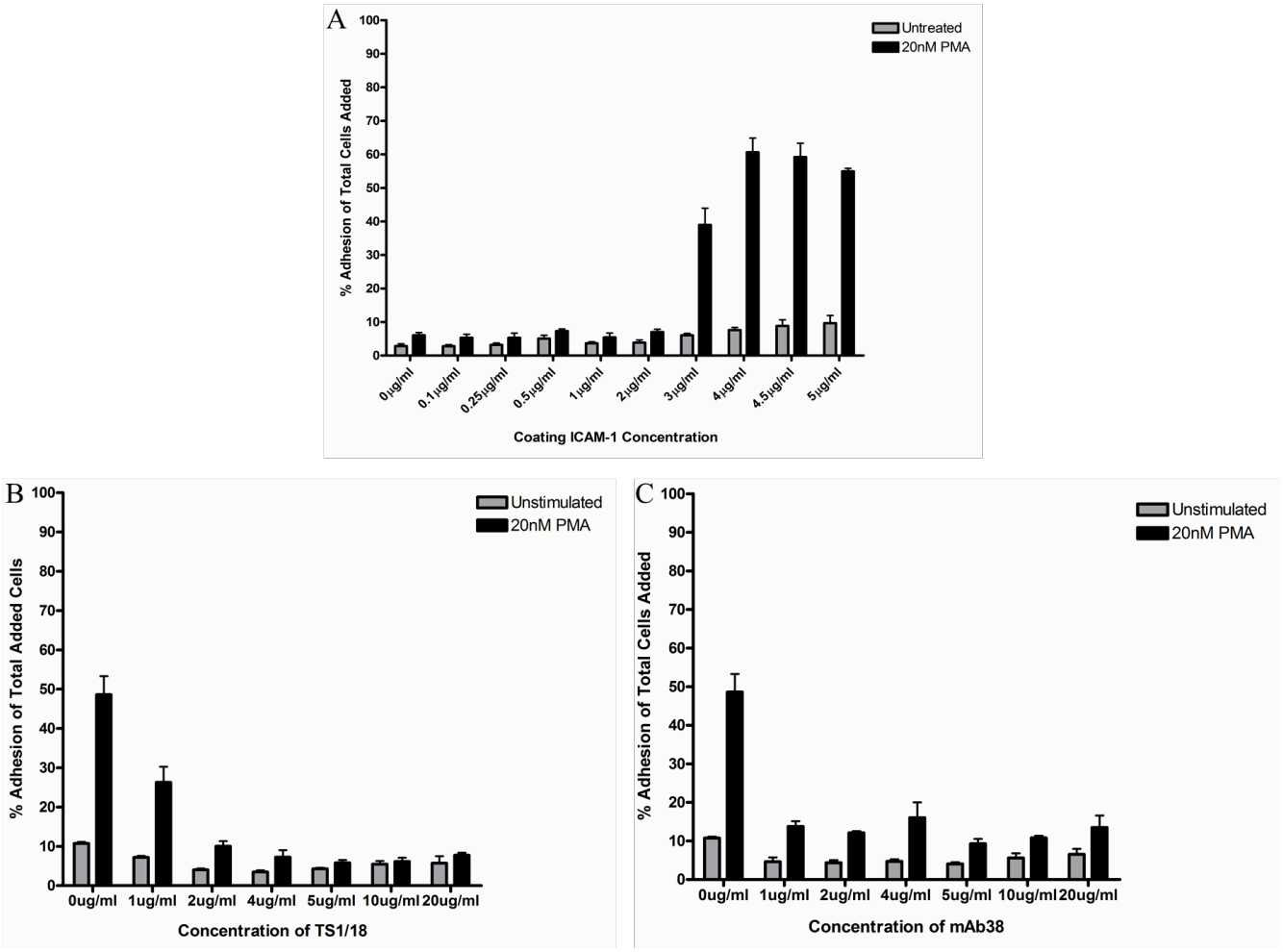


Figure 3.9: Optimisation of ICAM-1 adhesion assay. Adhesion to immobilised ICAM-1-Fc was used to evaluate β_2 integrin activation **(A)** $5x10^5$ fluorescently labelled T cells were added to wells coated with ICAM-1-Fc ranging from 0.1-5µg/ml, in the absence or presence of 20nM PMA. Adhesion was observed in wells coated with at least 3µg/ml ICAM-1-Fc. Increasing adhesion reached a plateau by 4µg/ml ICAM-1-Fc. 4.5µg/ml ICAM-1-Fc was used for further experiments. **(B)** $5x10^5$ fluorescently labelled T cells were added to wells in the absence or presence of 20nM PMA and 1-10µg/ml TS1/18, an anti-β_2 (CD18) blocking antibody. A dose-dependent inhibition of T cell adhesion was observed. **(C)** PMA-stimulated T cell adhesion was also assessed in the presence of 1-10µg/ml mAb38, an anti-α_L (CD11a) antibody. PMA-stimulated adhesion was inhibited from 1µg/ml mAb38. Data is presented as mean adhesion ±SD from three independent experiments.

3.5 Hypoxia reduces neutrophil adhesion to immobilised integrin ligands

Having optimised coating concentrations, I subsequently evaluated the effects of hypoxia upon neutrophil adhesion to immobilised fibrinogen, fibronectin and ICAM-1-Fc following 30 minutes and 8 hours exposure to normoxia or hypoxia. $5x10^5$ fluorescently labelled neutrophils were added to coated wells in the absence or presence of PMA and allowed to adhere for 30 minutes under normoxia or hypoxia. 30 minutes hypoxic exposure did not significantly affect neutrophil adhesion to immobilised fibrinogen, fibronectin or ICAM-1-Fc (Figure 3.10A-C).

Neutrophil adhesion was also assessed following 8 hours culture under normoxia or hypoxia. Adhesion of $5x10^5$ fluorescently labelled neutrophils in the absence or presence of PMA was evaluated. PMA-stimulated neutrophil adhesion following 8 hours culture under hypoxia was significantly reduced to immobilised fibrinogen ($p<0.01$), fibronectin ($p<0.05$) and ICAM-1-Fc ($p<0.01$) compared to normoxic controls (Figure 3.11A-C).

3.6 Hypoxia modulates endothelial adhesion molecule expression in response to LPS and TNF-α

Having found reduced adhesion to immobilised fibrinogen, fibronectin and ICAM-1-Fc following 8 hours culture under hypoxia, I then studied neutrophil adhesion to HUVEC. HUVEC viability was first assessed in response to hypoxia, to ensure that any modulation of endothelial adhesion molecule expression was not a result of cell death. HUVEC were cultured under normoxia or hypoxia for up to 24 hours and HIF-1α or HIF-2α expression confirmed by immunoblot (Figure 3.12A). HUVEC viability was not significantly different at 4 and 8 hours culture under normoxia or hypoxia. Following 24 hours culture however, OD values were significantly higher in HUVEC cultured under normoxia, indicating cell proliferation that was not observed in cells cultured under hypoxia ($p<0.05$) (Figure 3.12B).

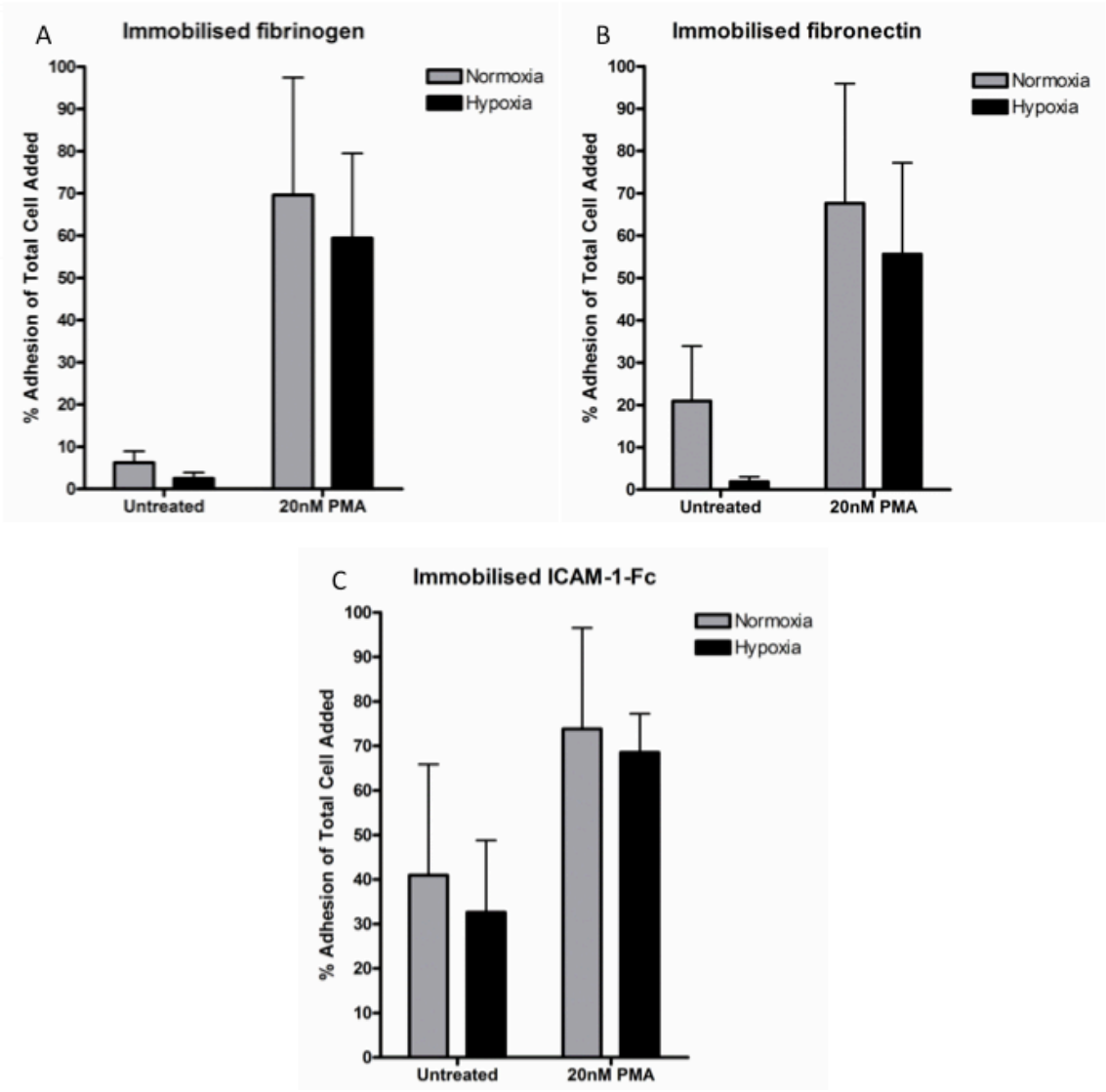


Figure 3.10: Neutrophil adhesion to immobilised integrin ligands is not affected by 30 minutes hypoxia. The effects of 30 minutes hypoxia upon neutrophil adhesion to immobilised **(A)** fibrinogen **(B)** fibronectin and **(C)** ICAM-1-Fc were studied. $5x10^5$ fluorescently labelled neutrophils were added to coated wells in the absence or presence of 20nM PMA and allowed to adhere for 30 minutes under normoxia or hypoxia. Hypoxia did not have a significant effect on neutrophil adhesion to immobilised integrin ligands, as determined by two-way ANOVA with Bonferroni's post-test. Data is presented as mean adhesion ±SD of three independent experiments.

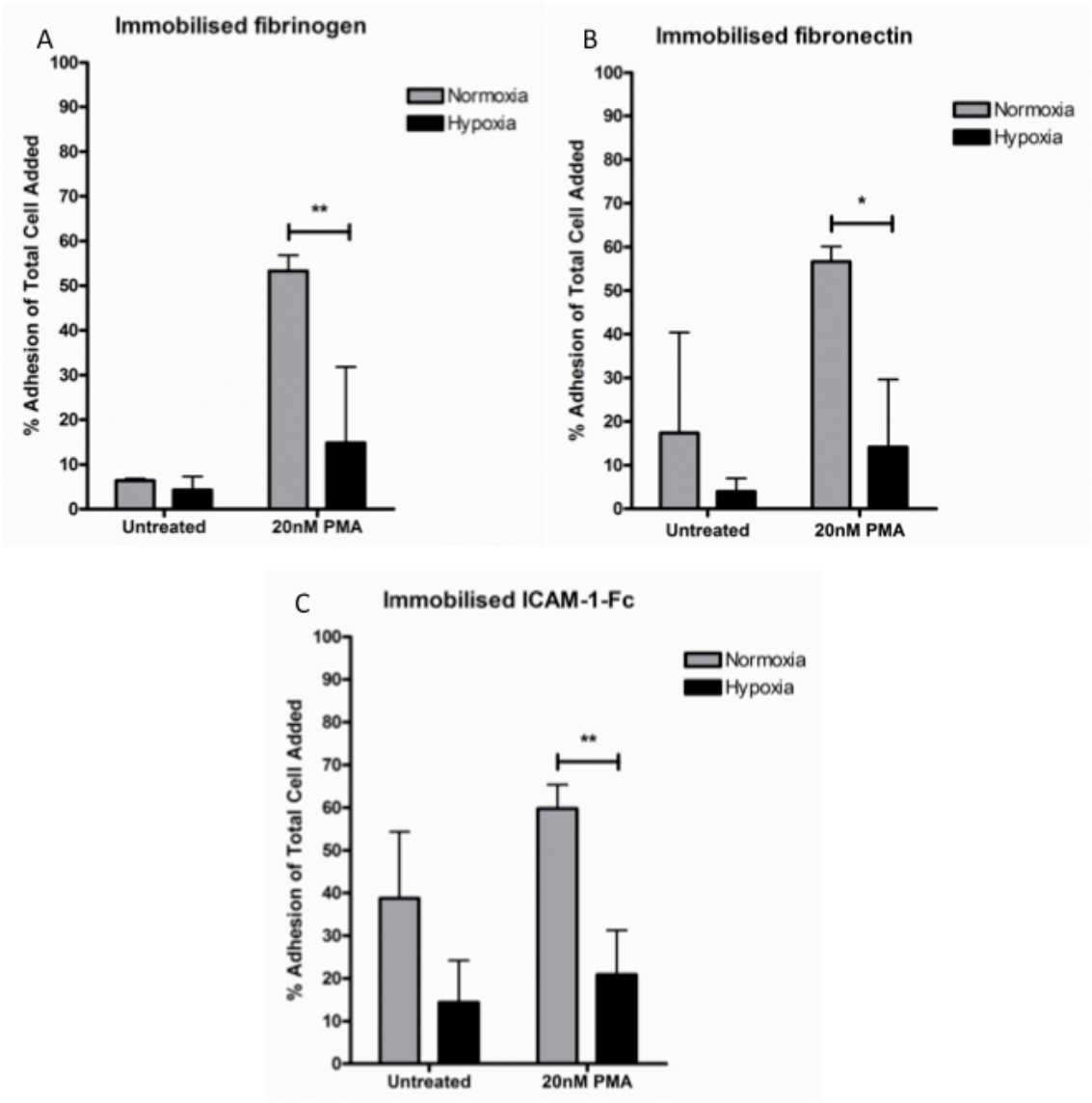
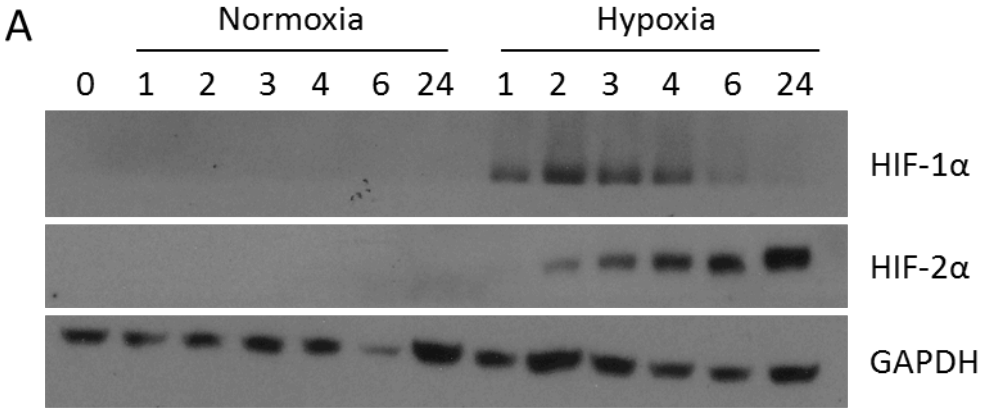


Figure 3.11: Neutrophils cultured under hypoxia for 8 hours have reduced adhesion to immobilised integrin ligands. The effects of hypoxia upon neutrophil firm adhesion to immobilised **(A)** fibrinogen **(B)** fibronectin and **(C)** ICAM-1-Fc following 8 hours culture were studied. Isolated neutrophils were cultured for 8 hours under normoxia or hypoxia. $5x10^5$ fluorescently labelled neutrophils were added to coated wells in the absence or presence of 20nM PMA and adhesion assessed. Neutrophils cultured under hypoxia for 8 hours had significantly reduced PMA-stimulated adhesion to immobilised integrin ligands, as determined by a two-way ANOVA analysis with a Bonferroni's post-test. Data is presented as mean adhesion ±SD of three independent experiments. *=$p<0.05$, **=$p<0.01$



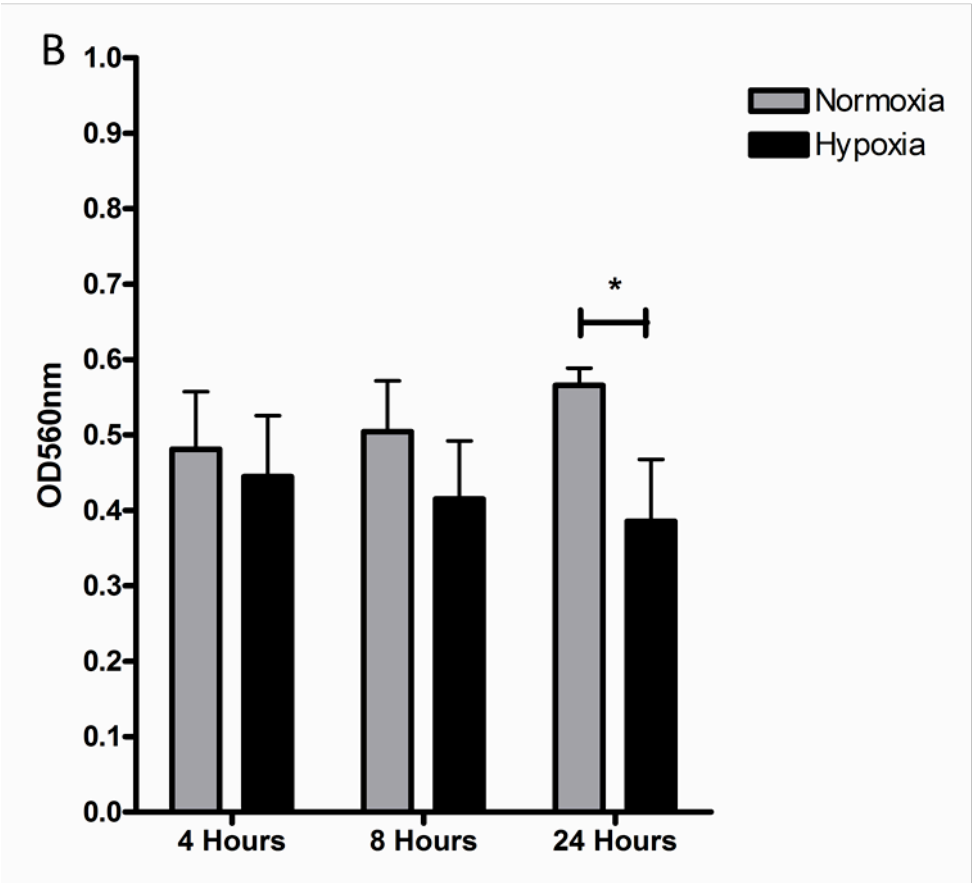


Figure 3.12: Hypoxia suppresses endothelial proliferation. MTT assays were performed to assess endothelial viability in response to hypoxia. $1.5x10^4$ HUVEC were cultured under normoxia or hypoxia for 4, 8 or 24 hours before being assessed for cell viability. OD values were not significantly different at 4 and 8 hours culture under normoxia or hypoxia. Following 24 hours, OD values obtained from HUVEC cultured under normoxia were significantly higher than cells cultured under hypoxia. Statistical analysis was determined by means of a two-way ANOVA with a Bonferroni's post-test. Data is presented as mean OD ±SD from three independent experiments. *=$p<0.05$

Prior to accessing neutrophil adhesion to HUVECs under hypoxia, I first examined the effects of hypoxia upon adhesion molecule expression in response to external stimuli. HUVEC were cultured in the absence or presence of LPS or TNF-α for 4, 8 and 24 hours under normoxia or hypoxia and accessed for surface expression of E-selectin, ICAM-1, ICAM-2 and VCAM-1 by flow cytometry.

In untreated cells, only ICAM-1 varied over time (Figure 3.13A-D). Minimal E-selectin expression was seen under both normoxia and hypoxia at all time points studied (Figure 3.13A). Induction of ICAM-1 expression was observed in untreated cells under normoxia, which peaked at 8 hours (Figure 3.13B). ICAM-1 expression was significantly higher under normoxia at all time points studied ($p<0.05$ at 4 hours, $p<0.001$ at 8 hours and $p<0.001$ at 24 hours). Untreated HUVEC had high basal expression of ICAM-2 and low VCAM-1 expression. Neither ICAM-2 nor VCAM-1 expression varied over time (Figure 3.13C, D).

LPS treatment induced E-selectin expression in HUVEC, which peaked at 4 hours stimulation (Figure 3.14A). E-selectin expression following 4 hours LPS stimulation was significantly lower in hypoxic cells ($p<0.001$). ICAM-1 and VCAM-1 expression was also induced by LPS, but there were no differences between HUVEC stimulated under normoxia and hypoxia (Figure 3.14B, D). LPS suppressed ICAM-2 expression under normoxia at all time points examined compared to baseline (Figure 3.14C). ICAM-2 expression was significantly higher in hypoxic HUVEC following 8 hours LPS stimulation ($p<0.05$) (Figure 3.14C).

TNF-α treatment induced E-selectin expression under normoxia, which peaked at 8 hours stimulation (Figure 3.15A). E-selectin expression was significantly lower in hypoxic HUVEC at all time points ($p<0.05$ at 4 hours, $p<0.001$ at 8 hours and $p<0.05$ at 24 hours). ICAM-1 expression was induced by TNF-α stimulation, which peaked at 8 hours under normoxia. Significantly higher levels of ICAM-1 expression however, were observed in HUVEC following 24 hours TNF-α stimulation under hypoxia compared to normoxia ($p<0.001$) (Figure 3.15B). ICAM-2 expression did not significantly vary between cells stimulated under normoxia and hypoxia (Figure 3.15C). There were no significant differences in TNF-α induced VCAM-1 expression between cells stimulated under normoxia and hypoxia (Figure 3.15D).

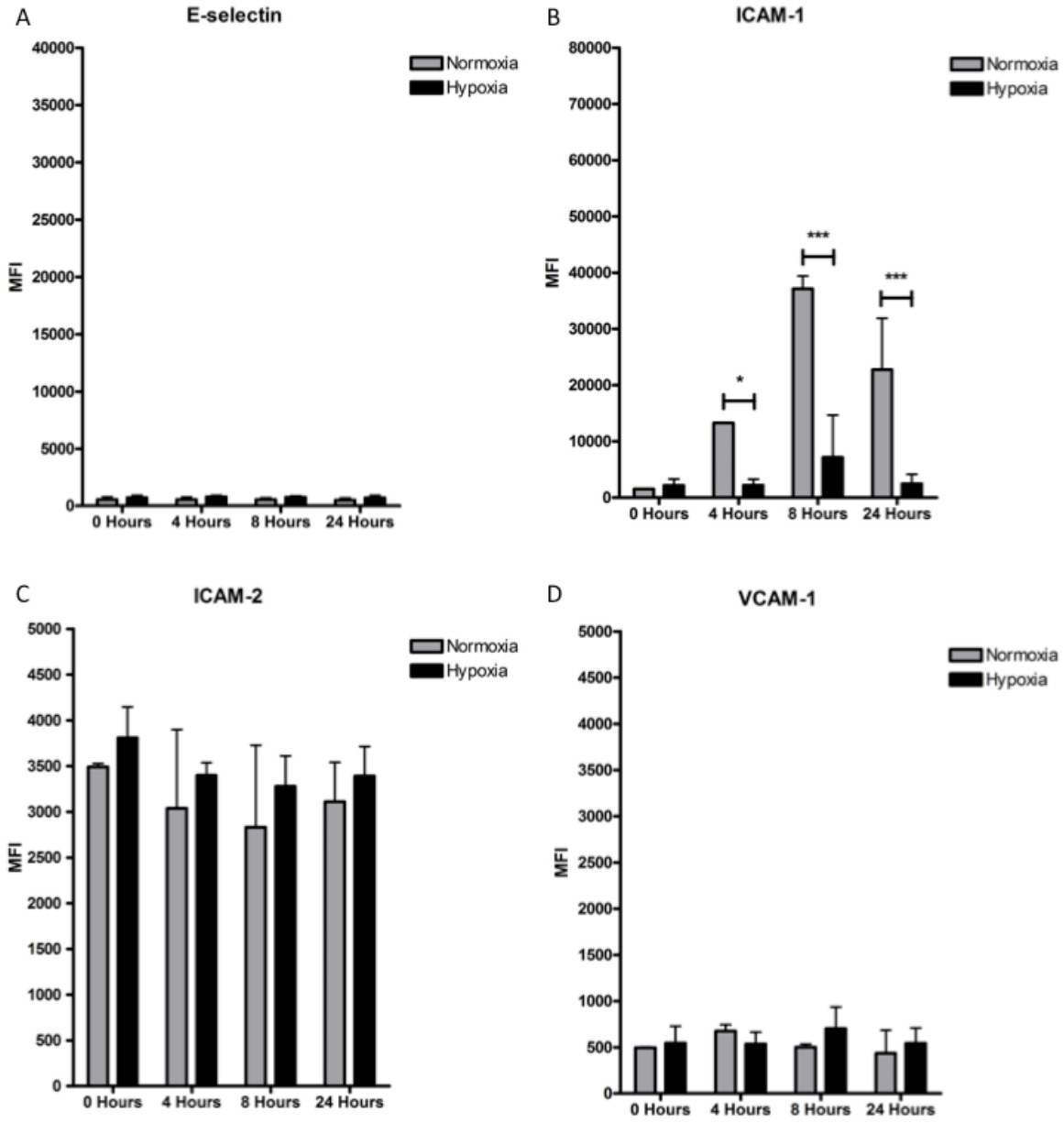


Figure 3.13: Hypoxia modulates ICAM-1 expression in untreated endothelial cells. To examine unstimulated endothelial responses to hypoxia, HUVEC were cultured under normoxia or hypoxia for 4, 8 or 24 hours and then examined for expression for E-selectin, ICAM-1, ICAM-2 and VCAM-1. **(A)** Low baseline expression E-selectin expression was observed. Expression did not vary over time and there were no significant differences between normoxic and hypoxic HUCEC. **(B)** Induction of ICAM-1 expression was observed under normoxia. ICAM-1 induction was significantly lower in hypoxic HUVEC. **(C)** ICAM-2 expression did not significantly change over time and was not affected by hypoxia. **(D)** VCAM-1 expression did not vary over time and was not modulated by hypoxia. Data is presented as mean fluorescence intensity (MFI) ±SD of three independent experiments. Statistical significance was determined by two-way ANOVA with a Bonferroni's post-test. *= $p<0.05$, ***= $p<0.001$

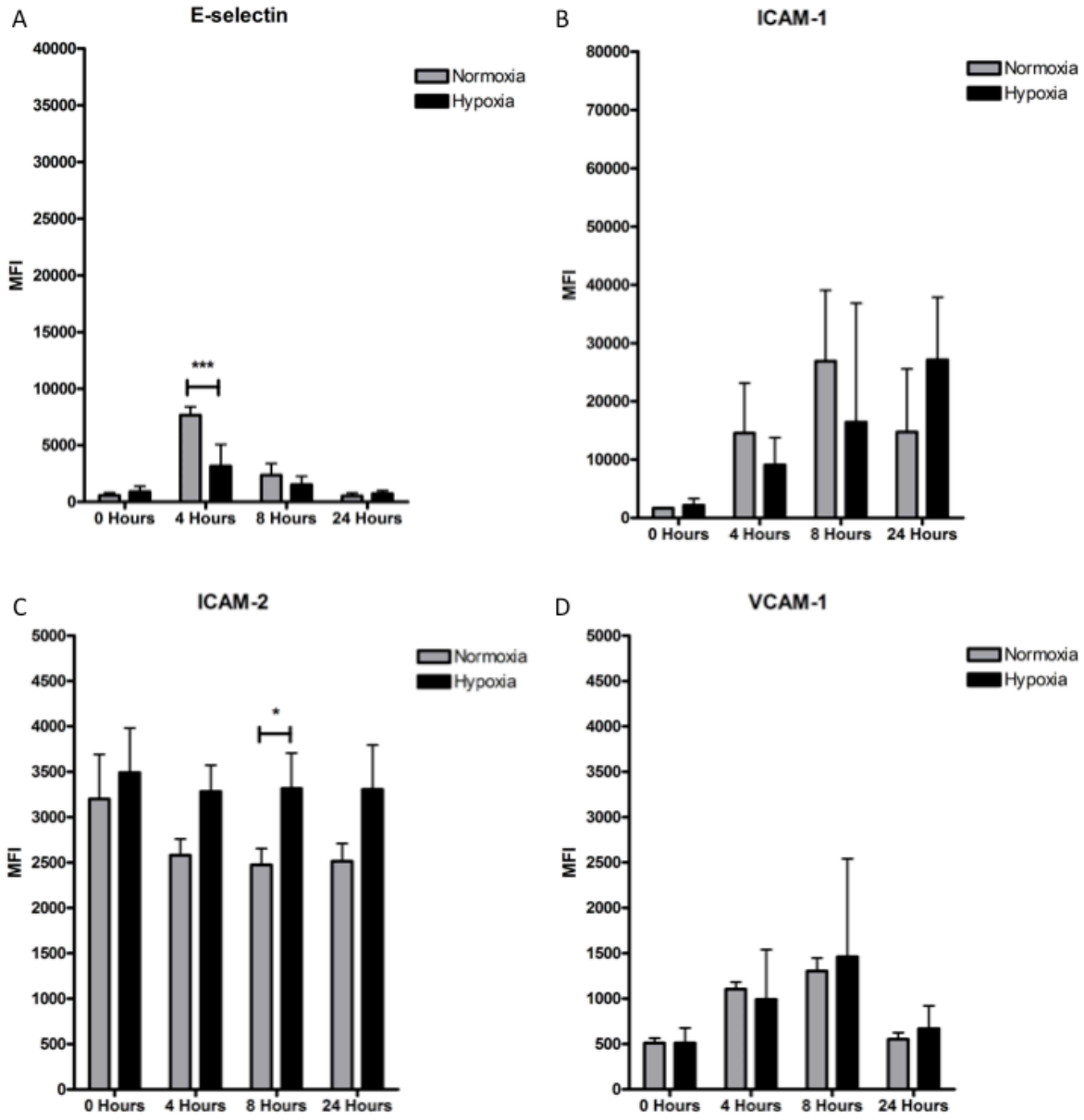


Figure 3.14: Hypoxia modulates E-selectin induction and ICAM-2 down regulation in LPS-stimulated HUVEC. Endothelial responses to LPS were studied under normoxia and hypoxia. HUVEC were cultured for 4, 8 or 24 hours in the presence of 100ng/ml LPS under normoxia or hypoxia and then examined for expression of E-selectin, ICAM-1, ICAM-2 and VCAM-1. **(A)** LPS induced E-selectin expression in normoxic HUVEC, which was significantly lower under hypoxia. **(B)** LPS upregulated ICAM-1 expression under both normoxia and hypoxia. Hypoxia did not significantly modulate ICAM-1 expression. **(C)** LPS stimulation reduced ICAM-2 expression in normoxic cells. There was a trend to increased ICAM-2 expression under hypoxia, with expression levels being significantly higher under hypoxia following 8 hours LPS stimulation. **(D)** VCAM-1 expression was observed in both normoxic and hypoxic cells treated with LPS. There were no significant differences between normoxia and hypoxia. Data is presented as mean fluorescence intensity (MFI) ±SD of three independent experiments. Statistical significance was determined by two-way ANOVA with a Bonferroni's post-test. *= $p<0.05$, ***= $p<0.001$

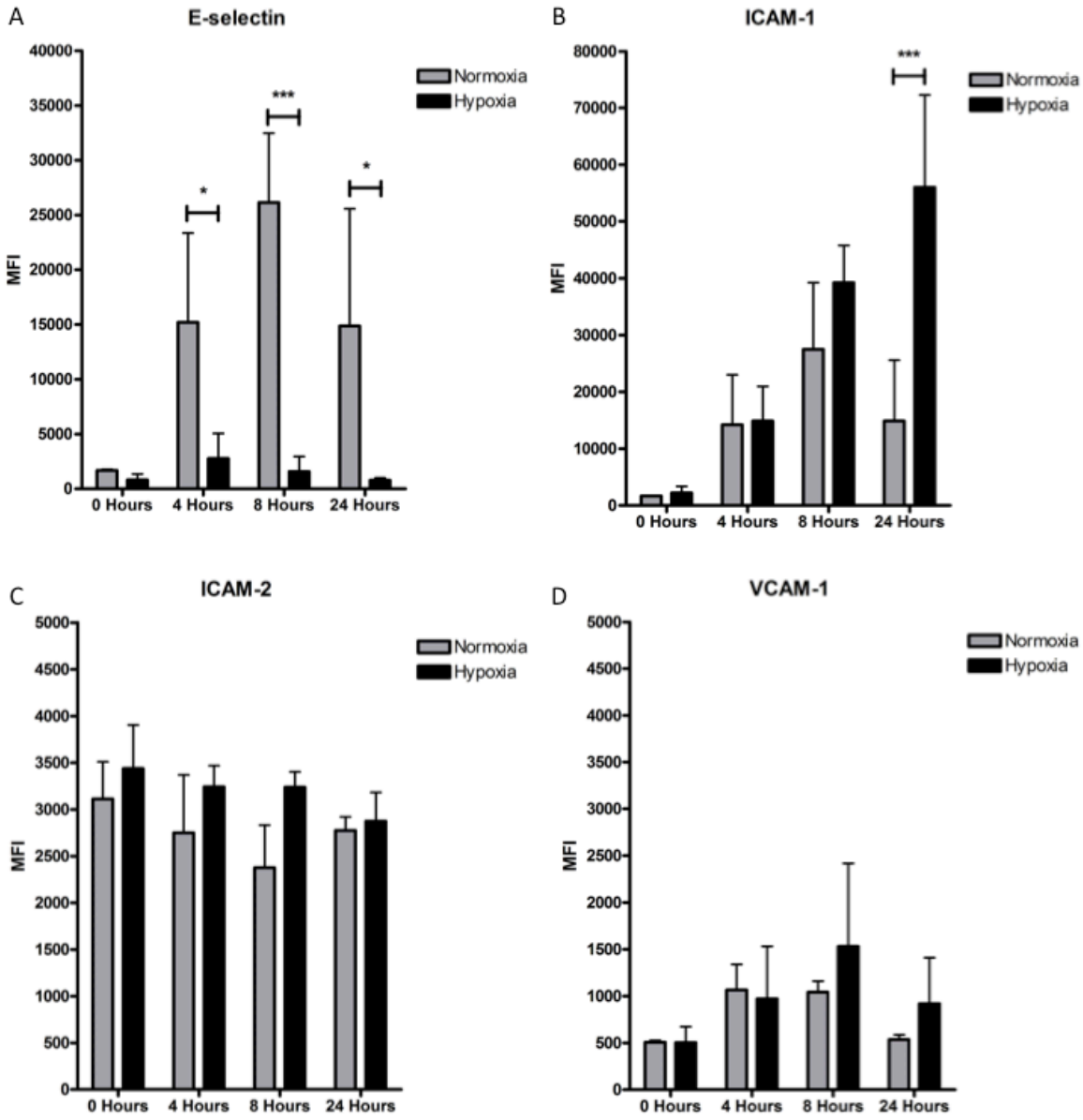


Figure 3.15: Hypoxia reduces E-selectin expression but increases ICAM-1 expression in TNF-α treated endothelial cells. Endothelial responses to TNF-α under normoxia and hypoxia were studied. ECs were treated with 10ng/ml TNF-α for 4, 8 or 24 hours under normoxia or hypoxia and examined for expression of E-selectin, ICAM-1, ICAM-2 and VCAM-1. **(A)** TNF-α stimulation induced expression of E-selectin under normoxia. This upregulation was significantly suppressed by hypoxia. **(B)** TNF-α induced ICAM-1 expression in normoxic ECs. ICAM-1 expression was modulated by hypoxia, with significantly higher expression at 24 hours stimulation. Moreover, whilst expression in normoxic HUVEC had begun to decrease at 24 hours, ICAM-1 expression in hypoxic cells was highest at 24 hours, suggesting a change in ICAM-1 expression dynamics. **(C)** ICAM-2 expression was not significantly different between normoxia and hypoxia. **(D)** VCAM-1 upregulation was observed in both normoxic and hypoxic HUVEC, but differences were not significant. Data is presented as mean fluorescence intensity (MFI) ±SD of three independent experiments. Statistical significance was determined by two-way ANOVA with a Bonferroni's post-test. *= $p<0.05$, ***= $p<0.001$

3.7 Hypoxia promotes neutrophil adhesion to endothelial monolayers

Having found a modulatory effect of hypoxia upon endothelial adhesion molecule expression in response to LPS and TNF-α, I then examined the effects of hypoxia upon neutrophil adhesion to ECs. HUVEC monolayers were cultured overnight under normoxia or hypoxia in the absence or presence of LPS or TNF-α. Neutrophils were isolated and also cultured under normoxia or hypoxia overnight. Following overnight cultures, neutrophils were fluorescently labelled and adhesion of $5x10^5$ neutrophils to HUVEC was evaluated in response to PMA, LPS or TNF-α. Adhesion was assessed with normoxic neutrophils to normoxic HUVEC and hypoxic neutrophils to hypoxic HUVEC.

Neutrophil adhesion to untreated HUVEC, in the absence of any stimuli, was significantly higher when both cells were cultured under hypoxia ($p<0.01$) (Figure 3.16). Hypoxia also significantly increased LPS-stimulated neutrophil adhesion to untreated HUVEC ($p<0.001$) (Figure 3.16). There were trends for increased neutrophil adhesion to HUVEC in response to PMA and TNF-α under hypoxia, however these differences did not reach significance (Figure 3.16).

Overnight culture of HUVEC and neutrophils under hypoxia did not have a significant effect upon unstimulated, LPS- or TNF-α-stimulated neutrophil adhesion to LPS-activated HUVEC (Figure 3.17). In contrast, PMA-stimulated neutrophil adhesion to endothelial monolayers stimulated with LPS overnight was significantly higher under hypoxia compared to normoxia ($p<0.01$) (Figure 3.17).

Hypoxia significantly increased both unstimulated and PMA-stimulated neutrophil adhesion to TNF-α-activated endothelial monolayers ($p<0.05$ and $p<0.001$ respectively) (Figure 3.18). There were trends to higher LPS- and TNF-α-stimulated neutrophil adhesion under hypoxia, however these differences did not reach statistical significance (Figure 3.18).

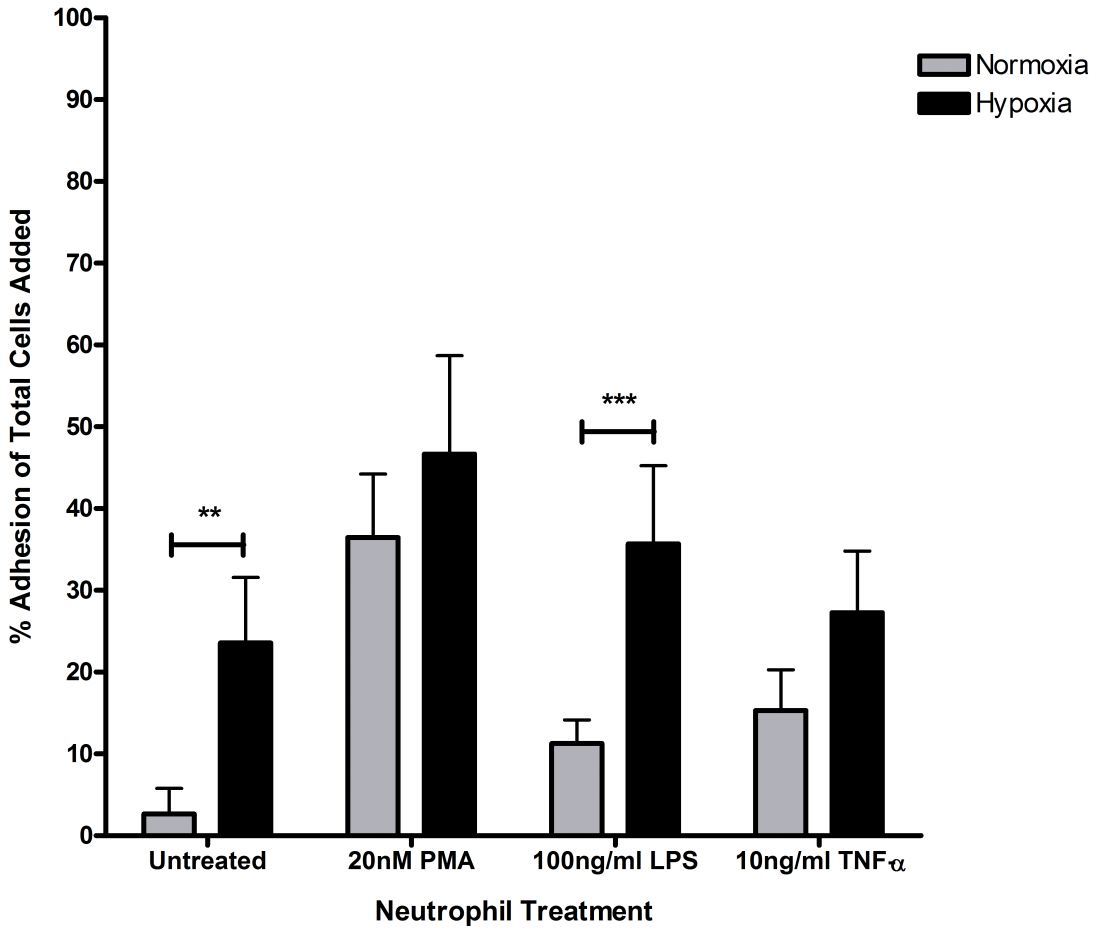


Figure 3.16: Hypoxia enhances unstimulated and LPS-stimulated neutrophil adhesion to untreated endothelial monolayers. The effects of hypoxia upon neutrophil adhesion to resting HUVEC were examined. Endothelial monolayers were cultured under normoxia or hypoxia overnight. Neutrophils were isolated from whole blood and cultured overnight under normoxia or hypoxia. $5x10^5$ fluorescently labelled neutrophils were added to endothelial monolayers in the absence or presence of 20nM PMA, 100ng/ml LPS or 10ng/ml TNF-α. Hypoxia significantly increased both unstimulated and LPS-stimulated neutrophil adhesion to resting HUVEC. Statistical significance was determined by means of a two-way ANOVA with a Bonferroni's post-test. Data is presented as mean adhesion ±SD of four independent experiments. **=$p<0.01$, ***=$p<0.001$

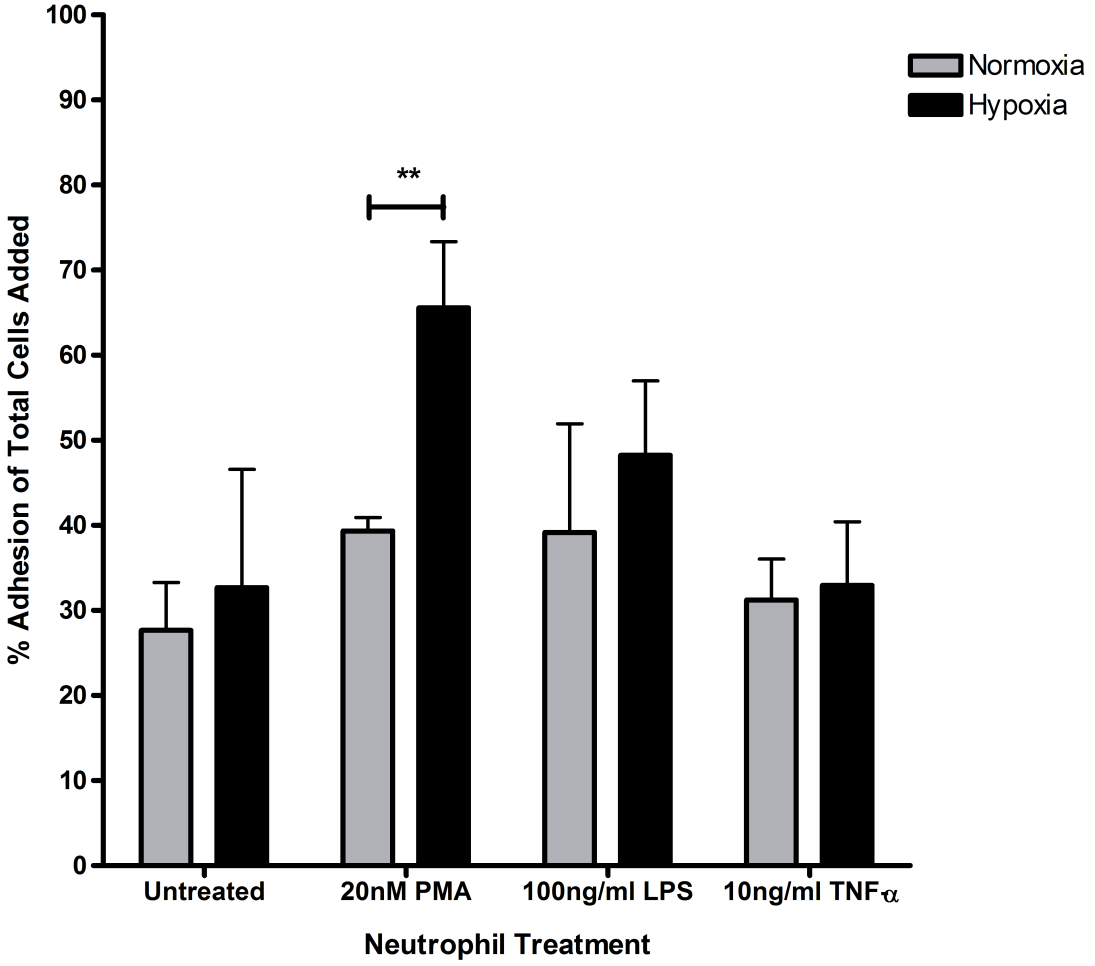


Figure 3.17: Hypoxia increases PMA-stimulated neutrophil adhesion to LPS-activated endothelial monolayers. The effects of hypoxia upon neutrophil adhesion to LPS-stimulated HUVEC were also studied. Endothelial monolayers were treated with 100ng/ml LPS overnight under normoxia or hypoxia. Neutrophils were isolated from whole blood and cultured overnight. $5x10^5$ fluorescently labelled neutrophils were added to endothelial monolayers in the absence or presence of 20nM PMA, 100ng/ml LPS or 10ng/ml TNF-α. Hypoxia significantly increased PMA-stimulated neutrophil adhesion to LPS-activated HUVEC. Statistical significance was determined by means of a two-way ANOVA with a Bonferroni's post-test. Data is presented as mean adhesion ±SD of four independent experiments. **=$p<0.01$

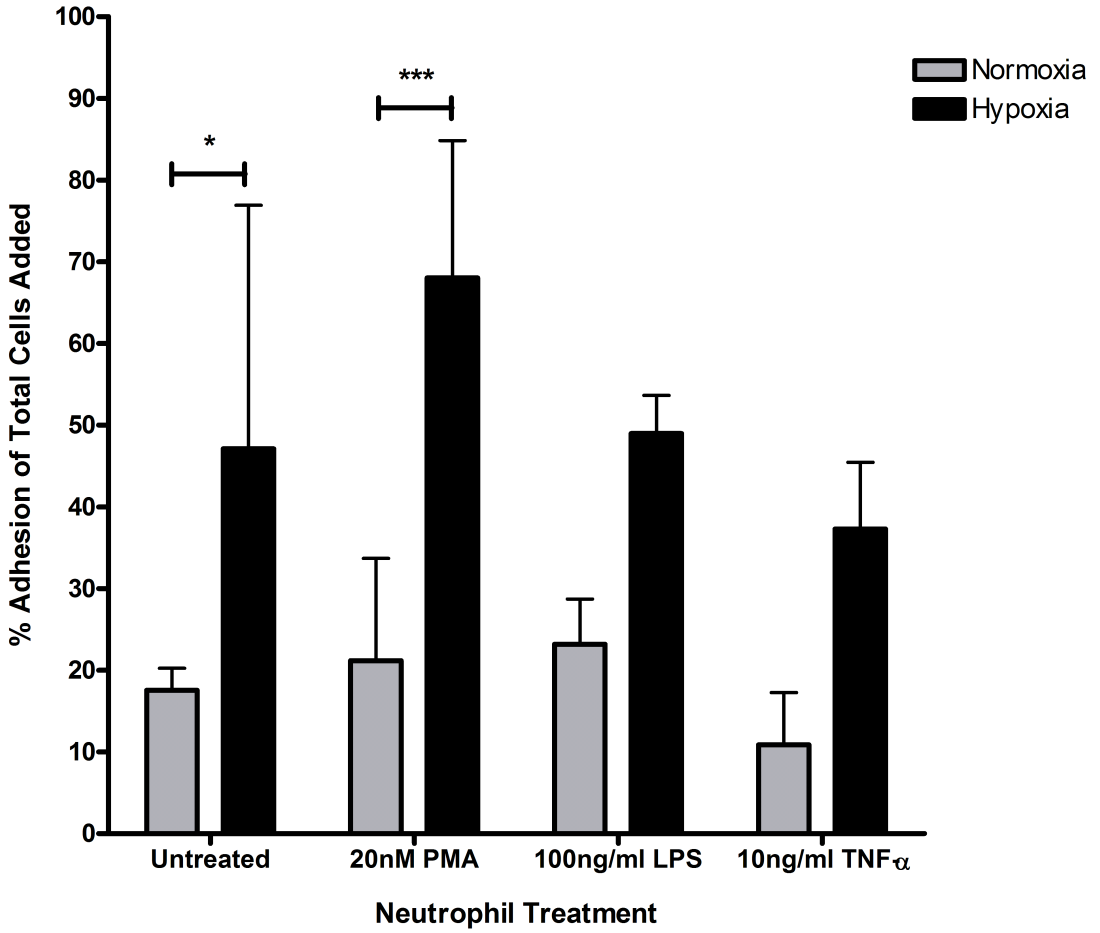


Figure 3.18: Hypoxia enhances unstimulated and PMA-stimulated neutrophil adhesion to TNF-α activated endothelial monolayers. The effects of hypoxia upon neutrophil adhesion to TNF-α activated ECs were examined. HUVEC were treated overnight with 10ng/ml TNF-α under normoxia or hypoxia. Neutrophils were isolated from whole blood and cultured overnight under normoxia or hypoxia. $5x10^5$ fluorescently labelled neutrophils were added to endothelial monolayers in the absence or presence of 20nM PMA, 100ng/ml LPS or 10ng/ml TNF-α. Unstimulated and PMA-stimulated neutrophil adhesion was significantly higher under hypoxia. Statistical significance was determined by means of a two-way ANOVA with a Bonferroni's post-test. Data is presented as mean adhesion ±SD of four independent experiments. *=$p<0.05$, ***=$p<0.001$

3.8 Hypoxia increases neutrophil trans-endothelial migration

Having studied the effects of hypoxia on integrin expression and integrin-mediated adhesion, I subsequently examined trans-endothelial migration under hypoxia. Low levels of neutrophil transmigration were observed in the absence of a chemoattractant. Neutrophil transmigration across HUVEC monolayers was promoted by the presence of IL-8 (Figure 3.19). In addition, hypoxia significantly increased IL-8-induced neutrophil transmigration across resting endothelial monolayers ($p<0.05$) (Figure 3.19A).

I also studied the effects of hypoxia upon neutrophil transmigration across activated ECs. HUVEC were cultured for 24 hours with TNF-α prior to transmigration. There was a trend to increased transmigration in the absence of a chemoattractant, however this difference did not reach statistical significance. Hypoxia significantly increased IL-8-induced neutrophil migration across TNF-α activated HUVEC ($p<0.05$) (Figure 3.19B).

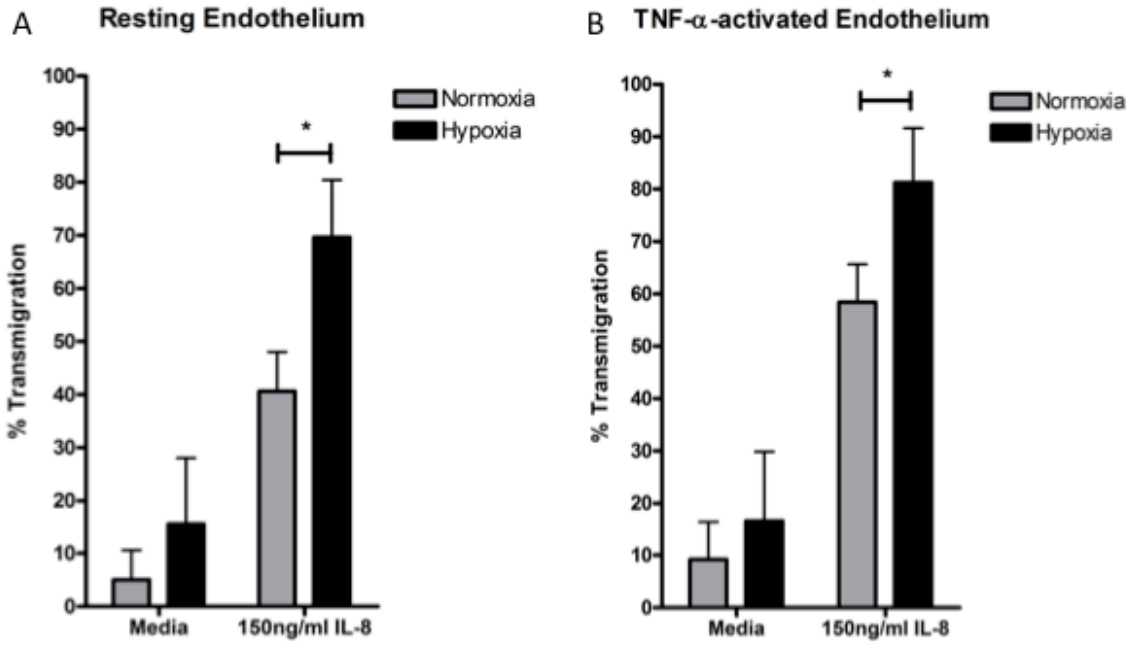


Figure 3.19: Hypoxia increases IL-8-induced neutrophil trans-endothelial migration. To examine the effects of hypoxia on trans-endothelial migration, neutrophil migration in response to IL-8 was studied. HUVEC were cultured overnight under normoxia or hypoxia in the absence or presence of 10ng/ml TNF-α. $1.1x10^6$ fluorescently labelled neutrophils were added to the apical side of HUVEC in the upper chamber and allowed to transmigrate for 90 minutes under normoxia or hypoxia. **(A)** Neutrophil migration towards IL-8 across resting HUVEC was significantly higher under hypoxia. **(B)** Greater migration was observed across TNF-α activated HUVEC. Hypoxia significantly increased neutrophil trans-endothelial migration towards IL-8 across TNF-α activated HUVEC. Statistical significance was determined by means of a two-way ANOVA with a Bonferroni's post-test. Data is presented as mean transmigration ±SD of three independent experiments. *=$p<0.05$

3.9 Discussion

In this chapter, I have shown that neutrophils express β_1 and β_2 integrins. Moreover, whilst β_1 integrin expression was not modulated by hypoxia, surface expression of LFA-1 ($\alpha_L\beta_2$), Mac-1 ($\alpha_M\beta_2$) and p150,95 ($\alpha_X\beta_2$) increased under hypoxia. Hypoxia modulated adhesion molecule expression in stimulated HUVEC by reducing E-selectin and enhancing ICAM-1 expression. Neutrophil adhesion to immobilised integrin ligands was reduced under hypoxia, but adhesion to HUVEC was increased by hypoxia. In addition, neutrophil trans-endothelial migration was enhanced under hypoxia. This section will discuss my results in context of published studies and highlight possible avenues for future work.

3.9.1 Effects of hypoxia upon neutrophil integrin expression

Published reports have documented the upregulation of β_1 integrins under hypoxia (Blaschke et al., 2002, Keely et al., 2009, Lee et al., 2011), however this effect was not replicated in my experimental data. Published studies demonstrated upregulation of β_1 integrins in embryonic stem cells, fibroblasts and smooth muscles cells cultured in hypoxia. In contrast, my work examined β_1 integrin expression in isolated neutrophils, which may not be subject to the same hypoxic regulation.

Studies have also shown increased β_2 expression under hypoxia (Kong et al., 2004). Whilst I did not find increased MFI, more neutrophils expressed the β_2 subunit under hypoxia. Key experimental differences may account for these discrepant results. Elevated β_2 expression was found in whole blood leukocytes cultured at 5% oxygen for 24 hours (Kong et al., 2004). Given that data was obtained from all leukocytes, the relative contribution from individual cell types cannot be determined. In contrast, my work examined integrin expression in isolated neutrophils cultured in 1% oxygen for 8 hours. Kong and colleagues also found that β_2 subunits associated with CD11 (Kong et al., 2004), however the precise α subunit was not determined. My experimental data show that the MFI for α_M and α_X, but not α_L, increased following 8 hours culture under hypoxia, which may indicate that hypoxia increases expression of Mac-1 ($\alpha_M\beta_2$) and p150,95 ($\alpha_X\beta_2$). Therefore, differences in cells studied, oxygen levels utilised and duration

of incubation, render comparisons between my own and published studies difficult and may account for discrepant results.

3.9.2 Effects of hypoxia upon neutrophil adhesion

There is conflicting evidence regarding the effects of hypoxia upon EC adhesion molecule expression. An early report found that LPS- and TNF-α-stimulated ECs under hypoxia had higher E-selectin expression (Zund et al., 1996). A more recent study found reduced E-selectin expression in ECs stimulated under hypoxia (Maurus et al., 2003). Several differences however, exist between these studies including origin of ECs, oxygen levels and assay to measure adhesion molecule expression. Zund and colleagues used bovine aortic ECs, whilst Maurus et al. examined human aortic ECs. Different oxygen levels were used between studies, with Zund et al. culturing cells at 2% oxygen whilst Maurus and colleagues subjected cells to 1% oxygen. In addition, Maurus et al. evaluated adhesion molecule expression by flow cytometry, whilst Zund et al. quantified E-selectin expression by cell ELISA. Therefore, a combination of species variation, differences in oxygen levels and techniques may explain the contrasting conclusions.

My observations are consistent with work published by Maurus and colleagues. I cultured human umbilical cord vein ECs at 1% oxygen and evaluated E-selectin expression by flow cytometry, which found reduced E-selectin expression in ECs stimulated under hypoxia. Whilst Maurus et al. examined arterial-derived ECs and I used venous-derived ECs, results are consistent between studies. Taken together, the evidence suggests that human ECs express lower levels of E-selectin on stimulation at 1% oxygen.

Published data show increased adhesion under hypoxia (Palluy et al., 1992, Rainger et al., 1995). Consistent with these reports, I found that hypoxia promoted neutrophil adhesion to HUVEC. Given my results from neutrophil integrin and endothelial adhesion molecule expression studies, increased neutrophil adhesion HUVEC under hypoxia may be attributed to enhanced interactions between Mac-1 and ICAM-1.

Interestingly, whilst neutrophil adhesion to HUVEC was enhanced under hypoxia, I found adhesion to immobilised fibrinogen, fibronectin and ICAM-1 was reduced. This observation may indicate that cellular crosstalk is required to promote adhesion under hypoxia. Alternatively, several integrin-ligand interactions may simultaneously be required for firm adhesion under hypoxia, which cannot be replicated when studying adhesion to single immobilised ligands.

3.9.3 Effects of hypoxia upon neutrophil transmigration

Early reports found that hypoxia promoted neutrophil trans-epithelial migration (Colgan et al., 1996). More recent evidence found neutrophil trans-endothelial migration was unaffected by hypoxia (McGovern et al., 2011), whilst other reports found increased chemotaxis under hypoxia (Wang and Liu, 2009). These differences arose due to differences in methodologies and chemoattractants used between studies. I found IL-8-induced neutrophil trans-endothelial migration across both resting and TNF-α-activated HUVEC was enhanced under hypoxia. Taken together, my data suggest that increased neutrophil integrin and EC adhesion molecule expression under hypoxia not only facilitate adhesion, but also promote transmigration.

3.9.4 Future work

With regards to integrin expression, examining integrin subunit mRNA levels in response to hypoxia would complement my cell surface expression data. Neutrophil adhesion studies could be expanded to include additional immobilised ligands, including ICAM-2 and VCAM-1. Adhesion to a combination of immobilised ligands could be examined to determine whether integrin crosstalk promotes adhesion under hypoxia. Endothelial adhesion assays could also be expanded to include functional blocking antibodies, targeting both neutrophil integrins and endothelial adhesion molecules, to dissect the molecular interactions mediating neutrophil adhesion. A similar approach would be beneficial in transmigration experiments, which would determine the cellular interactions promoting neutrophil trans-endothelial migration under hypoxia. Finally, having conducted static adhesion assays, it would be interesting to examine the effect of hypoxia upon neutrophil adhesion and transmigration under flow.

In this chapter, I observed differences in neutrophil integrin expression, adhesion and transmigration under hypoxia. Given that integrin activation not only mediates adhesion, but also promotes numerous cellular functions, in the next chapter, I examined the effects of hypoxia upon neutrophil activation.

Chapter Four:
The Effects of Hypoxia on Neutrophil Function

4.1 Introduction and aims

Investigations of the effects of hypoxia on ROS generation in neutrophils are limited and conflicting. Whilst one report has shown hypoxia increases ROS (Wang and Liu, 2009), another study found that GM-CSF primed neutrophils stimulated with fMLP or PMA produced lower levels of superoxide anions under hypoxia (McGovern et al., 2011). Similarly, limited studies have examined the effects of hypoxia upon NETosis. Increased bactericidal killing was reported in neutrophils treated with a HIF-1α agonist, which was mitigated by DNase treatment (Zinkernagel et al., 2008). Pharmacological stabilisation of HIF-1α has also been shown to promote NETosis (McInturff et al., 2012). Neither study however, examined NETosis cultured neutrophils under hypoxia. Therefore, I explored the effects of hypoxia upon neutrophil reactive nitrogen species generation, ROS production and NETosis.

4.2 Neutrophils do not produce detectable levels of reactive nitrogen species

Neutrophils were cultured for either 8 or 12 hours in the absence or presence of IFN-γ, a cytokine known to induce nitrite generation. Monocytes treated with IFN-γ for 12 hours were used as a positive control for nitrite production. Following incubation, cell supernatants were assessed for the presence of nitrites.

IFN-γ stimulation of $2x10^6$, $5x10^6$ and $1x10^7$ neutrophils did not produce detectable levels of nitrites (Figure 4.1A-C). $2x10^7$ neutrophils secreted nanomolar concentrations of nitrites following IFN-γ stimulation (Figure 4.1D). These results show that either the Griess assay is not sensitive enough to accurately measure nitrites produced by neutrophils or that neutrophils do not secrete nitrites on IFN-γ stimulation. Given these results, I did not explore the effects of hypoxia upon reactive nitrogen species further.

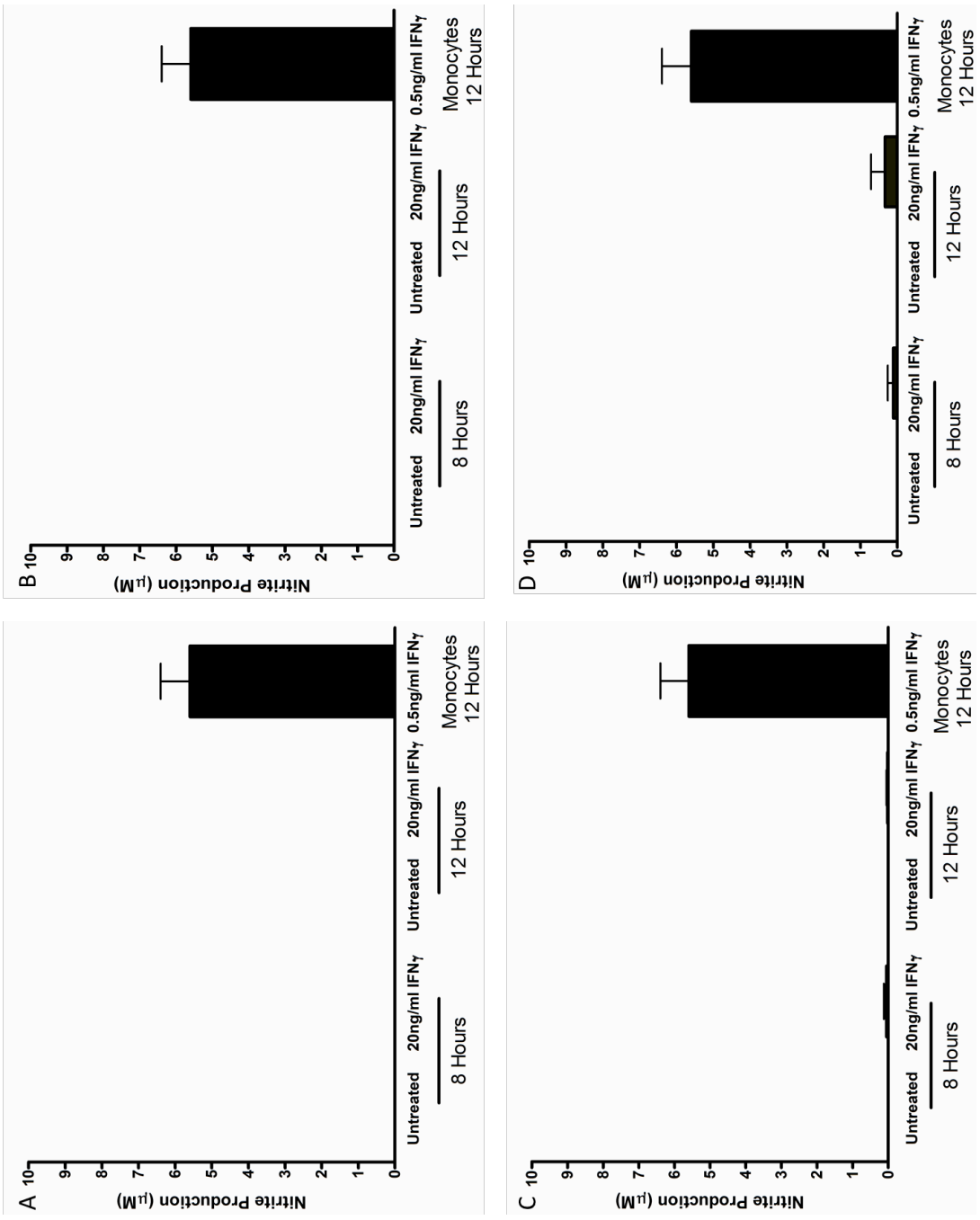


Figure 4.1: Neutrophils do not produce detectable levels of nitrites in response to interferon-γ. Neutrophils were isolated and **(A)** $2x10^6$, **(B)** $5x10^6$, **(C)** $1x10^7$ or **(D)** $2x10^7$ neutrophils were cultured in the absence or presence of 20ng/ml IFN-γ for 8 and 12 hours. $3x10^5$ monocytes were stimulated with 0.5ng/ml IFN-γ for 12 hours as a positive control. Cell supernatants were evaluated for the presence of nitrites using the Griess test. Nitrites were only detected in supernatants from $1x10^7$ or $2x10^7$ IFN-γ-stimulated neutrophils. Data is presented as the mean nitrite concentration ±SD of three independent experiments.

4.3 Neutrophils produce reactive oxygen species on PMA stimulation

Given the impractically large number of neutrophils required to study reactive nitrogen species, I examined ROS generation. A preliminary titration was performed with neutrophils isolated from two donors, which were stimulated with PMA concentrations ranging from 0-400nM. Hydrogen peroxide generation was assessed using the Amplex® UltraRed assay. A dose-dependent response was observed in both donors (Figure 4.2). Further experiments examining ROS generation were performed using 50nM PMA to ensure that both increased and decreased hydrogen peroxide production could be detected.

4.4 Hypoxia does not modulate hydrogen peroxide production by neutrophils

To examine the effects of hypoxia upon ROS generation, neutrophils were isolated from 8 healthy donors and cultured for 1 hour under normoxia or hypoxia. HRP and Amplex® UltraRed were added and rates of hydrogen peroxide generation measured in the absence or presence of PMA.

Unstimulated neutrophils produced minimal rates of hydrogen peroxide (Figure 4.3A). Unstimulated ROS generation was not altered under hypoxia ($p=0.5781$). Stimulation with PMA induced rapid hydrogen peroxide production. PMA-induced hydrogen peroxide generation was not affected by hypoxia ($p=0.2136$) (Figure 4.3B).

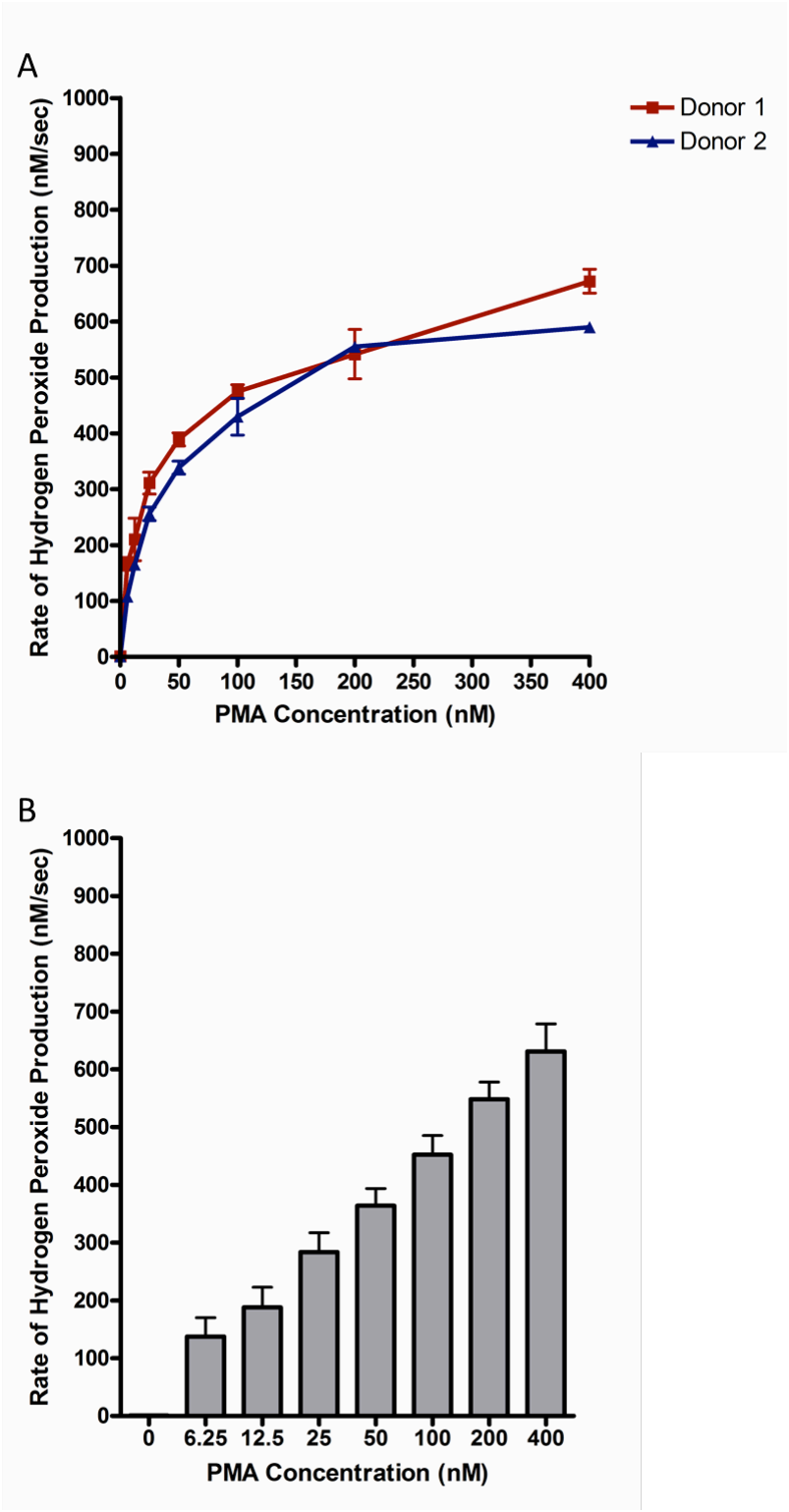


Figure 4.2: PMA-stimulated neutrophils produce hydrogen peroxide in a dose-dependent manner. Isolated neutrophils were cultured for 1 hour under normoxia before the addition of 0.5U/ml HRP and 60nM Amplex® UltraRed. $4x10^5$ neutrophils were treated with PMA at concentrations varying from 0-400nM and hydrogen peroxide generation assessed. **(A)** Individual rates of hydrogen peroxide from two donors. **(B)** Average rates obtained from both donors. Both donors show a dose-dependent response to PMA. Data is shown as mean rate ±SD of three independent experiments.

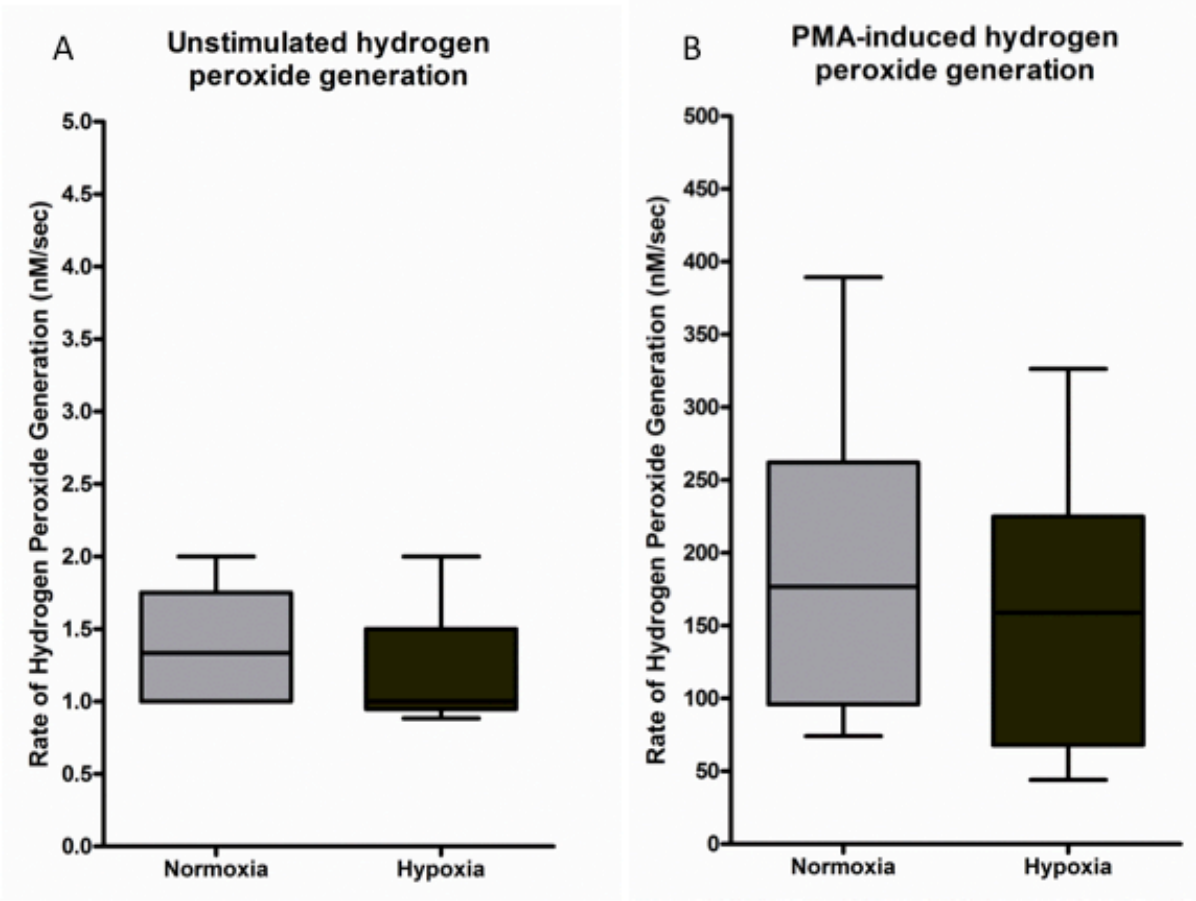


Figure 4.3: Hypoxia does not modulate neutrophil hydrogen peroxide generation. Neutrophils were isolated from whole blood donated from 8 different donors. Cells were cultured for 1 hour under either normoxia or hypoxia before the addition of 0.5U/ml HRP and 60nM Amplex® UltraRed. $4x10^5$ neutrophils were then treated with 50nM PMA and hydrogen peroxide generation evaluated. Rates of hydrogen peroxide production were determined using the Omega software package. **(A)** Minimal hydrogen peroxide was produced by unstimulated neutrophils. There were no significant differences between cells cultured under normoxia and those under hypoxia ($p=0.5781$, Wilcoxon matched pairs test). **(B)** Stimulation with 50nM PMA induced hydrogen peroxide production. Statistical analysis did not find a significant difference between normoxic and hypoxic neutrophils ($p=0.2136$, paired t test). Data presented as box plots of all eight matched observations.

4.5 Integrin blockade does not significantly affect rates of hydrogen peroxide generation under normoxia and hypoxia

Next, I assessed hydrogen peroxide generation in the presence of integrin blockade to determine whether integrins regulate ROS production. Neutrophils were isolated from 4 healthy individuals. Neutrophils were incubated for 1 hour in the absence or presence of 2LPM19c (Mac-1-specific blockade), mAb38 (LFA-1-specific blockade), P5D2 (β_1 integrin blockade) or mAb1976 ($\alpha_V\beta_3$-specific blockade). HRP and Amplex® UltraRed were added and hydrogen peroxide generation measured in response to PMA. Unstimulated neutrophils produced low rates of hydrogen peroxide production, whilst PMA-stimulated neutrophils in the absence of integrin blockade rapidly produced hydrogen peroxide (Figure 4.4). There was a trend for decreased hydrogen peroxide generation following Mac-1 blockade with 2LPM19c, but this difference was not significant ($p=0.0542$).

In addition, I examined the effects of hypoxia upon hydrogen peroxide generation following integrin blockade in 3 healthy donors. Neutrophils were cultured for 1 hour in the absence or presence of 2LPM19c, mAb38, P5D2 or mAb1976 under normoxia or hypoxia. HRP and Amplex UltraRed® were subsequently added and rates of hydrogen peroxide generation measured in response to PMA. There was a trend for decreased rates of hydrogen peroxide generation under hypoxia in the absence of integrin blockade (Figure 4.5A). This trend was more apparent in neutrophils subjected to integrin blockade (Figure 4.5B-E), however given the small number of donors tested, significance was not reached for any of the experimental conditions ($p=0.2500$).

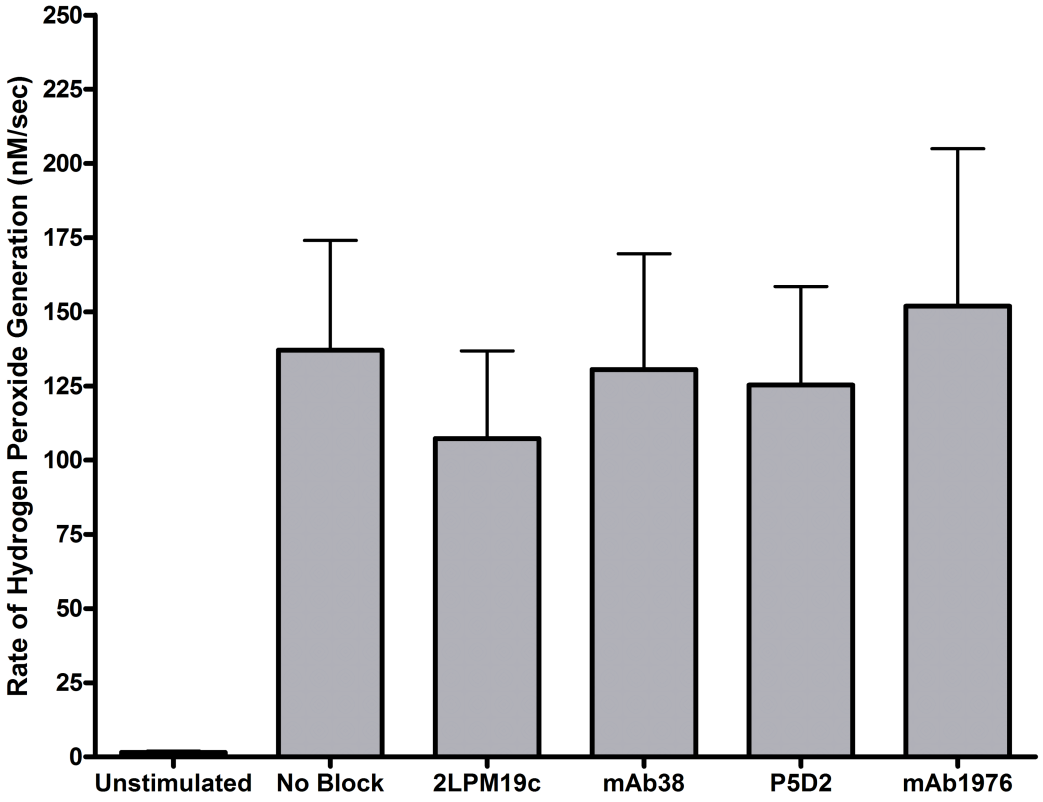


Figure 4.4: Integrin blockade does not significantly affect hydrogen peroxide generation. Neutrophils were isolated from 4 healthy donors. $2x10^6$ cells were cultured in the absence or presence of 10µg/ml 2LPM19c (Mac-1/$\alpha_M\beta_2$-specific blockade), mAb38 (LFA-1/$\alpha_L\beta_2$-specific blockade), P5D2 (β_1 integrin blockade) or mAb1976 ($\alpha_V\beta_3$-specific blockade) for 1 hour under normoxia. Following incubation, 0.5U/ml HRP and 60nM Amplex® UltraRed were added. $4x10^5$ neutrophils treated with 50nM PMA and hydrogen peroxide generation was assessed. Unstimulated neutrophils produced minimal hydrogen peroxide, whilst neutrophils treated with 50nM PMA rapidly produced hydrogen peroxide. Integrin blockade did not significantly affect rates of hydrogen peroxide generation ($p=0.0542$, Friedman test). Data is presented as the mean rate of hydrogen peroxide generation ±SD of all 4 donors.

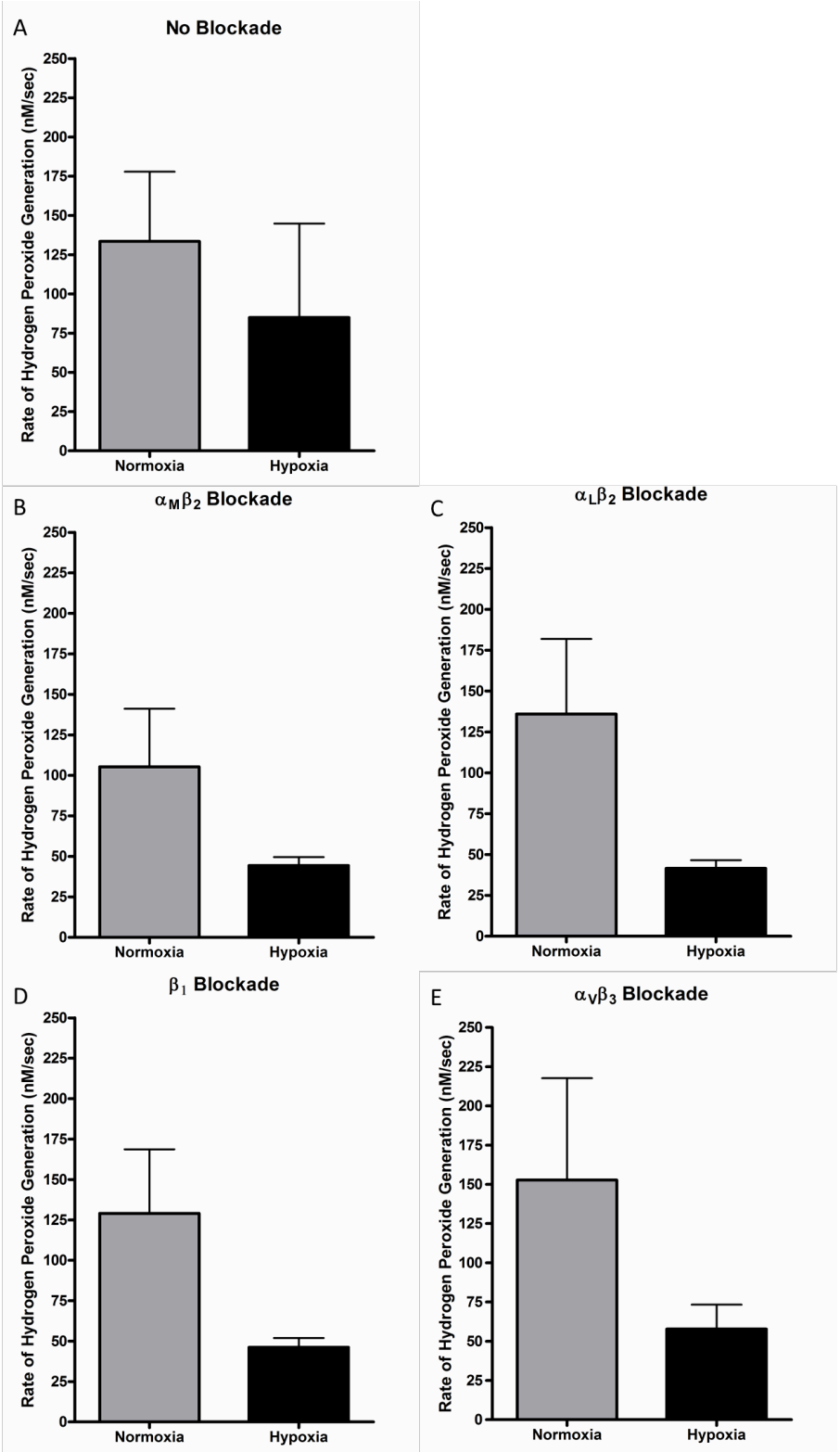


Figure 4.5: Hypoxia does not significantly affect integrin-mediated hydrogen peroxide generation. Neutrophils were isolated from 3 healthy donors. $2x10^6$ cells were cultured under normoxia or hypoxia for 1 hour in presence of **(A)** no blockade, **(B)** 2LPM19c (Mac-1/$\alpha_M\beta_2$-specific blockade), **(C)** mAb38 (LFA-1/$\alpha_L\beta_2$-specific blockade), **(D)** P5D2 (β_1 integrin blockade) or **(E)** mAb1976 ($\alpha_V\beta_3$-specific blockade). Following incubation, 0.5U/ml HRP and 60nM Amplex® UltraRed were added. $4x10^5$ neutrophils were then treated with 50nM PMA and hydrogen peroxide generation was assessed. None of the results were statistically significant (p=0.2500, Wilcoxon matched pairs test). Data is presented as the mean rate of hydrogen peroxide generation ±SD of all 3 different donors.

4.6 PMA stimulation induces neutrophil extracellular trap release

Having found no effect on hydrogen peroxide production, I examined the effects of hypoxia upon NETosis. Immunofluorescence microscopy was used to verify whether 4 hours stimulation with PMA was sufficient to induce NETosis. Immunofluorescence imaging using uncoated coverslips was unsuccessful as neutrophils failed to adhere to the glass coverslips (data not shown). Similarly, coating coverslips with poly-l-lysine produced high background and in some cases induced NETosis in unstimulated neutrophils (data not shown).

Fibrinogen-coated coverslips allowed neutrophils to adhere without inducing NETosis. Neutrophils were added to coverslips in the absence or presence of PMA for 4 hours. Cells were fixed and stained for histone H3 and mounted onto microscope slides with a DAPI mounting medium to stain DNA. Unstimulated cells had a punctate staining pattern, indicating nuclear localisation of both DNA and histone H3 (Figure 4.6). PMA stimulation induced the externalisation of both DNA and histone H3 (Figure 4.6), indicative of NETosis.

Having qualitatively demonstrated PMA-induced NETosis, quantitative measurement of NETosis was performed. The PicoGreen® dsDNA quantification kit is widely used to quantify NETs, as the PicoGreen® reagent fluorescently stains dsDNA. I also developed and optimised a capture ELISA to specifically measure NETs. Streptavidin-coated wells were coated with a biotin-conjugated anti-MPO capture antibody before incubation with PMA-stimulated neutrophil supernatants. NETs were detected by an anti-citrullinated histone H3 antibody followed by a HRP-conjugated secondary antibody. Titrations of all antibodies were conducted for optimal concentrations for the detection of NETs (data not shown).

Following optimisation of the NET capture ELISA, I compared the optimised capture ELISA with the PicoGreen® dsDNA quantification kit. Neutrophils were cultured in the absence or presence of PMA for 4 or 24 hours. Cell supernatants tested for the presence of NETs in both the NET capture ELISA and PicoGreen® dsDNA quantification kit.

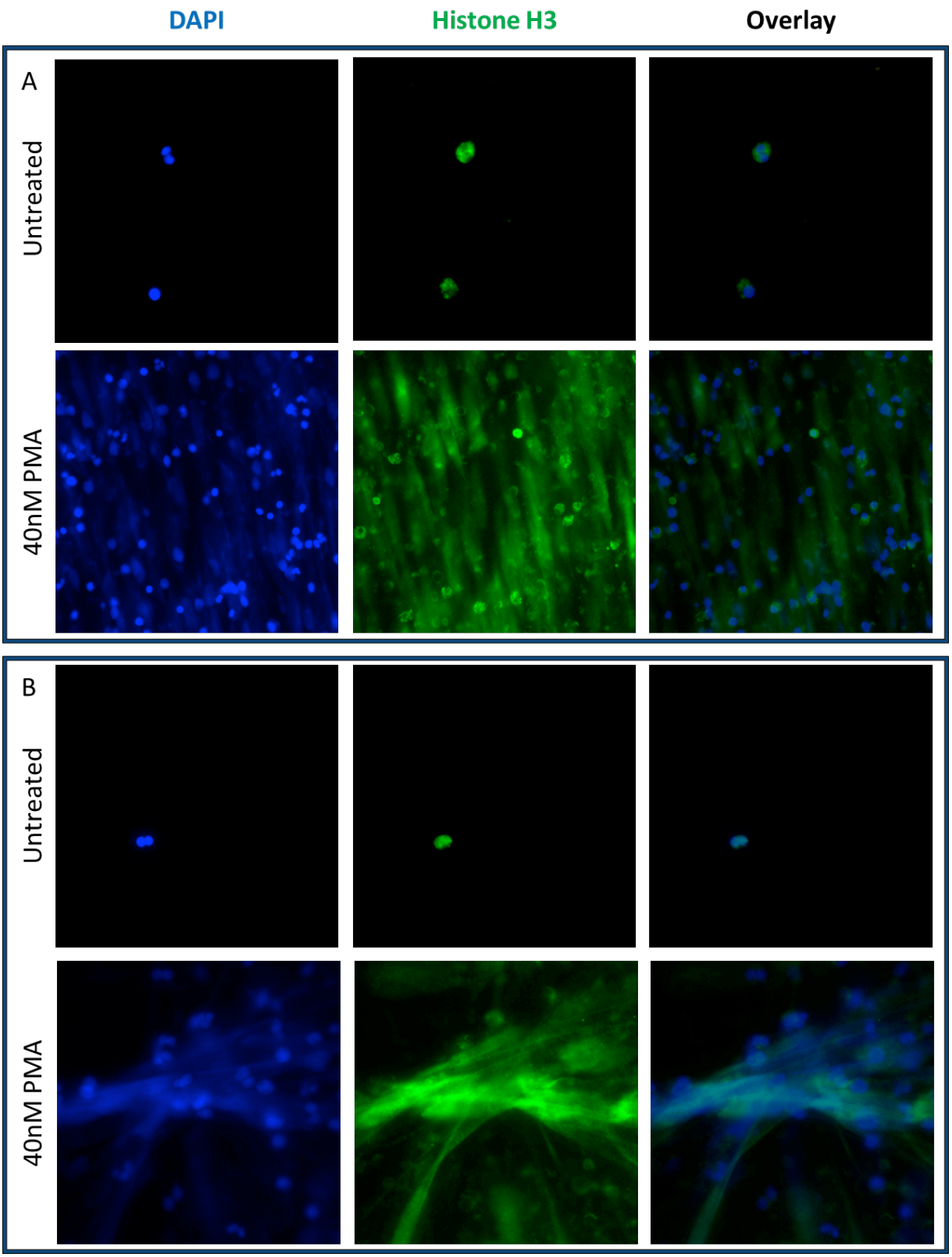


Figure 4.6: Neutrophils release NETs following PMA stimulation. $5x10^5$ neutrophils were added to fibrinogen-coated coverslips and cultured in the absence or presence of 40nM PMA for 4 hours. Cells were fixed and stained for histone H3 (green) and sealed with a DAPI mounting medium (blue). This was repeated with three different donors. Representative images can be seen for **(A)** donor 1 and **(B)** donor 2. PMA stimulation induced the externalisation of histone H3 and DNA, indicative of NETosis and was not seen in untreated cells. Images were taken at 20x magnification.

Neutrophil supernatants following PMA stimulation for 4 or 24 hours had significantly higher OD values compared to untreated neutrophils in the NET capture ELISA ($p<0.05$ and $p<0.01$ respectively) (Figure 4.7A). Supernatants assessed using the PicoGreen® dsDNA quantification kit displayed a similar pattern, however only 24 hours PMA stimulation produced significantly higher RFU values ($p<0.05$) (Figure 4.7B).

4.7 Hypoxia enhances PMA-stimulated NETosis

Having determined a quantitative method to evaluate NETs, I then studied the effects of hypoxia upon NETosis. Neutrophils were isolated and cultured overnight under normoxia or hypoxia. Following overnight incubation, cells were cultured in the absence or presence of PMA for 4 hours, after which supernatants were assessed for NETs.

Cell supernatants from PMA-stimulated neutrophils had significantly higher OD values compared to untreated cells. OD values from both untreated and PMA-stimulated neutrophil supernatants were significantly increased in hypoxia compared to normoxia ($p<0.05$ and $p<0.001$ respectively) (Figure 4.8).

4.8 NETosis is a cation-dependent process

Next, I conducted a preliminary study to determine whether NETosis is integrin-dependent. Immunofluorescence staining was performed in the absence or presence of EDTA, which chelates divalent cations that are essential for integrin function.

Untreated neutrophils had a punctate DAPI and histone H3 staining pattern (Figure 4.9A), whilst PMA stimulation externalised nuclear stains (Figure 4.9B). Neutrophils treated with PMA and EDTA also had a punctate staining pattern (Figure 4.9C), demonstrating a requirement for cations in NETosis. Manganese cations are able to activate cellular integrins. Neutrophils treated with manganese chloride had extracellular DAPI and histone H3 staining (Figure 4.9D), which was not observed in neutrophils treated with manganese chloride and EDTA (Figure 4.9E).

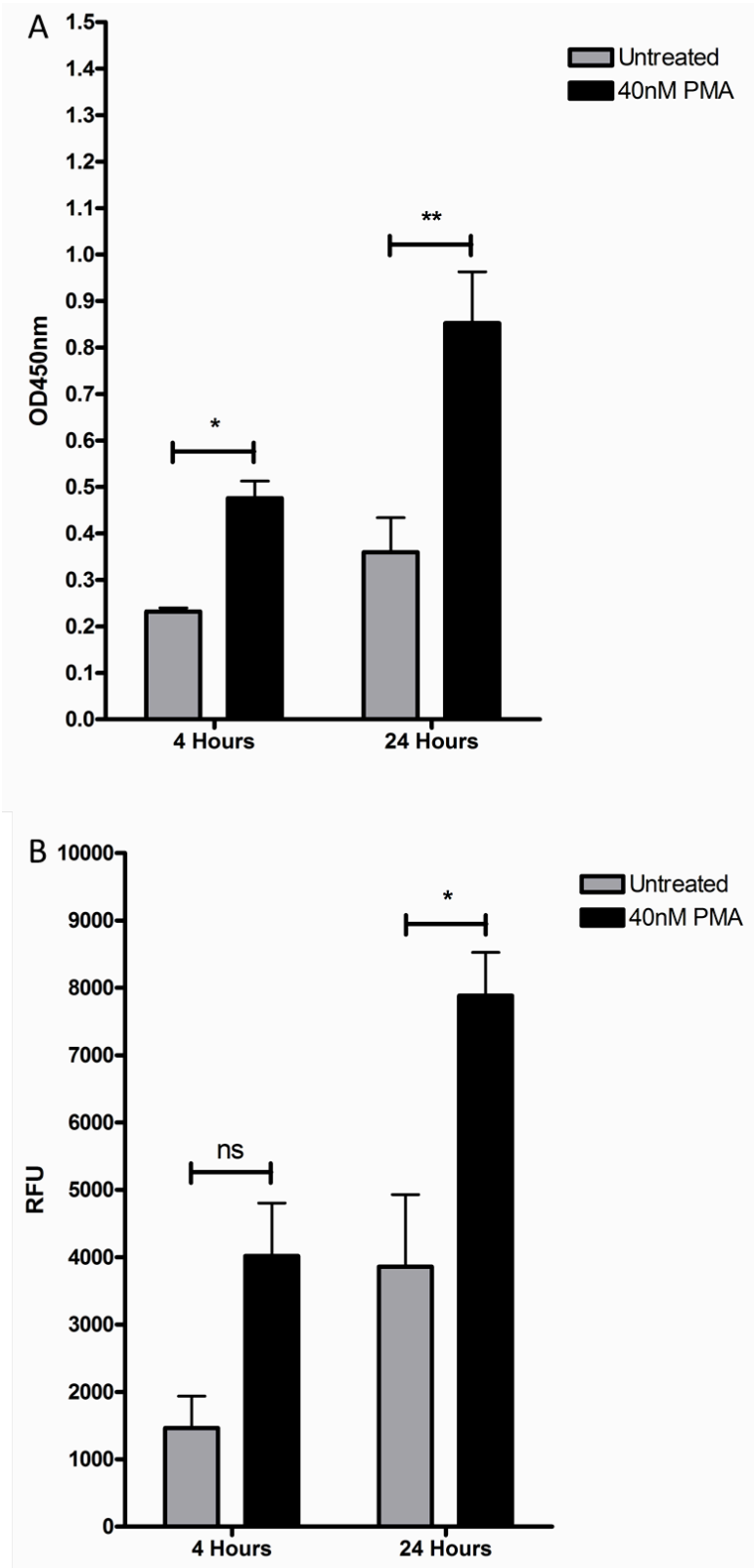


Figure 4.7: Similar results are obtained from a NET capture ELISA and PicoGreen® dsDNA quantification kit. $2x10^6$ neutrophils were cultured in the absence or presence of 40nM PMA for 4 or 24 hours. Cell supernatants were tested for presence of NETs using the **(A)** NET capture ELISA or **(B)** PicoGreen® dsDNA quantification kit. Readouts were significantly higher in the supernatant of neutrophils stimulated with 40nM PMA. Similar patterns were observed between assays. Statistics significance was tested using a 2-way ANOVA with a Bonferroni post-test analysis. Data is presented as mean ±SD of 3 independent experiments. ns = no significance, * = $p<0.05$, ** = $p<0.01$

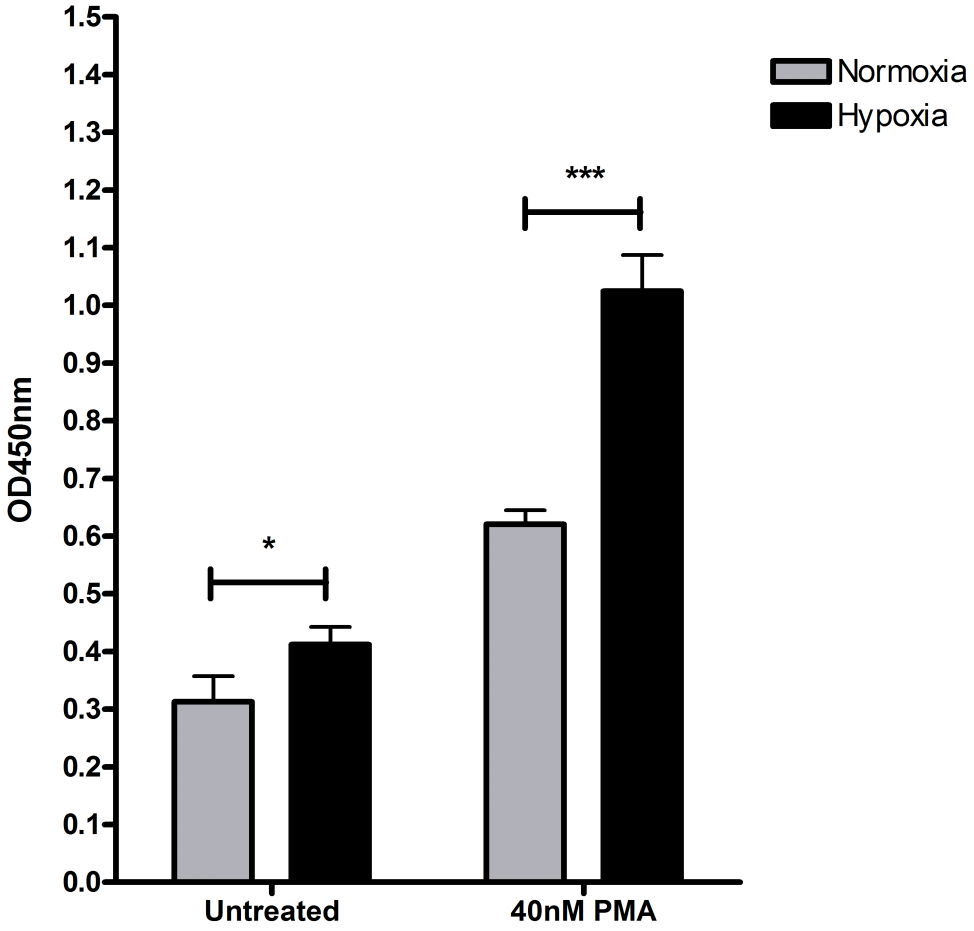


Figure 4.8: Hypoxia enhances NETosis of PMA-stimulated neutrophils. Neutrophils were isolated and incubated overnight under either normoxia or hypoxia. Cells were cultured for a further 4 hours in the absence or presence of 40nM PMA. Neutrophil supernatants were tested for the presence of NETs by capture ELISA. OD values were significantly higher in untreated and PMA-stimulated neutrophils supernatants under hypoxia. Statistical significance was tested by two-way ANOVA with a Bonferroni post-test analysis. Data is presented as the mean OD ±SD of three independent experiments *=$p<0.05$, *** = $p<0.001$

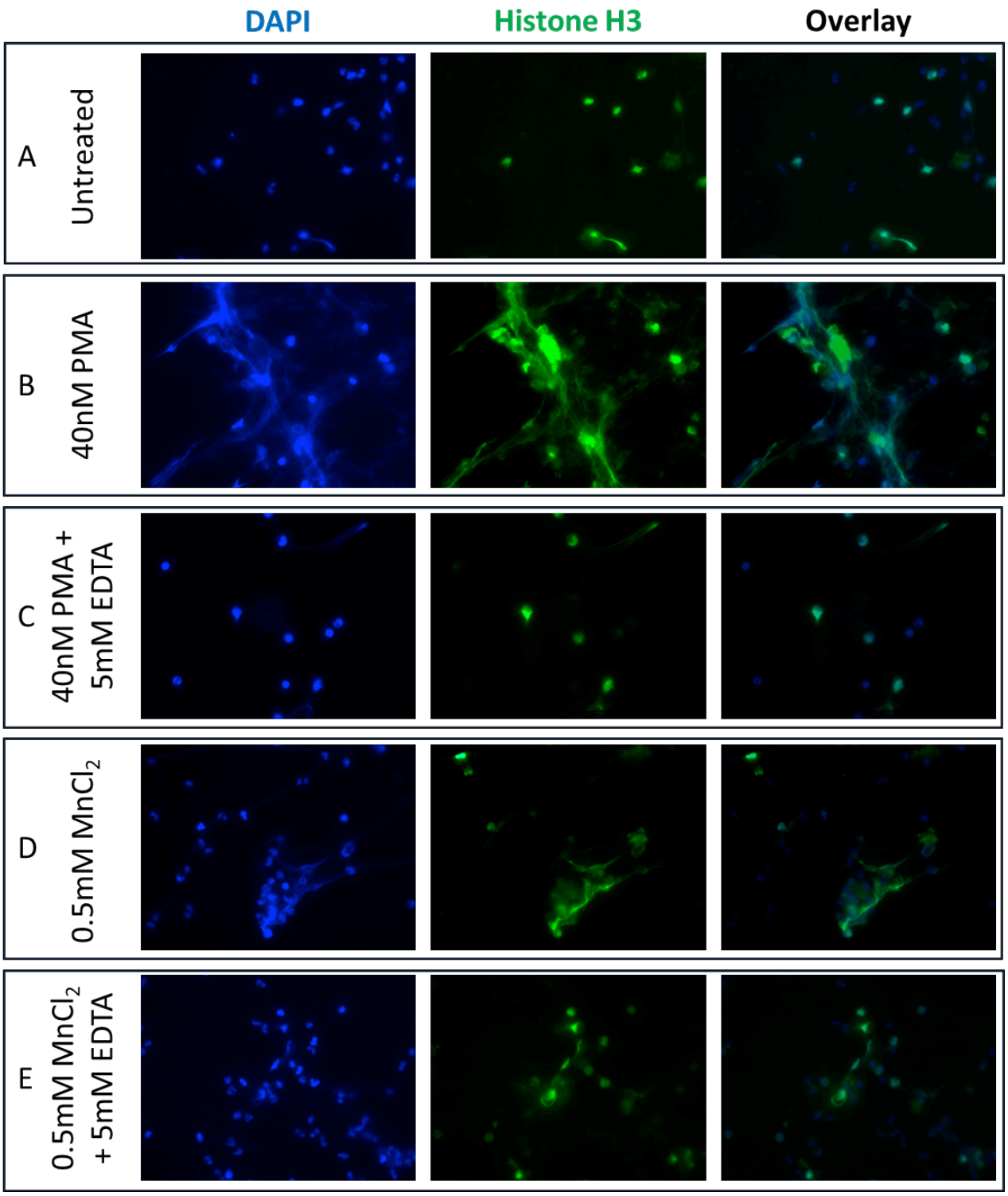


Figure 4.9: NETosis is inhibited by EDTA treatment and stimulated by manganese cations. $5x10^5$ neutrophils were added to fibrinogen-coated coverslips and incubated for 4 hours with **(A)** untreated media, **(B)** 40nM PMA, **(C)** 40nM PMA and 5mM EDTA, **(D)** 0.5mM manganese chloride or **(E)** 0.5mM manganese chloride and 5mM EDTA. Cells were fixed and stained for histone H3 (green) and sealed with a DAPI mounting medium (blue). Externalisation of nuclear stains was observed in PMA and manganese chloride treated cells, but not those treated with 5mM EDTA. Representative images from each slide are shown above. Images were taken at 20x magnification.

4.9 Mac-1 activation induces NETosis

Having shown NETosis is cation-dependent and can be induced with manganese cations, thus implicating integrin involvement, I subsequently studied the effects of Mac-1 activation on NETosis. Leukadherin-1 (LA-1) is a small molecule agonist, which specifically activates Mac-1 (Celik et al., 2013). Titrations of LA-1 induced neutrophil adhesion to immobilised fibrinogen in a dose-dependent manner (Figure 4.10).

Immunofluorescence studies were then conducted to study the effects of LA-1 treatment on NETosis. Neutrophils were treated with 10, 25, 100 or 200μM LA-1 for 4 hours and stained for NETs. Untreated and 10μM LA-1-treated cells displayed punctate staining (Figure 4.11A, B), however neutrophils treated with 25, 100 or 200μM LA-1 demonstrated evidence of NETosis (Figure 4.11C-E) similar to that of cells stimulated with 40nM PMA (Figure 4.11F).

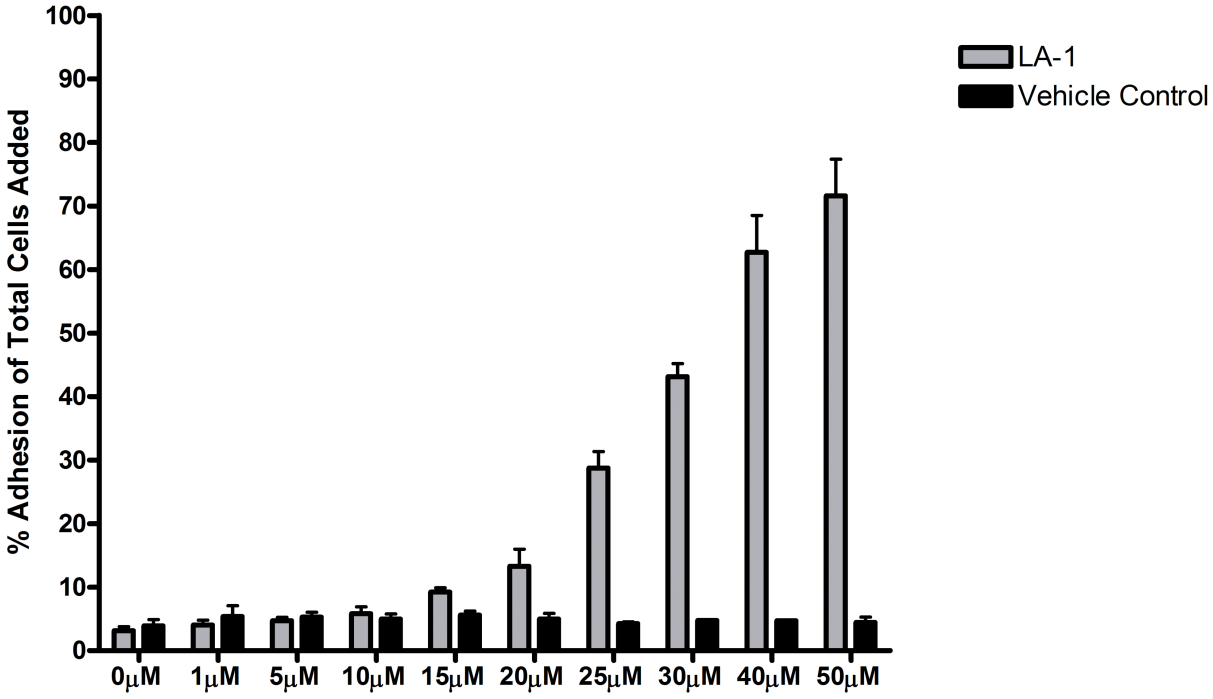


Figure 4.10: Leukadherin-1 promotes neutrophil adhesion to immobilised fibrinogen. Neutrophils were isolated from whole blood and fluorescently labelled with BCECF-AM. Neutrophil adhesion to immobilised fibrinogen was then measured in response to several concentrations of the small molecule Mac-1 agonist leukadherin-1 (LA-1). Increased adhesion compared to DMSO vehicle controls was observed from 15μM, which increased with greater LA-1 concentrations. Data is presented as mean adhesion ±SD of three independent experiments.

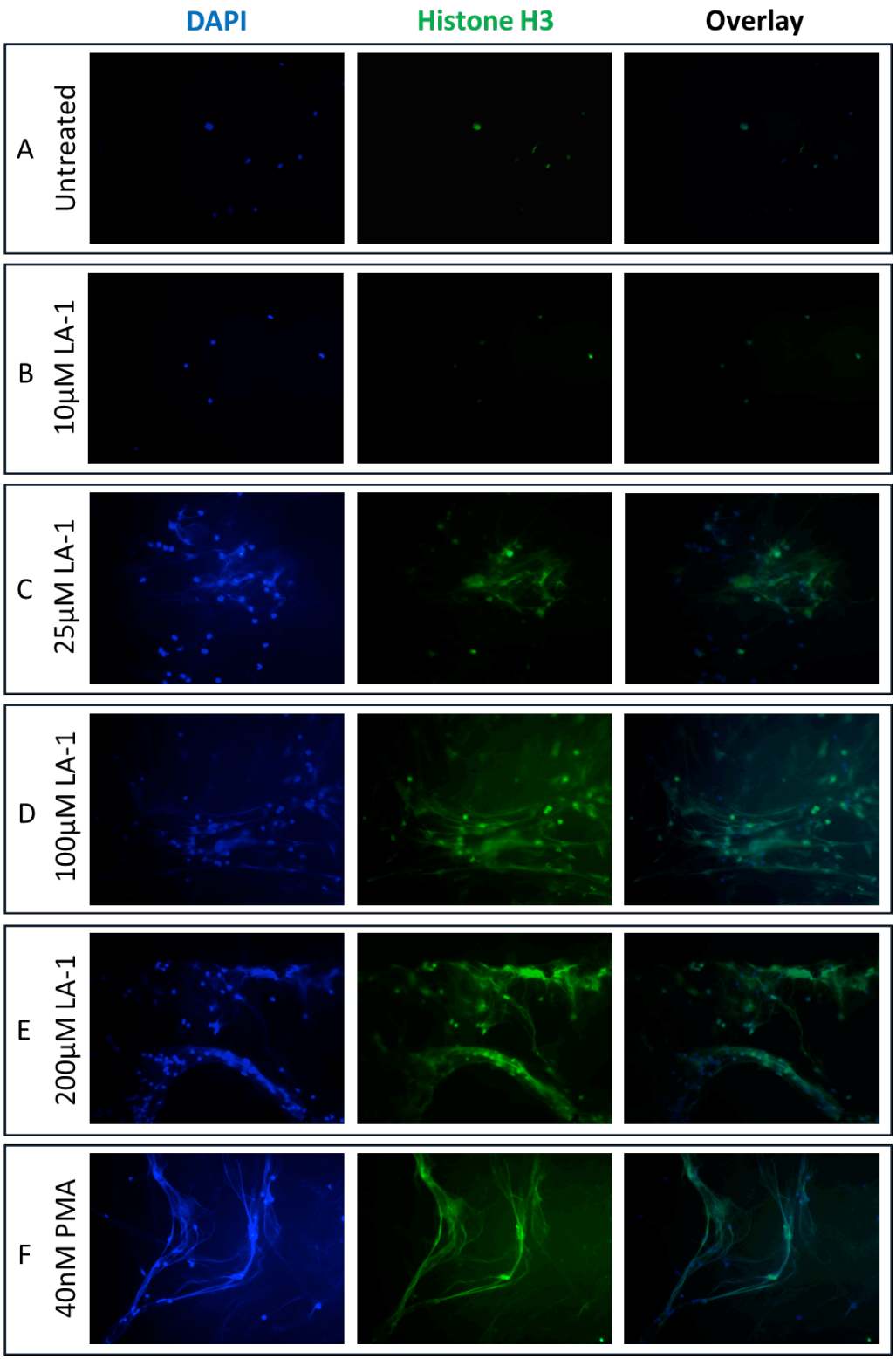


Figure 4.11: Mac-1 activation induces NETosis. $5x10^5$ neutrophils were added to fibrinogen-coated coverslips and incubated for 4 hours with **(A)** untreated media, **(B)** 10µM leukadherin-1 (LA-1), **(C)** 25µM LA-1, **(D)** 100µM LA-1, **(E)** 200µM LA-1, or **(F)** 40nM PMA. Cells were fixed and stained for histone H3 (green) and sealed with a DAPI mounting medium (blue). Externalisation of nuclear stains was observed in neutrophils cultured with LA-1 concentrations of 25µM and greater. PMA stimulation also induced extracellular histone H3 and DAPI staining. Representative images from each slide are shown above of 2 independent experiments. Images were taken at 20x magnification.

4.10 Endothelial co-culture may modulate neutrophil responses to PMA

As integrin activation induced NETosis, I next examined neutrophil responses during endothelial co-culture. A preliminary experiment was conducted to explore the effects of neutrophil-endothelial interactions upon ROS generation under normoxia. HUVEC were seeded into 96-well plates and grown to confluence. Prior to experimentation, cells were cultured overnight in the absence or presence of TNF-α. Neutrophils were isolated and treated with HRP and Amplex® UltraRed before addition of neutrophils to empty wells or wells containing resting or TNF-α-activated endothelial monolayers.

PMA-induced hydrogen peroxide production was reduced in neutrophils stimulated in the presence of resting HUVEC monolayers compared to empty wells (Figure 4.12). Interestingly, rates of hydrogen peroxide generation were higher in neutrophils stimulated in the presence of TNF-α-activated endothelial monolayers compared to resting HUVEC, similar to rates obtained from empty wells (Figure 4.12). Integrin blockade also had greater effects in wells containing HUVEC. Whilst each condition was tested in triplicate, the experiment was only conducted once so statistical significance cannot be determined.

I also studied the contribution of endothelial monolayers to NETosis. Neutrophils were added to empty wells or wells containing endothelial monolayers. Neutrophils were cultured in the absence or presence of PMA for 4 hours, following which, cell supernatants were assessed for NETs by capture ELISA. NETs were detected in the supernatants of PMA-stimulated neutrophils, but not HUVEC (Figure 4.13). Neutrophils stimulated in the presence of HUVEC had significantly higher levels of NETs compared to neutrophils stimulated in the absence of endothelial monolayers ($p<0.01$) (Figure 4.13).

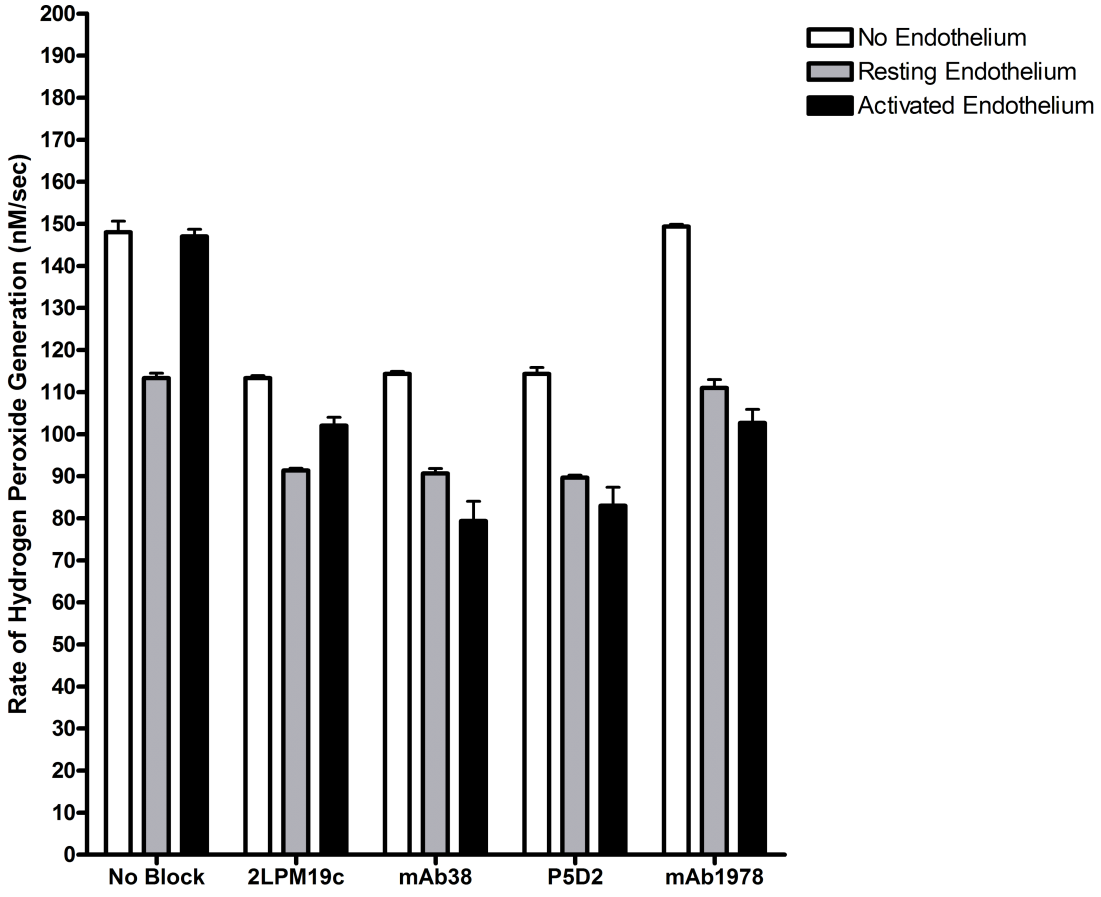


Figure 4.12: The effects of endothelial co-culture on neutrophil ROS generation. HUVEC were grown to confluence. Prior to experimentation, EC monolayers were cultured for 24 hours in the absence or presence of 10ng/ml TNF-α. Neutrophils were isolated and cultured in the absence or presence of 10μg/ml 2LPM19c (Mac-1/$\alpha_M\beta_2$-specific blockade), mAb38 (LFA-1/$\alpha_L\beta_2$-specific blockade), P5D2 (β_1 integrin blockade) or mAb1976 ($\alpha_V\beta_3$-specific blockade) for 1 hour under normoxia, after which 0.5U/ml HRP and 60nM Amplex® UltraRed were added. $4x10^5$ neutrophils were added to empty wells, resting HUVEC monolayers or activated HUVEC monolayers. Cells were treated with 50nM PMA and hydrogen peroxide generation was then assessed. Neutrophils stimulated in the presence of resting ECs had lower rates of hydrogen peroxide generation, compared to empty wells and wells containing TNF-α-activated ECs. Integrin blockade had greater effects in the presence of EC monolayers. Statistical significance could not be determined as this preliminary experiment was only conducted once. Data is presented as the mean rate of hydrogen peroxide generation ±SD of triplicate wells.

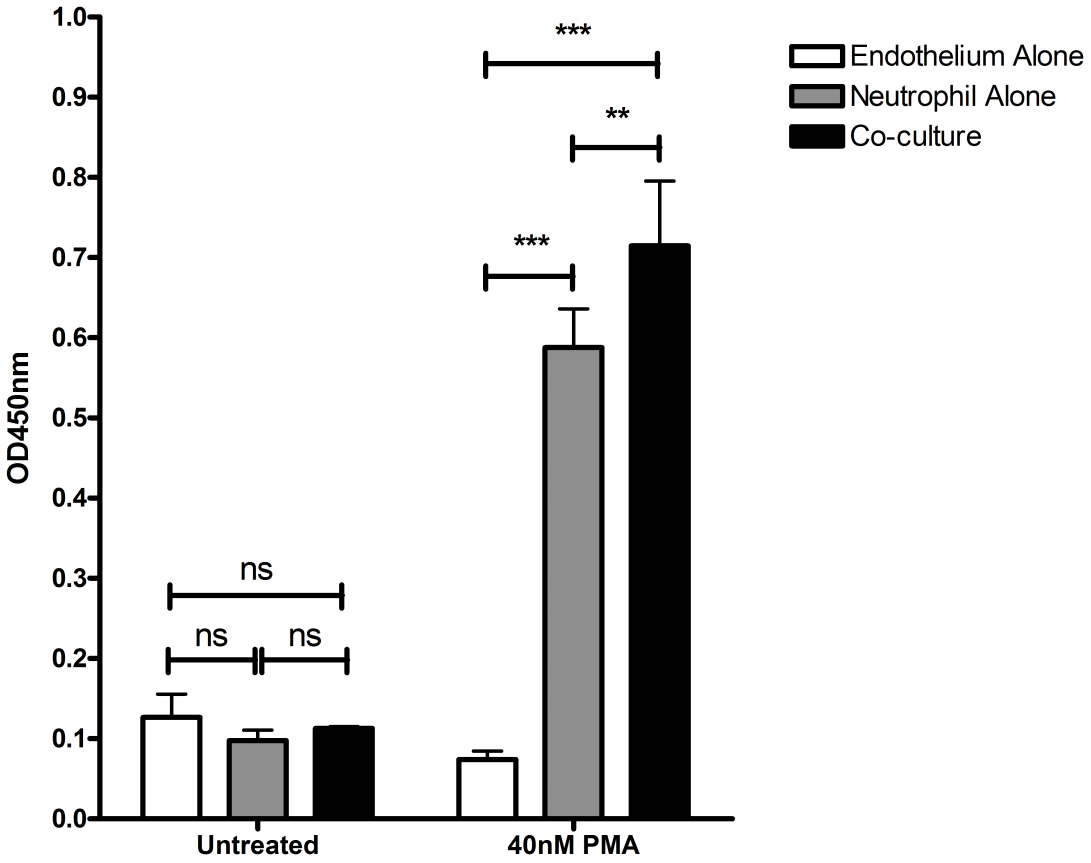


Figure 4.13: Endothelial co-culture enhances NETosis. HUVEC were grown to confluence into endothelial monolayers. Neutrophils were isolated and $2x10^6$ cells were added to either empty wells or wells containing EC monolayers. Cells were then cultured for 4 hours in the absence or presence of 40nM PMA. Following incubation, cell supernatants were tested for the presence of NETs by capture ELISA. Only PMA-stimulated neutrophil supernatants tested positive for NETs. Neutrophils stimulated in the presence of endothelial monolayers had significantly higher levels of NETs compared to neutrophils stimulated alone. Statistical significance was determined by two-way ANOVA with a Bonferroni post-test analysis. Data is presented as the mean OD ±SD of three independent experiments. ns= no significance, **=$p<0.01$, *** = $p<0.001$

4.11 Hypoxia alters protein expression in neutrophils

Having found functional effects of hypoxia upon neutrophil activation, I began to explore the mechanisms underpinning neutrophil activation. Given the importance of MPO and PAD-4 in NETosis, preliminary experiments were conducted to determine the expression and intracellular localisation of both proteins in response to hypoxia and PMA stimulation.

Neutrophils were cultured under normoxia or hypoxia for 4 hours in the absence or presence of PMA. Following incubation, cells were lysed using a ProteoExtract® subcellular proteome extraction kit (Millipore, Ireland), to obtain cytosolic, membrane, nuclear and cytoskeletal cell lysate fractions. Cell lysates from each fraction were resolved by gel electrophoresis and transferred to a PVDF membrane, which was probed for MPO, PAD-4 and GAPDH by immunoblot (Figure 4.14A).

MPO was predominately found in nuclear cell fractions. Unstimulated neutrophils cultured under normoxia had greater MPO expression than unstimulated hypoxic cells (Figure 4.14B). In contrast, greater MPO expression in PMA-stimulated neutrophils was seen under hypoxia compared to normoxia (Figure 4.14C).

PAD-4 expression was only seen in cytosolic cell fractions. Unstimulated hypoxic neutrophils had twice as much PAD-4 expression compared to normoxic cells (Figure 4.14D). PMA stimulation increased expression in normoxic cells, however expression was still higher in hypoxic cells (Figure 4.14E).

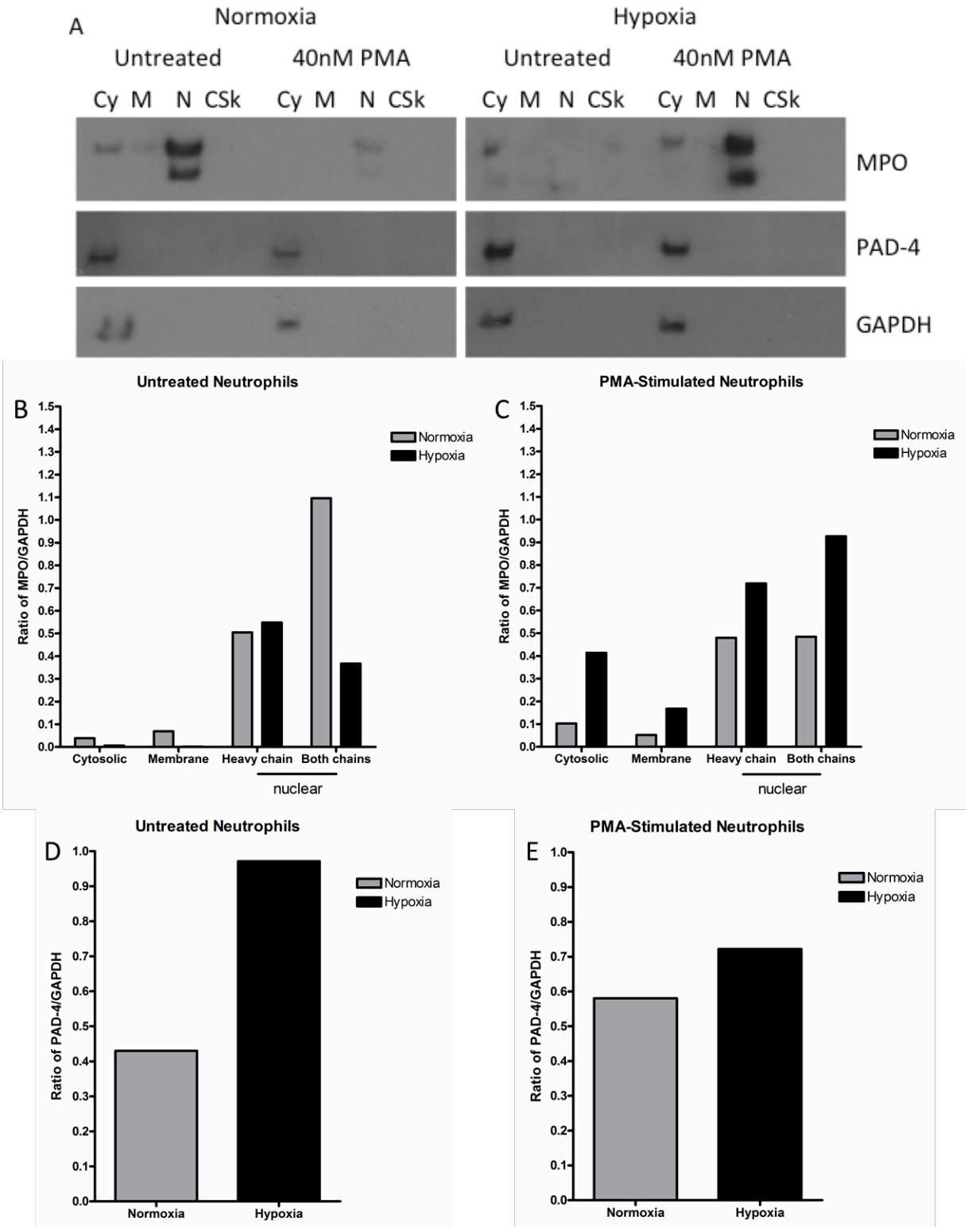


Figure 4.14: Hypoxia alters intracellular expression of PAD-4 and MPO in neutrophils. Neutrophils cultured under normoxia or hypoxia in the absence or presence of 40nM PMA for 4 hours, after which cells were subjected to fractional lysis. **(A)** Resolved proteins were transferred to a PVDF membrane, which were probed for MPO and PAD-4, which were normalised to GAPDH. **(B)** Unstimulated hypoxic neutrophils had lower MPO levels. **(C)** Greater MPO levels were observed in PMA-stimulated neutrophils under hypoxia. **(D)** PAD-4 levels were higher in unstimulated hypoxic neutrophils. **(E)** Hypoxic stimulated neutrophils had lower levels than unstimulated cells, but had higher levels than normoxic counterparts. Cy= cytosolic, M= membrane, N= nuclear, CSk=cytoskeletal

4.12 Inhibition of p38 MAPK, but not mTOR, reduces PMA-induced NETosis

Before determining whether hypoxia modulates signalling pathways regulating NETosis, I conducted preliminary experiments to highlight the key pathways involved in PMA-induced NETosis. Given that p38 MAPK signalling has been implicated in PMA-induced NETosis (Riyapa et al., 2012) as well as the importance of mTOR and HIF-1α in the regulation of NET release (McInturff et al., 2012), I studied the effects of mTOR and p38 MAPK inhibition on NETosis.

Neutrophils were incubated with varying concentrations of rapamycin or SB203580, which inhibit mTOR and p38 MAPK signalling respectively, for 2 hours and then treated with PMA for a further 4 hours. Following incubation, supernatants were assessed for the presence of NETs. There was a trend for decreased NETosis with increasing concentrations of rapamycin, but these differences did not reach significance (Figure 4.15A). In contrast, a significant dose-dependent reduction in NETosis was observed in neutrophils treated with 10μM and 20μM SB203580 (Figure 4.15B).

4.13 Hypoxia induces transient endothelial PAD-4 expression and activity

In addition to studying the effects of hypoxia upon neutrophil protein expression, I also examined the endothelial PAD-4 and citrullinated histone H3 expression. A preliminary time course experiment was conducted in which HUVEC were exposed to hypoxia for up to 24 hours, before cells were lysed and proteins resolved by gel electrophoresis. Resolved proteins were transferred to a membrane and probed for HIF-1α, PAD-4, citrullinated histone H3 and β-tubulin (Figure 4.16A). HIF-1 α protein levels increased by 3.1 fold compared to baseline following 4 hours in hypoxia, before declining back to baseline (Figure 4.16B). PAD-4 expression increased by 1.2 fold following 4 hours of hypoxic culture (Figure 4.16C). Levels of citrullinated histone H3 also rapidly increased by 15 fold above baseline after 4 hours incubation in hypoxia (Figure 4.16D). As this experiment was only conducted once, statistical significance could not be determined.

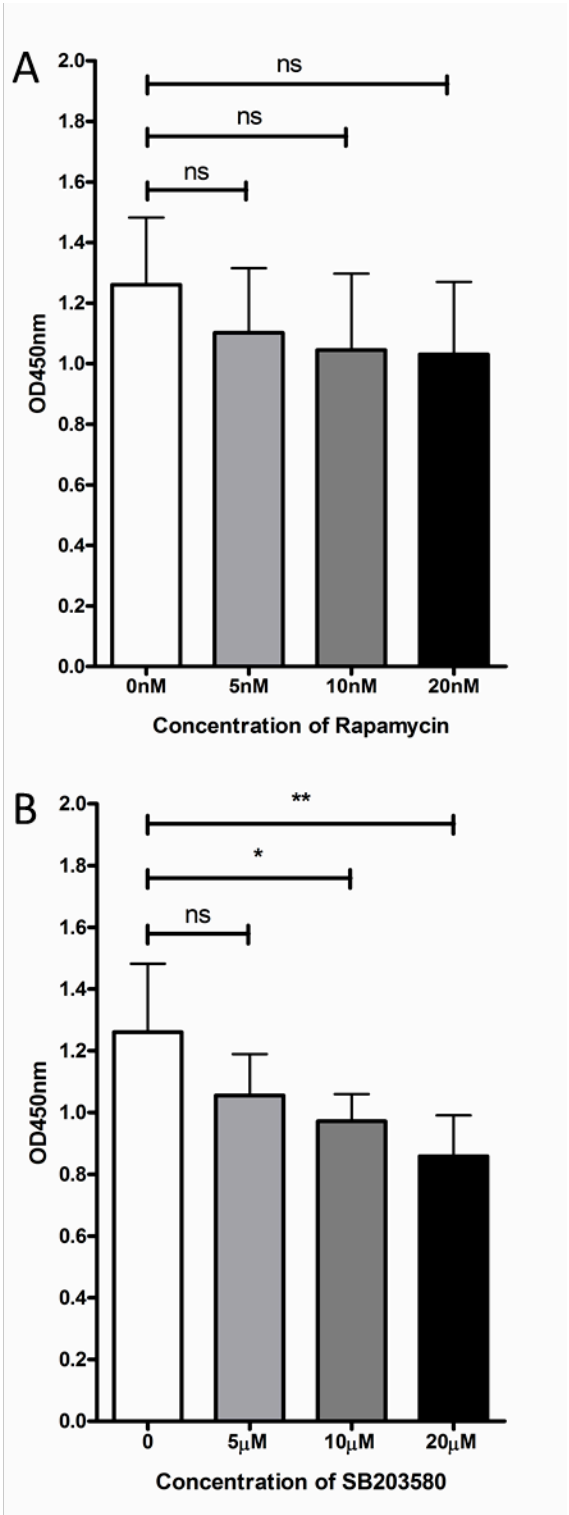


Figure 4.15: Inhibition of p38 MAPK, but not mTOR signalling significantly reduces NETosis. Neutrophils were isolated and treated with varying concentrations of rapamycin or SB203580 for 2 hours and then stimulated with 40nM PMA for 4 hours and supernatants assessed for NETs by capture ELISA. **(A)** A marginal decrease was observed with increasing rapamycin concentrations, however this difference was not significant. **(B)** PMA-stimulated NETosis significantly decreased with increasing concentrations of SB203580. Statistical significance was tested by one-way ANOVA with a Bonferroni multiple comparison test. Data is presented as the mean OD ±SD of two independent experiments. ns= no significance, *=$p<0.05$, ** = $p<0.01$

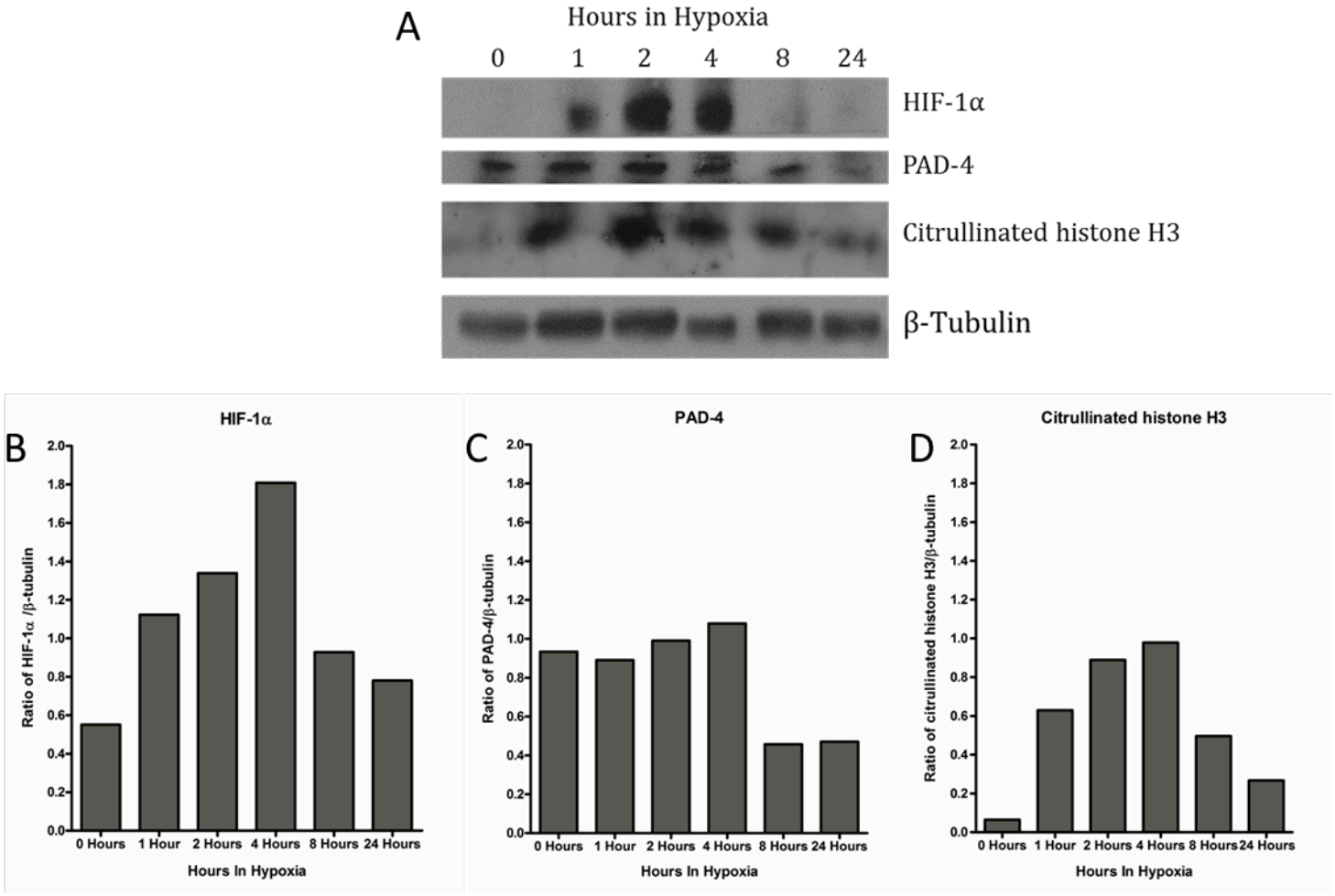


Figure 4.16: Hypoxia induces transient PAD-4 expression and activity in endothelial cells. HUVEC were seeded into 6-well tissue culture plates and cultured under hypoxia and lysed at set time points. **(A)** Resolved proteins were transferred to a PVDF membrane, which were probed for HIF-1α and PAD-4, which were normalised to β-tubulin. **(B)** HIF-1α expression peaked at 4 hours hypoxic exposure and then declined. **(C)** A marginal increase in PAD-4 expression was observed by 4 hours hypoxic culture, which then dropped. **(D)** Levels of citrullinated histone H3 dramatically increased from 1 hour hypoxia, which peaked at 4 hours before decreasing.

4.14 Discussion

In this chapter, I have shown that PMA induces neutrophil hydrogen peroxide production, which is neither modulated by hypoxia nor integrin blockade. PMA stimulation induces NETosis, which is enhanced under hypoxia and is cation-dependent. Preliminary work has implicated Mac-1 activation in the generation of NETs. Co-culture of neutrophils with ECs appears to modulate neutrophil responses, however further work is required to validate preliminary findings. Hypoxia increases PAD-4 expression in neutrophils, with increased PAD-4 and citrullinated histone H3 also being observed in HUVEC. In this section I will discuss my findings within the context of published reports, evaluate limitations of my work and explore avenues of future work.

4.14.1 Effects of hypoxia upon ROS generation

There is conflicting evidence regarding the effects of hypoxia on neutrophil ROS generation. Whilst Wang and Lui reported increased ROS production when neutrophils were exposed to 12% oxygen (Wang and Liu, 2009), McGovern et al. found defective ROS production when neutrophils primed with GM-CSF were stimulated under 3% oxygen (McGovern et al., 2011). The latter study is most similar to my work, but I cultured neutrophils at 1% oxygen and did not prime cells prior to treatment. I did not find a significant difference in hydrogen peroxide production by neutrophils stimulated under normoxia and hypoxia.

Conflicting evidence may be explained by considering the cellular effects of the varying oxygen levels. Jiang and colleagues had previously demonstrated that HIF-1α protein expression and DNA-binding activity was only seen in HeLa S3 nuclear extracts when exposed to 5% oxygen or lower (Jiang et al., 1996). It is therefore possible that the neutrophils exposed to 12% oxygen by Wang and Lui lacked HIF-mediated signalling. In contrast, as both my own work and that of McGovern et al. had cultured neutrophils below 5% oxygen, responses are likely to be mediated by HRE-mediated transcription. Given the discrepant findings and variations in experimental conditions, further work is required to determine the precise effects of hypoxia upon ROS generation in neutrophils.

4.14.2 Effects of hypoxia upon NETosis

Two studies have examined the role of HIF-1α in NETosis, both of which implicate HIF-1α signalling with enhanced NETosis. Neither study subjected neutrophils to hypoxia, but instead treated cells with hypoxia mimetics. Neutrophils treated with the HIF-1α agonist mimosine displayed increased bactericidal killing, which was mitigated by the degradation of extracellular DNA via DNase treatment (Zinkernagel et al., 2008). In addition, mTOR-dependent HIF-1α stabilisation promoted NETosis, whilst inhibition of either mTOR or HIF-1α reduced NETosis (McInturff et al., 2012). My work conducted under low oxygen levels is consistent with these published reports using hypoxia mimetics. Whilst these studies show that pharmacological stabilisation of HIF-1α enhances NET release, my results demonstrate that culture under hypoxia, which stabilises HIF-1α expression, promotes NETosis in human neutrophils. Therefore, an important factor in NETosis is the stabilisation of HIF-1α.

4.14.3 Molecular mechanisms and cell signalling underlying neutrophil activation

Conflicting evidence exists regarding the molecular and signalling events regulating neutrophil activation under hypoxia. Several studies have shown Mac-1 blockade reduces NETosis (Neeli et al., 2009, Raftery et al., 2014, Rossaint et al., 2014, Yalavarthi et al., 2015), but did not dissect integrin involvement further. Similar observations have been reported with β_1 integrin blockade (Lavigne et al., 2007, Gillenius and Urban, 2015). My preliminary data shows that NETosis is cation-dependent, which indirectly supports these published reports. Moreover, my data showing LA-1, which specifically activates Mac-1 via allosteric conformational changes (Celik et al., 2013), induces NETosis complements the findings of published reports showing reduced NETosis following Mac-1 inhibition.

My preliminary data show that endothelial co-culture, particularly with activated ECs, may modulate neutrophil hydrogen peroxide generation. NETosis was also elevated in neutrophils cultured with resting ECs. Published reports demonstrate that activated ECs can induce NETosis (Gupta et al., 2010), therefore cellular interactions with ECs may promote NET

production and release. Further work evaluating NETosis in the presence of activated ECs may provide data more consistent with published reports.

I found increased PAD-4 expression in neutrophils following 4 hours culture under hypoxia. There was also an increase in PAD-4 expression in HUVEC exposed to hypoxia, however as there were no EC normoxic comparisons, it is impossible to say whether this finding represents increased expression in response to hypoxia. A published report has documented increased PAD-2 expression and activity in astrocytes exposed to hypoxia (Sambandam et al., 2004), however the effects of hypoxia upon PAD-4 expression have not been studied. My novel finding demonstrates that PAD-4 expression is also under hypoxic regulation. Increased citrullinated histone H3 under hypoxia was noted in ECs, which indicates enhanced PAD-4 activity, however due to the lack of normoxic controls, this observation cannot be conclusively attributed to the effects of hypoxia.

There is conflicting evidence regarding the contribution of mTOR in NETosis. In one report, mTOR signalling promotes NETosis via HIF-1α stabilisation (McInturff et al., 2012), whilst in another publication, mTOR inhibited NETosis through suppression of autophagy (Itakura and McCarty, 2013). My preliminary data found that mTOR inhibition with rapamycin did not significantly affect PMA-induced NETosis. In these published reports, neutrophils were stimulated with either LPS or fMLP, whilst in my work neutrophils were stimulated with PMA. Therefore, it is possible that mTOR does not regulate NETosis in response to PMA stimulation. In contrast, treatment with the p38 MAPK inhibitor SB203580 significantly reduced NET release in PMA-stimulated neutrophils. This observation is consistent with published reports, which have also found that PMA-induced NETosis was reduced following p38 MAPK inhibition (Riyapa et al., 2012).

4.14.4 Future work

There are many avenues in which my work could be expanded. With regards to hydrogen peroxide generation, as my work only examined the effects of hypoxia after 1 hour exposure, additional time points up until 8 hours could be studied to explore the effects of

longer culture under hypoxia upon ROS generation. Additional experiments could also be conducted to examine the effects of integrin blockade on hydrogen peroxide generation by neutrophils in the presence of immobilised ligands. Further investigation is required to expand on my preliminary data exploring the effects of endothelial co-culture upon neutrophil ROS generation. In addition, whilst the Amplex® UltraRed assay is a robust assay, it may be useful to assess ROS generation using flow cytometry-based assays that allow for the identification of several species of both reactive oxygen and nitrogen species, which would provide a better understanding of how hypoxia affects neutrophil activation.

I would have also liked to conduct further endothelial co-culture experiments. Studying NETosis in co-culture with resting and activated endothelium would validate and expand upon my preliminary data. Inclusion of functional blocking antibodies against both neutrophil integrins and endothelial adhesion molecules would build upon published studies and identify the cellular interactions that promote NETosis in co-culture. Examining the effects of hypoxia on cellular interactions and how they modulate NETosis would provide further insight into neutrophil biology.

Further work is also required to complete studies examining the effects of hypoxia upon protein expression and subcellular localisation in both neutrophil and ECs. Earlier time points would highlight the dynamics of protein expression and subcellular localisation, which may indicate how hypoxia affects protein trafficking. Given that the proteins examined are integral to NET structure and function, assessing cell supernatants for MPO, citrullinated histone H3, and even PAD-4 (Spengler et al., 2015), would contribute to our understanding of the kinetics and dynamics of NETosis under hypoxia, from protein synthesis to release within NETs.

Finally, further dissection of the signalling pathways involved may highlight important signalling molecules important to NETosis during hypoxia. For example, as p38 MAPK inhibition exhibited a dose-dependent inhibitory effect in NETosis, it would be interesting to examine the effects of hypoxia upon p38 MAPK expression and activity. Having explored the effects of hypoxia upon neutrophil activation, in the next chapter, I will evaluate the modulatory role of IgG upon neutrophil function.

Chapter Five:
The Effects of Purified Immunoglobulin G on Neutrophil Function

5.1 Introduction and aims

Neutrophil dysfunction has been implicated in the pathology of RA, SLE and APS, but few studies have examined the effects of purified IgG upon neutrophil adhesion and activation. SLE- and APS-IgG enhance monocyte adhesion to ECs (Simantov et al., 1995, Del Papa et al., 1999), but the effects upon adhesion of other leukocytes have not been explored. RA-IgG has been found to modulate *in vitro* NETosis (Khandpur et al., 2013), with a similar effect being observed with SLE-IgG in mouse models (Knight et al., 2013). Recent studies have also shown that APS-IgG enhances NETosis (Yalavarthi et al., 2015). Therefore, I carried out work in this chapter to examine how purified IgG affects neutrophil adhesion, ROS generation and NETosis.

5.2 IgG purification from the serum of patients with RA, SLE and APS

To examine the effects of IgG upon neutrophil activation, I first purified whole IgG fractions from the sera of HCs and patients with RA, SLE or APS. Demographic details of these IgG samples are listed in Table 5.1.

The RA cohort had a mean age of 51 ±13.4 years, which was significantly older than SLE, APS and HC cohorts ($p<0.05$). All 9 RA patients had inflammatory arthritis, 1 patient was hypertensive, 1 had ILD and 1 had rheumatoid nodules. There was a range of immunomodulatory medications within the RA cohort: 5 patients were prescribed HCQ; 3 patients received methotrexate; 1 patient was prescribed prednisolone; and 1 patient was prescribed sulfasalazine. In addition, 2 patients had been administered rituximab and 1 patient had stopped humira between 3-6 months before the time of serum preparation.

The SLE cohort had a mean age of 32 ±7.4 years. Within the cohort, 2 patients had arthralgia, 4 had nephritis and 2 had a lupus-associated rash. SLE patients were also prescribed a range of immunomodulatory treatments: 5 patients were prescribed mycophenolate mofetil; 5 patients were prescribed prednisolone; 4 patients were taking HCQ; and 1 patient was prescribed tacrolimus. Three patients were also prescribed aspirin.

		Healthy Control	RA	SLE	APS
Sample Size		9	9	5	9
Age (±SD)		30 ±4.5	51 ±13.4	32 ±7.4	39 ±10.1
Sex (M:F)		2:7	2:7	0:5	2:7
ACPA (U/ml)		2.16 ±0.10	124.74 ±106.12	2.38 ±0.34	2.11 ±0.11
ANCA	PR3	0.79 ±0.15	0.73 ±0.07	0.75 ±0.04	0.75 ±0.05
	MPO	0.75 ±0.04	0.74 ±0.07	0.74 ±0.02	0.76 ±0.03
VT		0	0	0	7
VT/PM		0	0	0	2
PM		0	0	0	0
Clinical Manifestations	CVD	0	1	0	0
	Joint	0	9	2	0
	Lung	0	1	0	0
	Renal	0	0	4	0
	Skin	0	1	2	0
Treatment	Thromboprophylaxis	N/A	N/A	Aspirin (3)	Aspirin (1), Warfarin (9)
	Immunomodulatory	N/A	HCQ (5), Humira (1), MTX (3), Pred (1), RTX (2), SSZ (1)	HCQ (4), MMF (5), Pred (5), Tacrolimus (1)	Pred (1)

Table 5.1: Demographics table of purified IgG patient cohorts. Clinical details were recorded for all subjects. At the time of serum preparation, 1 RA patient was hypertensive, 9 RA had inflammatory arthritis, 2 SLE patients had arthralgia, 1 RA patient had interstitial lung disease, 1 RA patient presented with rheumatoid nodules, 2 SLE patients had rashes and 4 SLE patients had nephritis. Purified IgG were assessed for ACPA and ANCA positivity by ELISA. Abbreviations: CVD, cardiovascular disease; HCQ, hydroxychloroquine; MMF, mycophenolate mofetil; MTX, methotrexate; Pred, prednisolone; PM, pregnancy morbidity; RTX, rituximab; SSZ, sulfasalazine; VT, venous thrombosis

The APS cohort had a mean age of 39 ±10.1 years. 7 patients had venous thrombotic clinical events, whilst the remaining 2 had pregnancy morbidity. 1 patient was prescribed prednisolone. All 9 patients were taking warfarin and 1 was prescribed aspirin as thromboprophylaxis.

The HC cohort had a mean age of 39 ±4.5 years. None of these subjects had any clinical complications and were not taking any prescribed medications.

ACPA and ANCA titres were determined by ELISA, with positivity defined as ACPA titres of 5 U/ml or greater and ANCA ratios greater than 4.0, as determined by manufacturer protocol. 8 of 9 RA-IgG samples were ACPA positive (20.46-299.3 U/ml) and one was ACPA negative (1.57 U/ml). All other IgG samples were ACPA negative: SLE-IgG (2.04-2.99 U/ml); APS-IgG (2.03-2.40 U/ml); and HC-IgG (2.00-2.32 U/ml). All IgG samples were negative for both PR3- and MPO-ANCA: RA-IgG (PR3-ANCA: 0.66-0.90; MPO-ANCA: 0.67-0.90); SLE-IgG (PR3-ANCA: 0.69-0.79; MPO-ANCA: 0.70-0.76); APS-IgG (PR3-ANCA: 0.69-0.83; MPO-ANCA: 0.72-0.81); and HC-IgG (PR3-ANCA: 0.69-1.18; MPO-ANCA: 0.70-0.82).

5.3 APS-IgG enhances PMA-induced neutrophil adhesion to fibronectin

To examine the effects of IgG upon integrin activation, I assessed neutrophil adhesion to immobilised integrin ligands in the presence of purified IgG. First the effects of IgG upon β_1 integrin activation were investigated by examining neutrophil adhesion to immobilised fibronectin. Neutrophils were allowed to adhere to immobilised fibronectin in the absence or presence of PMA and purified IgG.

Unstimulated neutrophils displayed a 1.5 to 2.5 fold increase in adhesion to fibronectin in the presence of IgG, however increased adhesion was not significant compared to no IgG controls (Figure 5.1A). Stimulation with PMA increased neutrophil adhesion in the presence and absence of IgG. Neutrophils treated with HC-IgG had similar binding to no IgG controls (Figure 5.B). APS-IgG significantly increased PMA-stimulated adhesion to immobilised fibronectin compared to HC-IgG ($p<0.05$). Although there was a trend for increased adhesion in

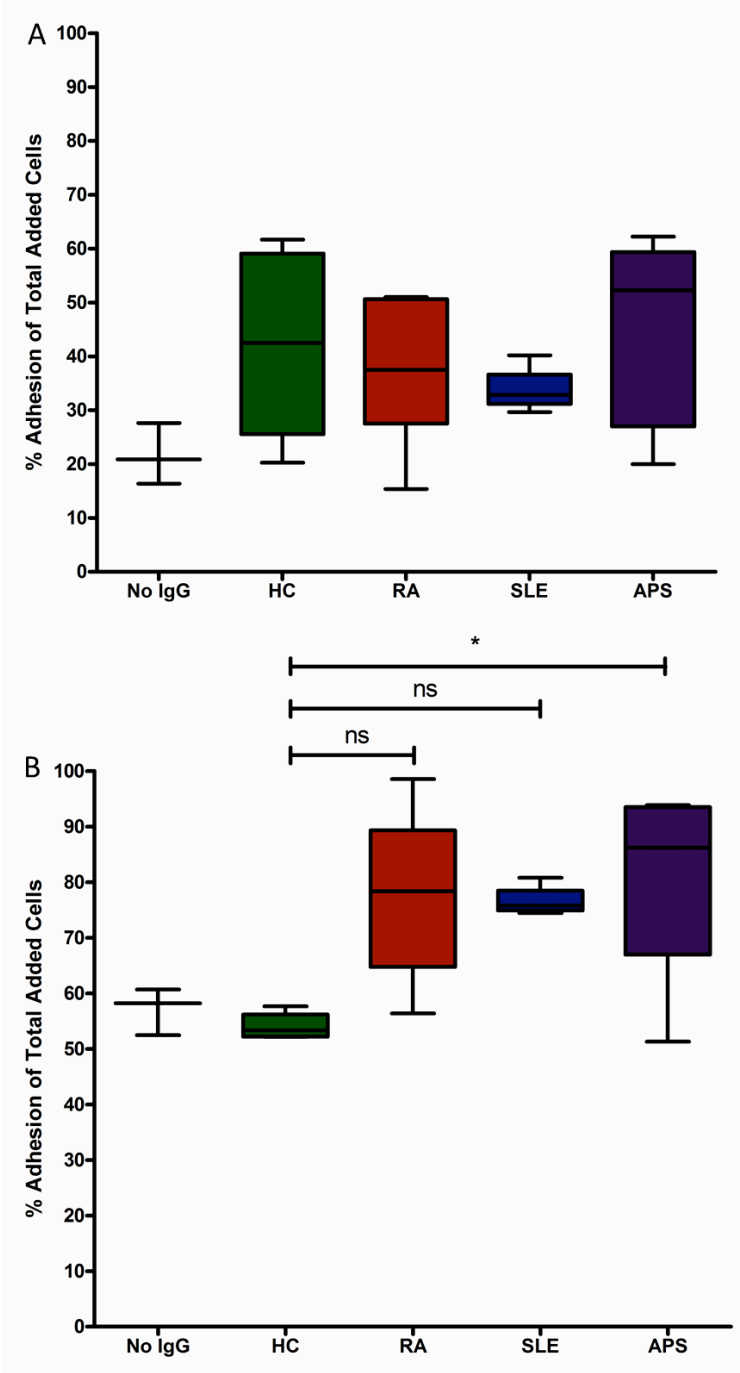


Figure 5.1: APS-IgG significantly enhances PMA-stimulated neutrophil adhesion to immobilised fibronectin. To examine the modulatory effects of IgG upon β_1 integrin activation, neutrophil adhesion to immobilised fibronectin in the presence of IgG was studied. $5x10^5$ fluorescently labelled neutrophils were added to fibronectin-coated wells in the absence or presence of 20nM PMA and 200µg/ml of purified RA-IgG (n=7), SLE-IgG (n=4), APS-IgG (n=6) or HC-IgG (n=6). **(A)** The presence of purified IgG increased adhesion, however this increase was not significant compared to no IgG **(B)** APS-IgG significantly increased PMA-induced adhesion to fibronectin compared to HC-IgG. Statistical significance was determined by a Kruskal-Wallis test with a Dunn's multiple comparison test. Data is presented as box plots, with averaged results for each IgG samples from three independent experiments. Abbreviations: HC, healthy control; RA, rheumatoid arthritis; SLE, systemic lupus erythematosus; APS, antiphospholipid syndrome. ns= no significance *=$p<0.05$

PMA-stimulated neutrophils treated with RA- and SLE-IgG, these differences failed to reach statistical significance.

To determine whether the increased PMA-stimulated adhesion to fibronectin was β_1 integrin-dependent, adhesion assays were conducted in the absence or presence of P5D2, which specifically inhibits β_1 integrin-mediated adhesion. For all IgG groups, incubation with P5D2 significantly reduced adhesion, demonstrating that the adhesion observed in the presence of IgG was mediated by β_1 integrin activation (Figure 5.2A-D).

5.4 RA- and SLE-IgG enhance Mac-1-mediated neutrophil adhesion

Having found differential β_1 integrin activation by APS-IgG, I then studied the effects of purified IgG upon β_2 integrin-mediated adhesion. To interrogate activation of Mac-1, rather than LFA-1, neutrophil adhesion to immobilised fibrinogen was examined. Neutrophils were added to fibrinogen-coated wells and adhesion assessed in the absence or presence of PMA and HC-, RA-, SLE- or APS-IgG.

Low binding to fibrinogen was observed by unstimulated neutrophils. Adhesion was increased by the presence of purified IgG, however these differences were not significant (Figure 5.3A). In contrast, PMA stimulation significantly increased neutrophil adhesion to fibrinogen, which was further modulated by the presence of certain ARD-IgG (Figure 5.3B). HC-IgG did not significantly alter adhesion compared to no IgG controls. Significant increases in neutrophil adhesion to immobilised fibrinogen were observed in the presence of RA-IgG ($p<0.05$) and SLE-IgG ($p<0.01$) but not APS-IgG ($p>0.05$) compared to HC-IgG.

To determine whether the increased PMA-stimulated adhesion observed was Mac-1-dependent, neutrophil adhesion was also examined in the absence or presence of the Mac-1-specific blocking antibody 2LPM19c. For all IgG groups, PMA-induced neutrophil adhesion was significantly reduced by the presence of 2LPM19c (Figure 5.4A-D). Therefore, RA- and SLE-IgG modulate PMA-stimulated neutrophil adhesion to fibrinogen by promoting Mac-1 activation.

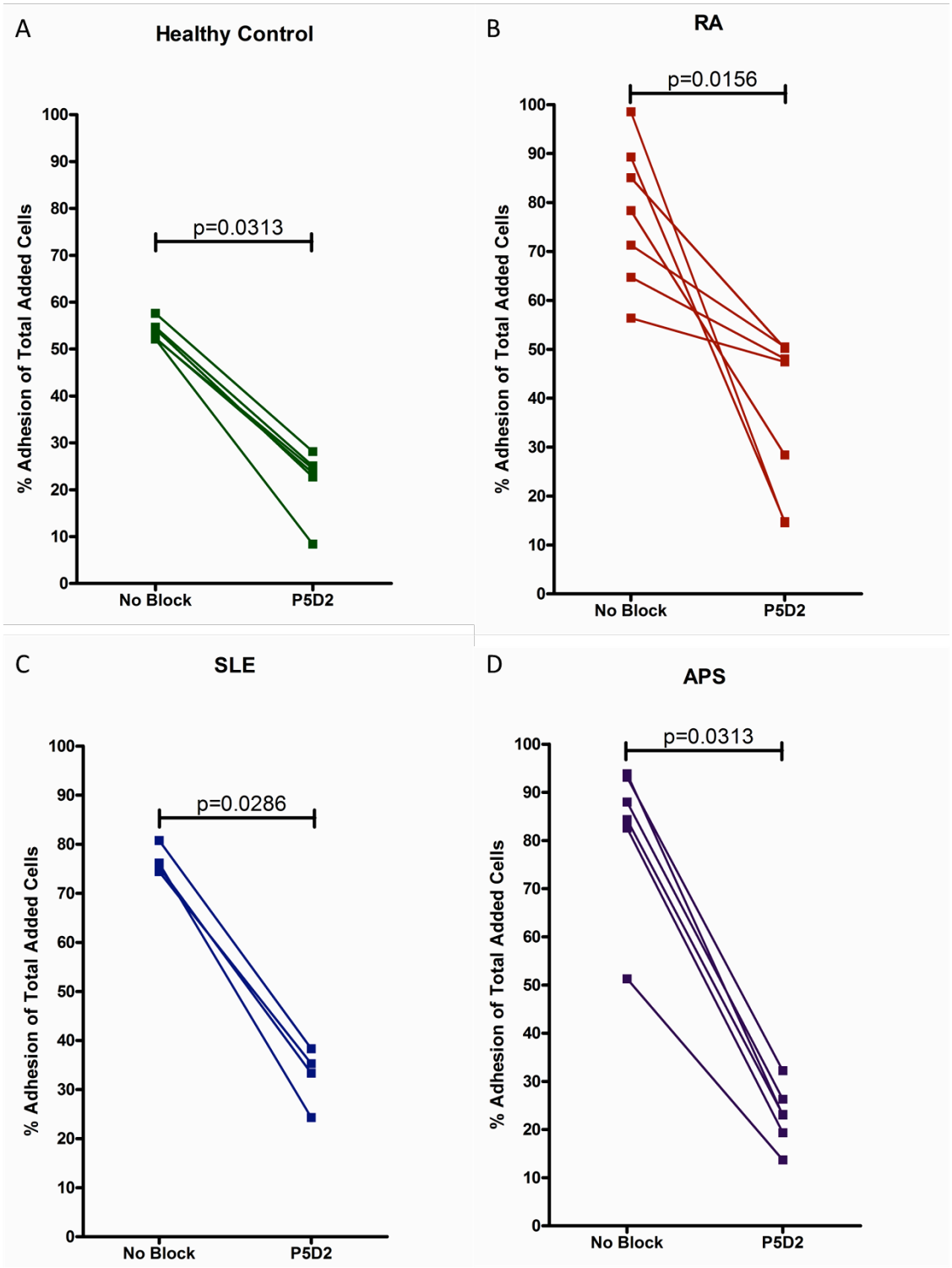


Figure 5.2: Increased PMA-stimulated neutrophil adhesion to fibronectin is inhibited by β_1 integrin blockade. To assess whether increased adhesion to fibronectin was integrin mediated, adhesion assays were performed in the absence or presence of 10µg/ml of the pan-β_1 integrin inhibitor P5D2. $5x10^5$ fluorescently labelled neutrophils were allowed to adhere to immobilised fibronectin with 20nM PMA and 200µg/ml of **(A)** HC-IgG (n=6) **(B)** RA-IgG (n=7) **(C)** SLE-IgG (n=4) or **(D)** APS-IgG (n=6). P5D2 significantly reduced adhesion for all groups by Wilcoxon matched pairs tests. Data is presented as matched observations with averaged results from three independent experiments. Abbreviations: HC, healthy control; RA, rheumatoid arthritis; SLE, systemic lupus erythematosus; APS, antiphospholipid syndrome.

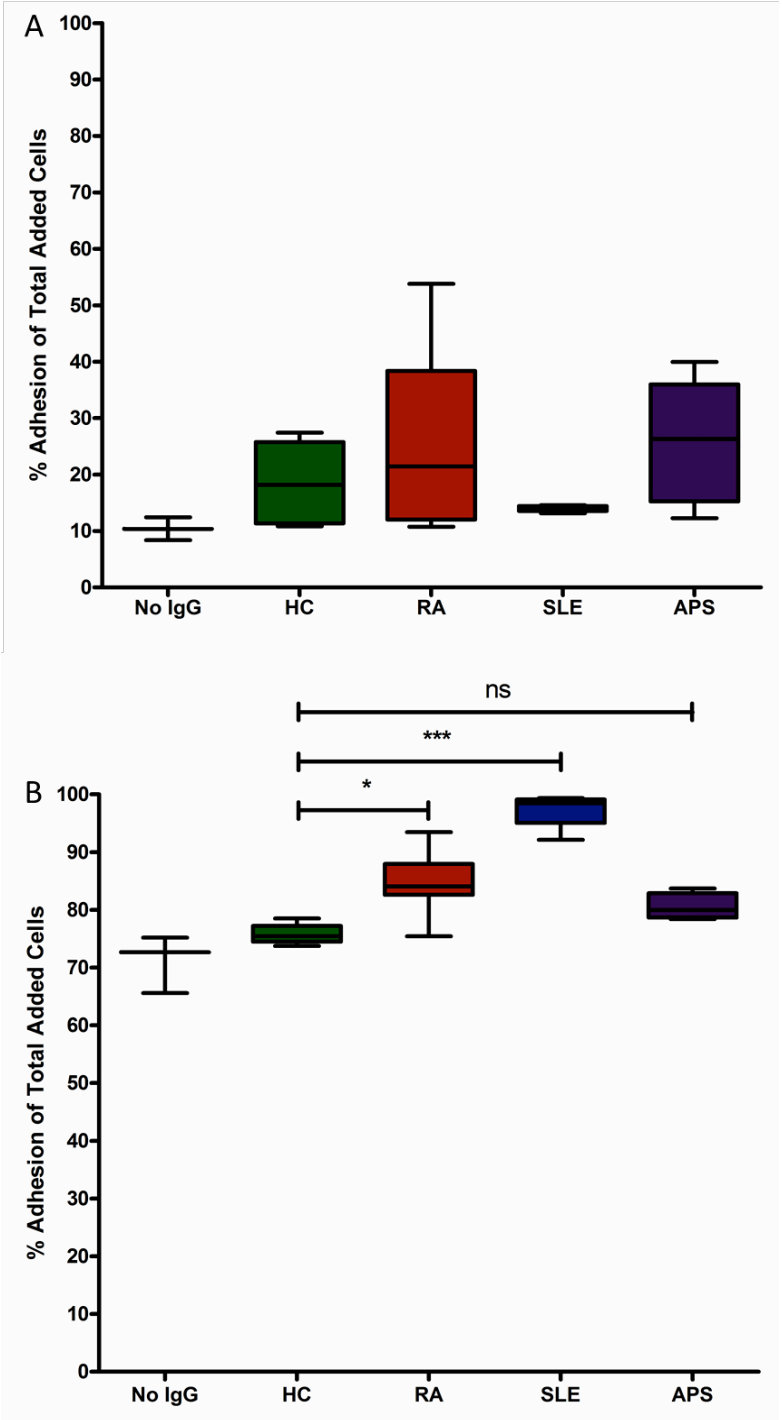


Figure 5.3: RA- and SLE-IgG enhance PMA-stimulated neutrophil adhesion to immobilised fibrinogen. To examine the effects of IgG upon β_2 integrin activation, neutrophil adhesion to immobilised fibrinogen in the presence of IgG was assessed. $5x10^5$ fluorescently labelled neutrophils were added to fibrinogen-coated wells in the absence or presence of 20nM PMA and 200µg/ml of purified RA-IgG (n=7), SLE-IgG (n=4), APS-IgG (n=6) or HC-IgG (n=6). **(A)** The presence of IgG did not significantly increase neutrophil adhesion in the absence of PMA **(B)** Significant increases in adhesion in the presence of PMA was observed in the presence of RA-IgG and SLE-IgG. Statistical significance was determined by a Kruskal-Wallis test with a Dunn's multiple comparison test. Data is presented as box plots, with averaged results for each IgG samples from three independent experiments. Abbreviations: HC, healthy control; RA, rheumatoid arthritis; SLE, systemic lupus erythematosus; APS, antiphospholipid syndrome. ns= no significance *=$p<0.05$, ***=$p<0.001$

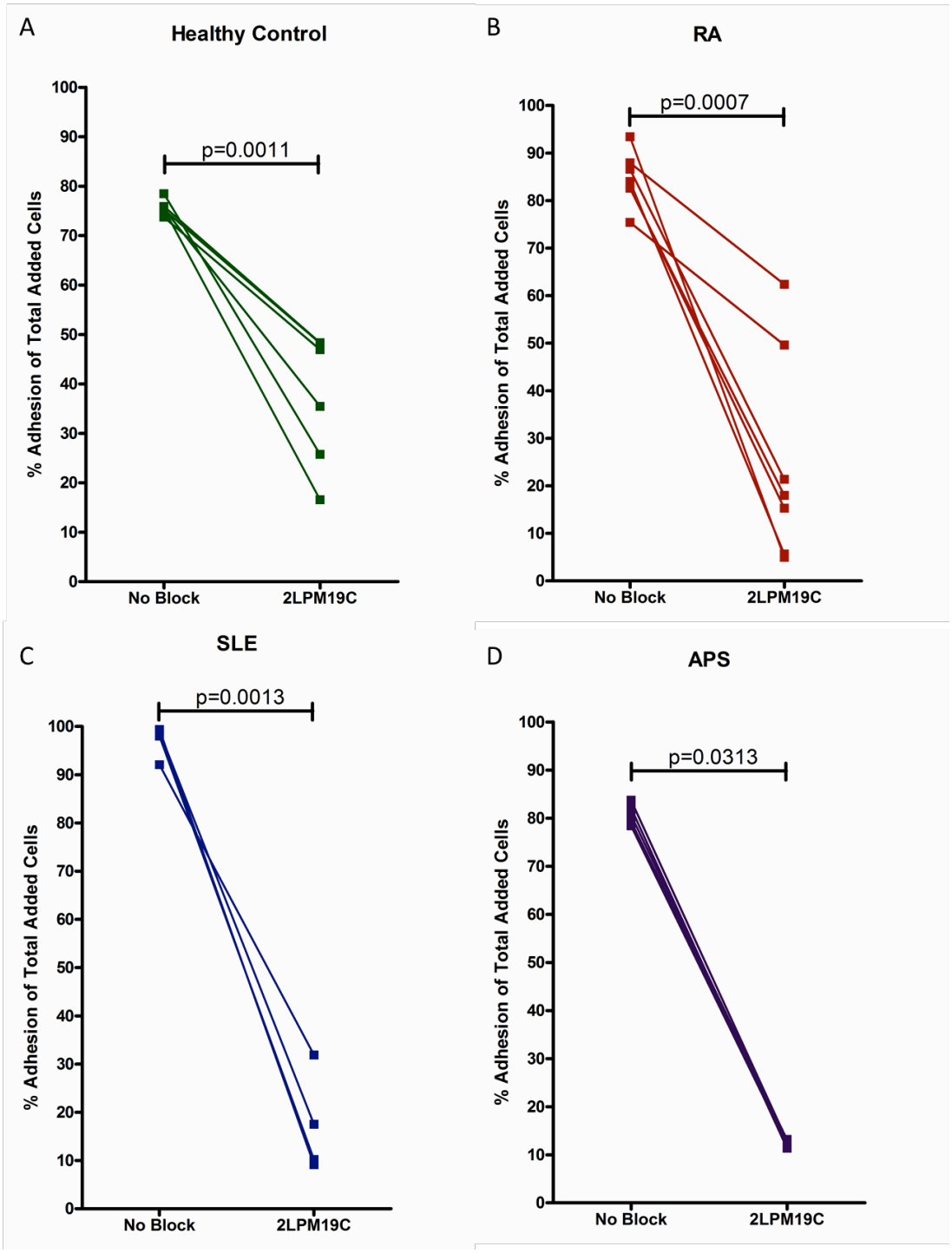


Figure 5.4: Enhanced PMA-induced neutrophil adhesion to fibrinogen is inhibited by Mac-1 blockade. To determine whether adhesion to fibrinogen was integrin mediated, assays were performed in the absence or presence of 10µg/ml of the Mac-1 ($\alpha_M\beta_2$) inhibitor 2LPM19C. $5x10^5$ fluorescently labelled neutrophils were allowed to adhere to immobilised fibrinogen with 20nM PMA and 200µg/ml of **(A)** HC-IgG (n=6) **(B)** RA-IgG (n=7) **(C)** SLE-IgG (n=4) or **(D)** APS-IgG (n=6). Paired t tests found significant decreases in adhesion with 2LPM19C treatment in panels A and B. Wilcoxon matched pairs tests found 2LPM19C significantly reduced adhesion in panels C and D. Data is presented as matched observations with averaged results from three independent experiments. Abbreviations: HC, healthy control; RA, rheumatoid arthritis; SLE, systemic lupus erythematosus; APS, antiphospholipid syndrome.

5.5 RA-, SLE- and APS-IgG bind neutrophils

Having found an effect of purified IgG upon neutrophil adhesion to immobilised integrin ligands, I examined whether purified IgG bound neutrophils. To exclude non-specific effects of FcR binding, neutrophils were cultured in the absence or presence of a FcR blocking reagent (Miltenyi, UK) with IgG pooled from either healthy volunteers or patients with RA, SLE or APS characterised in Table 5.1. Following incubation, neutrophils were fixed and stained for CD15 and human IgG, to determine the extent to which purified IgG bound neutrophils.

$CD15^+$ neutrophils incubated with either no IgG or HC-IgG had low positivity for human IgG (Figure 5.5A), demonstrating low binding to neutrophils. Significantly higher levels of $CD15^+IgG^+$ neutrophils were observed when cells were incubated with RA-, SLE or APS-IgG (Figure 5.5A), indicating increased binding of ARD-IgG to neutrophils. Furthermore, neutrophils incubated with RA-, SLE- or APS-IgG in the absence of FcγR blockade had significantly higher binding compared to cells treated with HC-IgG ($p<0.01$) (Figure 5.5B). Interestingly in the presence of FcγR blockade, significantly more neutrophils bound RA-IgG ($p<0.001$) and APS-IgG ($p<0.05$) compared to HC-IgG (Figure 5.5C). Incubation with SLE-IgG however, failed to produce significantly higher numbers of IgG^+ neutrophils compared to HC-IgG (Figure 5.5C).

Binding of pooled IgG was also compared in the absence or presence of FcγR blockade (Figure 5.6). Binding of HC-, RA- and APS-IgG was not significantly affected by FcγR blockade (Figure 5.6A, B, D), however significantly lower numbers of neutrophils were positive for human IgG when cultured with SLE-IgG in the presence of FcγR blockade (Figure 5.6C). Having shown that purified IgG can directly bind neutrophils and modulate neutrophil adhesion to fibronectin and fibrinogen, I then examined the effects of purified IgG upon other aspects of neutrophil activation.

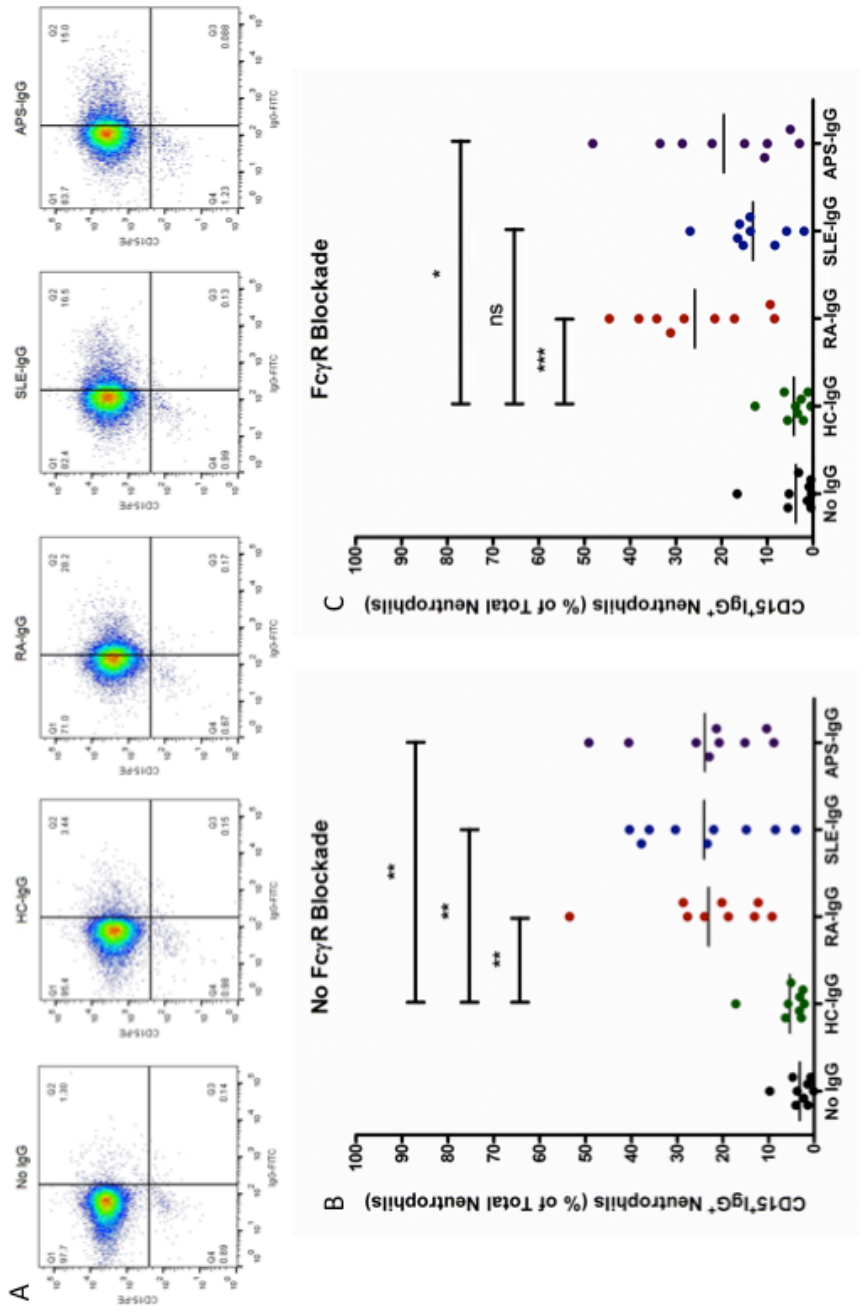


Figure 5.5: ARD-IgG bind neutrophils. To determine whether purified IgG bound neutrophils, $2x10^5$ isolated neutrophils from 9 donors were cultured for 2 hours in the absence or presence of 200µg/ml pooled HC-, RA-, SLE- or APS-IgG (IgG pools comprised of 5 IgG samples previously characterised in Table 5.1). Following incubation, cells were fixed and stained for the neutrophil cell surface marker CD15 and for human IgG. Stained cells were analysed by flow cytometry and assessed for positivity for both CD15 and human IgG. **(A)** Representative flow cytometry plots for neutrophils cultured with the various IgG groups. **(B)** Neutrophils cultured in the absence of FcγR blockade had significantly higher numbers of $CD15^+$ neutrophils that were also positive for RA-, SLE- and APS-IgG compared to HC-IgG (Kruskal-Wallis test with a Dunn's multiple comparison test). **(C)** Neutrophils cultured in the presence of FcγR blockade had significantly higher binding of RA- and APS-IgG compared to HC-IgG (one-way ANOVA with a Bonferroni's multiple comparison test). Abbreviations: HC, healthy control; RA, rheumatoid arthritis; SLE, systemic lupus erythematosus; APS, antiphospholipid syndrome. ns= no significance *=p<0.05, **=p<0.01, ***=p<0.001

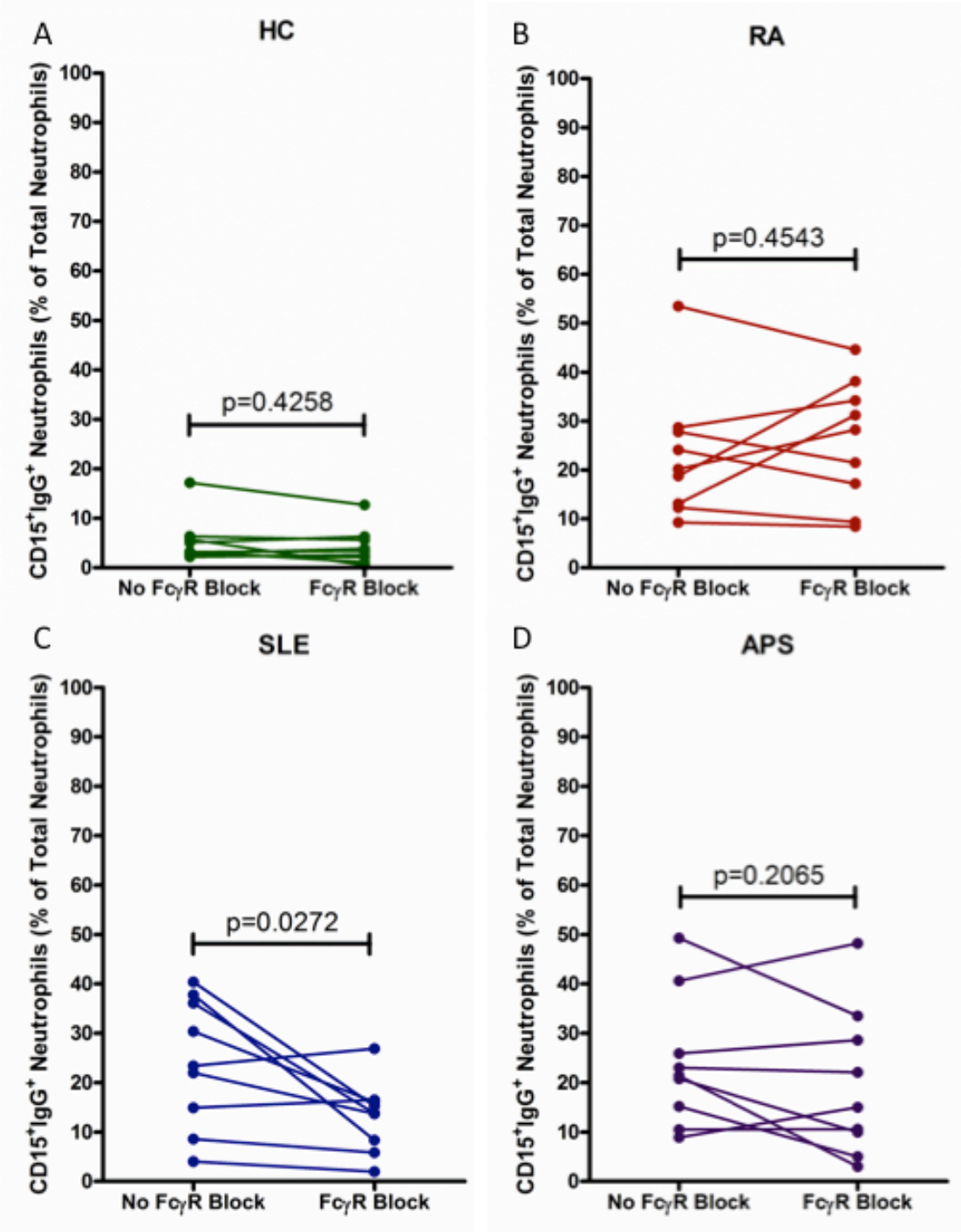


Figure 5.6: Binding of SLE-, but not HC-, RA- or APS-IgG to neutrophils is reduced by FcγR blockade. IgG binding was compared in the absence or presence of FcγR blockade. $2x10^5$ neutrophils from 9 donors were cultured for 2 hours with 200μg/ml pooled HC-, RA-, SLE- or APS-IgG (IgG pools were comprised of 5 IgG samples previously characterised in Table 5.1). After incubation, cells were stained for CD15 and human IgG and then analysed by flow cytometry. **(A)** Binding of HC-IgG was not significantly affected by FcγR blockade (Wilcoxon matched pairs test). **(B)** RA-IgG binding was not affected by the presence of FcγR blockade (paired t test). **(C)** Binding of SLE-IgG was significantly lower in the presence of FcγR blockade (paired t test). **(D)** APS-IgG was not significantly affected by FcγR blockade (Wilcoxon matched pairs test). Abbreviations: HC, healthy control; RA, rheumatoid arthritis; SLE, systemic lupus erythematosus; APS, antiphospholipid syndrome

5.6 SLE patient-derived neutrophils have lower rates of hydrogen peroxide generation

To explore the effects of purified IgG upon neutrophil activation, I began by examining the effects of IgG upon neutrophil hydrogen peroxide generation. First, to determine whether there were intrinsic differences in ROS production between control and patient populations, I compared hydrogen peroxide generation in neutrophils isolated from separate cohorts of HC and patients with RA, SLE or APS, demographics shown in Table 5.2.

RA patients had a mean age of 62 ±4.1 years, which was significantly older than SLE ($p<0.05$), APS ($p<0.05$) and HC cohorts ($p<0.001$). All 7 RA patients had inflammatory arthritis, 1 patient was hypertensive, 3 had ILD and 1 had rheumatoid nodules. There was a range of immunomodulatory medications within the RA cohort: 5 patients were prescribed HCQ; 1 patient received methotrexate; and, 1 patient was prescribed prednisolone. In addition, 1 patient had received rituximab 6 months prior to blood donation.

The SLE cohort had a mean age of 40 ±4.5 years. Within the cohort, 3 patients had arthralgia, 6 had nephritis, 2 had ILD and 2 had a lupus-associated rash. SLE patients were also prescribed a range of immunomodulatory treatments: 12 patients were prescribed mycophenolate mofetil; 12 patients were prescribed prednisolone; 8 patients were taking HCQ; and 2 patients were prescribed tacrolimus. Five patients with SLE were also prescribed aspirin.

APS patients had a mean age of 44 ±13.7 years. 5 patients had venous thrombotic clinical events, 2 patients had pregnancy morbidity and 3 had both thrombotic and pregnancy manifestations. At the time of blood donation, 3 patients were prescribed prednisolone. Five patients were taking warfarin and 8 were taking aspirin as thromboprophylaxis.

The HC cohort had a mean age of 36 ±8.2 years. None of the subjects had any clinical complications and were not taking any prescribed medications at the time of blood donation.

		Healthy Control	RA	SLE	APS
Sample Size		12	7	12	10
Age (±SD)		36 ±8.2	62 ±4.1	40 ±4.5	44 ±13.7
Sex (M:F)		5:7	2:5	2:10	1:9
VT		0	0	0	5
VT/PM		0	0	0	3
PM		0	0	0	2
Clinical Manifestations	CVD	0	1	0	0
	Joint	0	7	3	0
	Lung	0	3	2	0
	Renal	0	0	6	0
	Skin	0	1	2	0
Treatment	Thromboprophylaxis	N/A	Aspirin (1)	Aspirin (5)	Aspirin (8), Warfarin (5)
	Immunomodulatory	N/A	HCQ (5), MTX (1), Pred (1), RTX (1)	HCQ (8), MMF (12), Pred (12), Tacrolimus (2)	Pred (3)

Table 5.2: Demographic information of subjects who donated blood for neutrophil hydrogen peroxide generation assays. Clinical details were recorded for all subjects. At the time of blood donation 1 RA patient was hypertensive, 7 RA patients had inflammatory arthritis, 3 SLE patients had arthralgia, 3 RA patients and 2 SLE patients had interstitial lung disease, 1 RA patient presented with rheumatoid nodules, 2 SLE patients had rashes and 6 SLE patients had nephritis. Abbreviations: CVD, Cardiovascular disease; HCQ, hydroxychloroquine; MMF, mycophenolate mofetil; MTX, methotrexate; Pred, prednisolone; PM, pregnancy morbidity; RTX, rituximab; SSZ, sulfasalazine; VT, venous thrombosis

Neutrophils were isolated from patients with RA (n=7), SLE (n=12) or APS (n=10) and HC (n=12). Rates of hydrogen peroxide were assessed in the absence or presence of PMA. Low levels of hydrogen peroxide were seen in unstimulated neutrophils, which were not significantly different between patient and control populations (Figure 5.7A). PMA stimulation induced hydrogen peroxide generation in all groups, however rates of production were significantly lower in SLE-derived neutrophils compared to neutrophils isolated from patients with RA ($p<0.05$), APS ($p<0.001$) and HC ($p<0.05$) (Figure 5.7B). There was a trend to faster rates of hydrogen peroxide production by neutrophils derived from APS patients compared to HC, however this difference did not reach statistical significance.

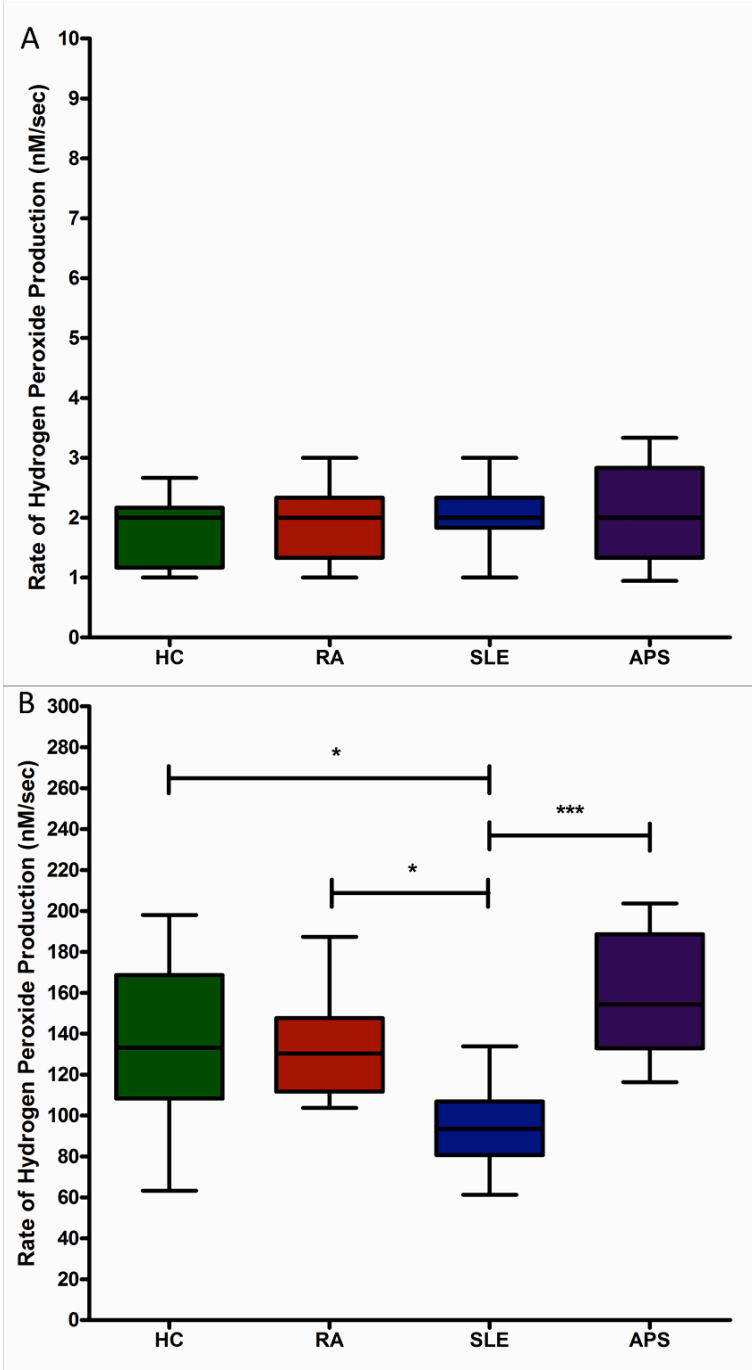


Figure 5.7: Neutrophils isolated from SLE patients display slower rates of hydrogen peroxide generation. To examine whether there were differences in reactive oxygen species generation between patient and control populations, neutrophils were isolated patients with RA (n=7), SLE (n=12) or APS (n=10) and healthy volunteers (n=12) and rates of hydrogen peroxide production from $4x10^5$ neutrophils were assessed in the absence or presence of 50nM PMA. **(A)** Neutrophils produce low rates of hydrogen peroxide production in the absence of PMA. Rates were similar across all groups. **(B)** SLE-derived neutrophils has slower rates of PMA-stimulated hydrogen peroxide generation compared to RA, APS and HC. Statistical significance was determined by one-way ANOVA with a Bonferroni's multiple comparison test. Data is presented as box and whisker plots from averaged observations from each patient. Abbreviations: HC, healthy control; RA, rheumatoid arthritis; SLE, systemic lupus erythematosus; APS, antiphospholipid syndrome. *=$p<0.05$, ***=$p<0.001$

5.7 RA- and SLE-IgG elevate rates of hydrogen peroxide generation

Having found reduced rates of hydrogen peroxide in neutrophils of patients with SLE, I then examined the effects of purified IgG upon ROS production by HC neutrophils. A preliminary experiment was conducted to determine whether incubation of neutrophils with IgG induced FcR-mediated modulation of neutrophil activation. HC neutrophils were isolated and incubated with HC-IgG (n=6) or RA-IgG (n=9) in the absence or presence of a FcR blocking reagent (Miltenyi, UK). Rates of hydrogen peroxide generation were then measured in response to PMA (Figure 5.8A). In the absence of FcγR blockade, rates of hydrogen peroxide generation were not significantly different between neutrophils incubated with HC- and RA-IgG ($p=0.0813$). In contrast, RA-IgG significantly increased hydrogen peroxide generation compared to HC-IgG in the presence of FcγR blockade ($p=0.0008$). FcγR blockade significantly reduced hydrogen peroxide production rates in neutrophils incubated with HC-IgG ($p=0.0169$) (Figure 5.8B). In contrast, no significance difference was observed in RA-IgG treated cells in the absence or presence of FcγR blockade ($p=0.2348$) (Figure 5.8C). Given these observations, FcγR blockade was used for all further functional neutrophil activation assays.

To examine the effects of purified IgG upon ROS generation, HC neutrophils were pre-incubated with HC-IgG (n=9), RA-IgG (n=9), SLE-IgG (n=4) or APS-IgG (n=9) for 1 hour. Following incubation, rates of hydrogen peroxide production were measured in the absence or presence of PMA. Unstimulated neutrophils produced low levels of hydrogen peroxide, which were similar across all IgG groups (data not shown). PMA stimulation induced hydrogen peroxide generation, with ARD-IgG conferring a modulatory effect. Pre-incubation with HC-IgG did not significantly affect rates of PMA-induced hydrogen peroxide production compared to no IgG controls (Figure 5.9). Rates of hydrogen peroxide generation were significantly increased following pre-incubation with RA-IgG ($p<0.01$) and SLE-IgG ($p<0.001$) compared to HC-IgG. A trend for increased hydrogen peroxide generation was observed following pre-incubation with APS-IgG, but differences failed to reach statistical significance.

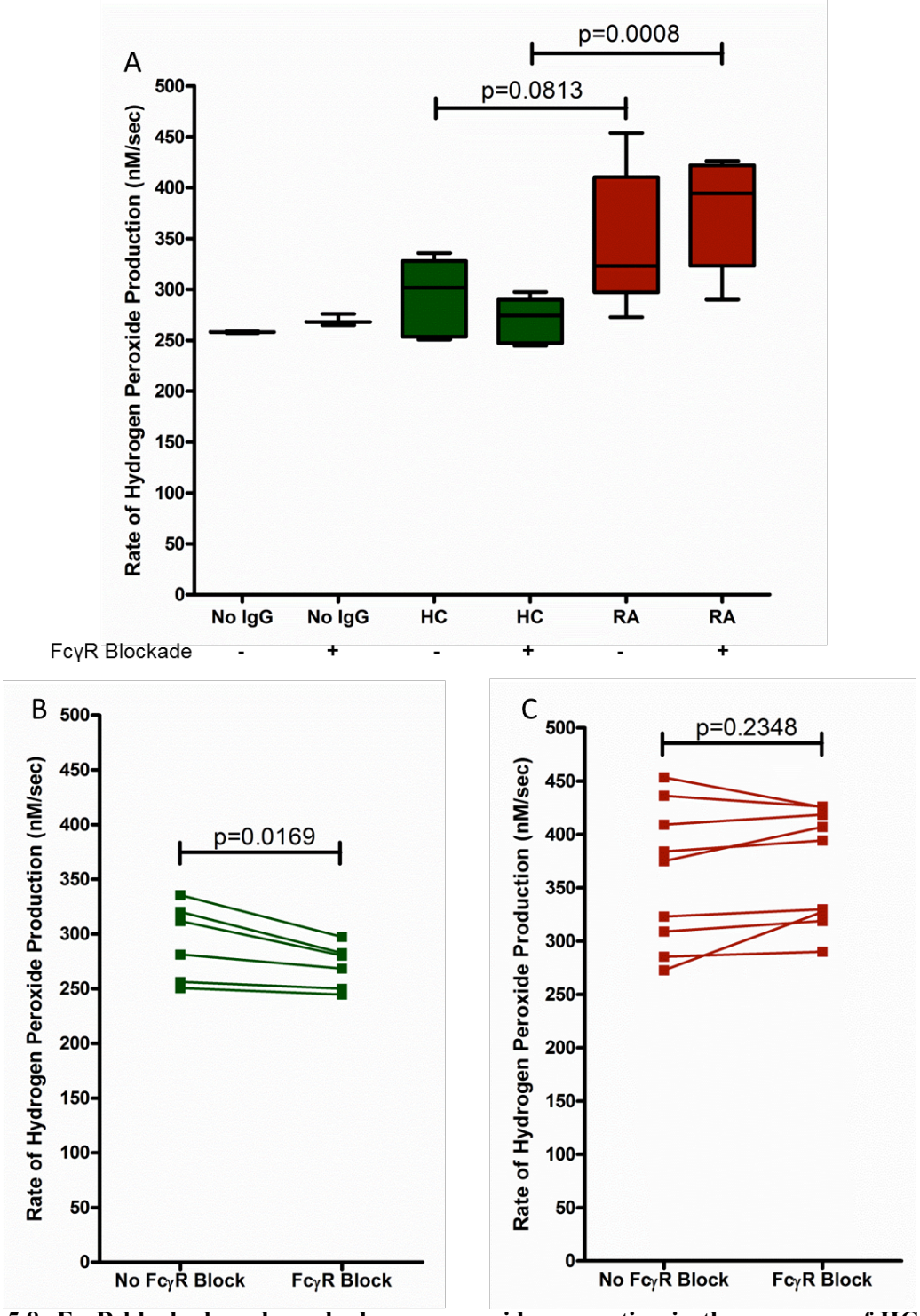


Figure 5.8: FcγR blockade reduces hydrogen peroxide generation in the presence of HC-IgG but not RA-IgG. Hydrogen peroxide generation by $4x10^5$ neutrophils was measured in the absence or presence of FcγR blockade and 200µg/ml HC-IgG (n=6) or RA-IgG (n=9) in response to 50nM PMA. **(A)** HC-IgG produced similar rates of hydrogen peroxide generation as no IgG controls. In the absence of FcγR blockade, HC- and RA-IgG were not significantly different (p=0.0813, unpaired t test), whereas in the presence of FcγR blockade, differences were significantly different (p=0.0008, unpaired t test). **(B)** FcγR blockade significantly reduced hydrogen peroxide production in the presence of HC-IgG (p=0.0169, paired t test). **(C)** FcγR blockade did not significantly affect hydrogen peroxide generation in the presence of RA-IgG (p=0.2348, paired t test). Data is presented as box and whisker plots or matched observations from each IgG sample. Abbreviations: HC, healthy control; RA, rheumatoid arthritis

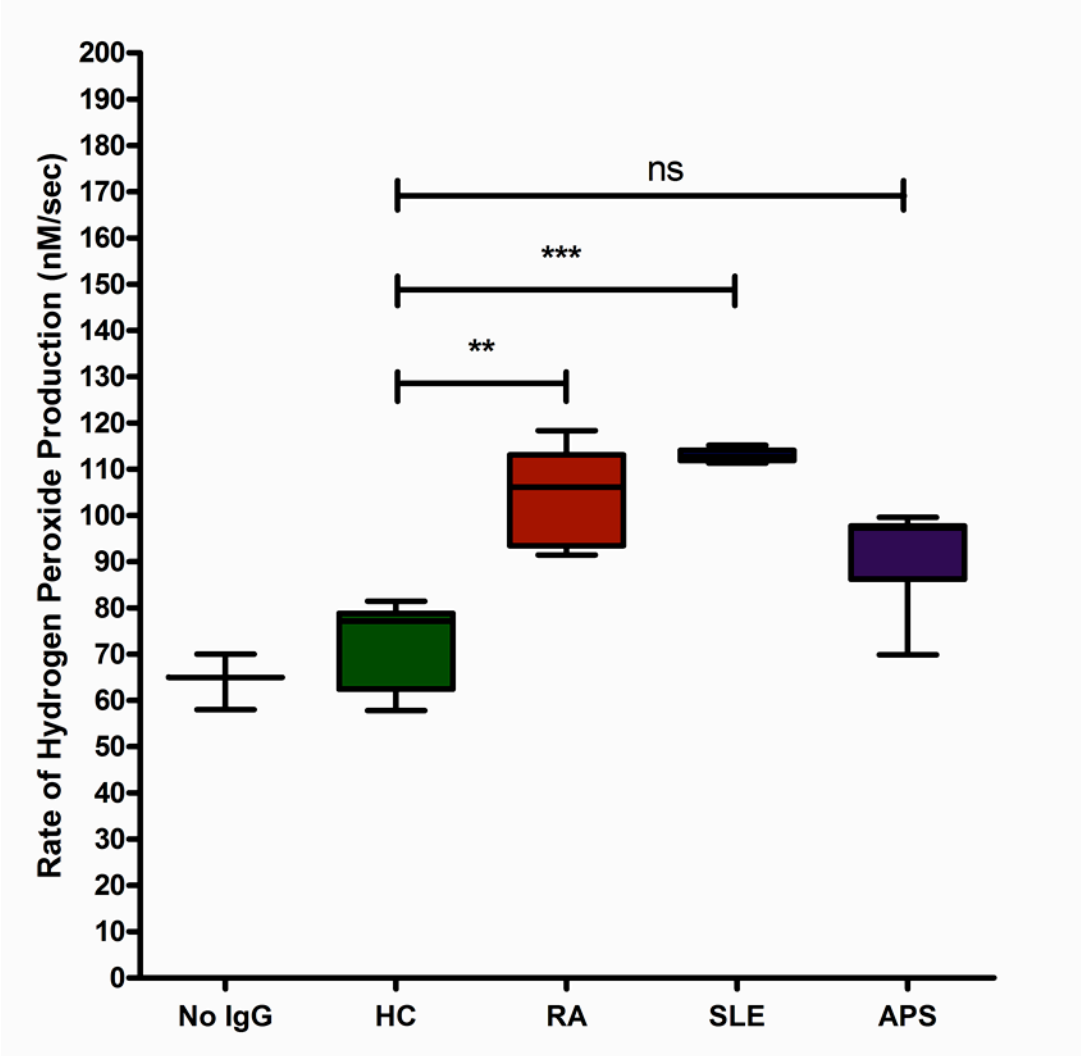


Figure 5.9: Neutrophils treated with RA- or SLE-IgG display elevated rates of hydrogen peroxide generation. Healthy control neutrophils were isolated and treated with FcγR blockade. Cells were then incubated for 1 hour with 200µg/ml purified RA-IgG (n=9), SLE-IgG (n=4), APS-IgG (n=9) or HC-IgG (n=9). Following incubation, $4x10^5$ neutrophils were added to the wells of a 96-well black tissue culture plate and rates of hydrogen peroxide generation evaluated in response to 50nM PMA. Significantly higher rates of hydrogen peroxide production were observed in neutrophils incubated with RA- or SLE-IgG compared to HC-IgG. Statistical significance was determined by a Kruskal-Wallis test with a Dunn's multiple comparison test. Data is presented as box and whisker plots from averaged observations made in three independent experiments. Abbreviations: HC, healthy control: RA, rheumatoid arthritis; SLE, systemic lupus erythematosus; APS, antiphospholipid syndrome. ns= no significance, **=$p<0.01$, ***=$p<0.001$

5.8 RA- and SLE-IgG modulate NETosis in control neutrophils

Having demonstrated a modulatory effect of purified ARD-IgG upon neutrophil adhesion and ROS generation, I subsequently explored the effects of purified IgG upon NETosis.

HC neutrophils were isolated and pre-incubated with purified HC-IgG (n=5), RA-IgG (n=5), SLE-IgG (n=5) or APS-IgG (n=5) for 1 hour in the presence of FcγR blockade. To examine spontaneous NETosis, following pre-incubation cells were incubated for a further 4 hours. Some neutrophils were treated with PMA or LPS for 4 hours to examine whether IgG modulates PMA-induced or LPS-induced NETosis. Following incubation, neutrophil supernatants were obtained by centrifugation and assessed for the presence of NETs using the Quanti-iT™ PicoGreen® dsDNA kit.

Unstimulated neutrophils cultured with HC-IgG had similar levels of NETs compared to no IgG controls. Significant increases in cell-free DNA were observed in neutrophils cultured with RA-IgG ($p<0.05$) and SLE-IgG ($p<0.01$) (Figure 5.10). APS-IgG did not significantly increase spontaneous NETosis in HC neutrophils compared to HC-IgG.

Addition of PMA stimulation increased cell-free DNA in all neutrophils (Figure 5.11). HC-IgG did not significantly modulate PMA-induced NETosis further, compared with neutrophils stimulated in the absence of purified IgG. Pre-incubation with RA-IgG, prior to PMA stimulation significantly increased NETosis compared to HC-IgG ($p<0.01$) (Figure 5.11). Pre-incubation with SLE- or APS-IgG did not significantly affect PMA-induced NETosis compared to HC-IgG.

A similar pattern was obtained with LPS treatment that also increased cell-free DNA (Figure 5.12). Pre-incubation with HC-IgG produced similar results as neutrophils stimulated with LPS in the absence of IgG. Significant increases in cell-free DNA however, were observed in neutrophils cultured with both RA-IgG ($p<0.05$) and SLE-IgG ($p<0.01$) (Figure 5.12). APS-IgG did not significantly increase LPS-induced NETosis compared to HC-IgG.

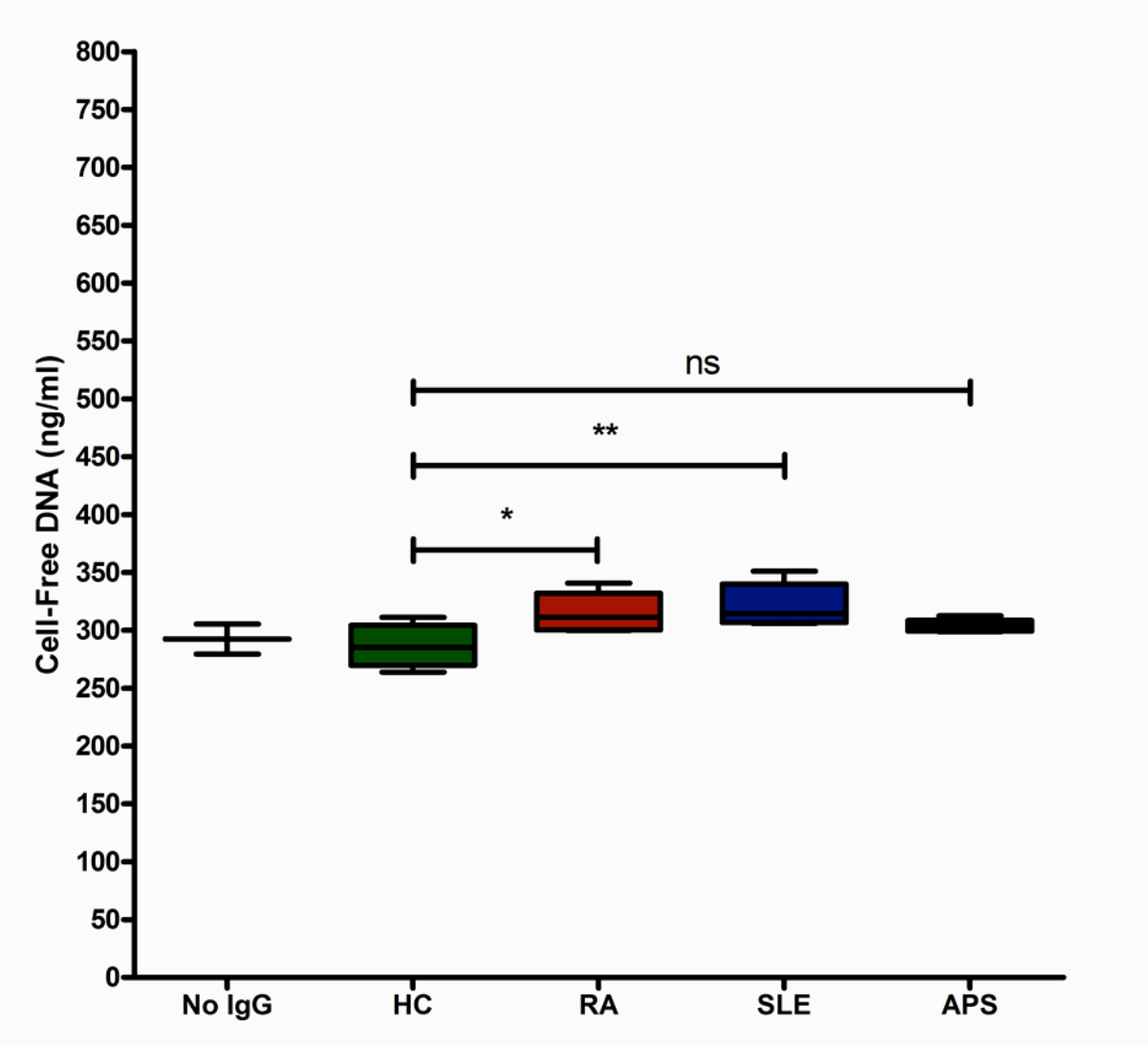


Figure 5.10: RA- and SLE-IgG induce greater levels of spontaneous NETosis compared to HC-IgG. Control neutrophils were isolated and treated with FcγR blockade. Cells were then incubated for 1 hour with 200μg/ml purified RA-IgG (n=5), SLE-IgG (n=5), APS-IgG (n=5) or HC-IgG (n=5). Neutrophils were incubated for a further 4 hours in the absence of additional stimuli. Cell supernatants were assessed for the presence of NETs using the Quanti-iT™ PicoGreen® dsDNA kit. Significantly higher levels of cell-free DNA were found in supernatants from neutrophils incubated with RA- or SLE-IgG compared to HC-IgG. Statistical significance was determined by one-way ANOVA with a Tukey post-test analysis. Data is presented as box and whisker plots from averaged observations from each IgG sample in two independent experiments. Abbreviations: HC, healthy control; RA, rheumatoid arthritis; SLE, systemic lupus erythematosus; APS, antiphospholipid syndrome. ns= no significance, *=$p<0.05$, **=$p<0.01$

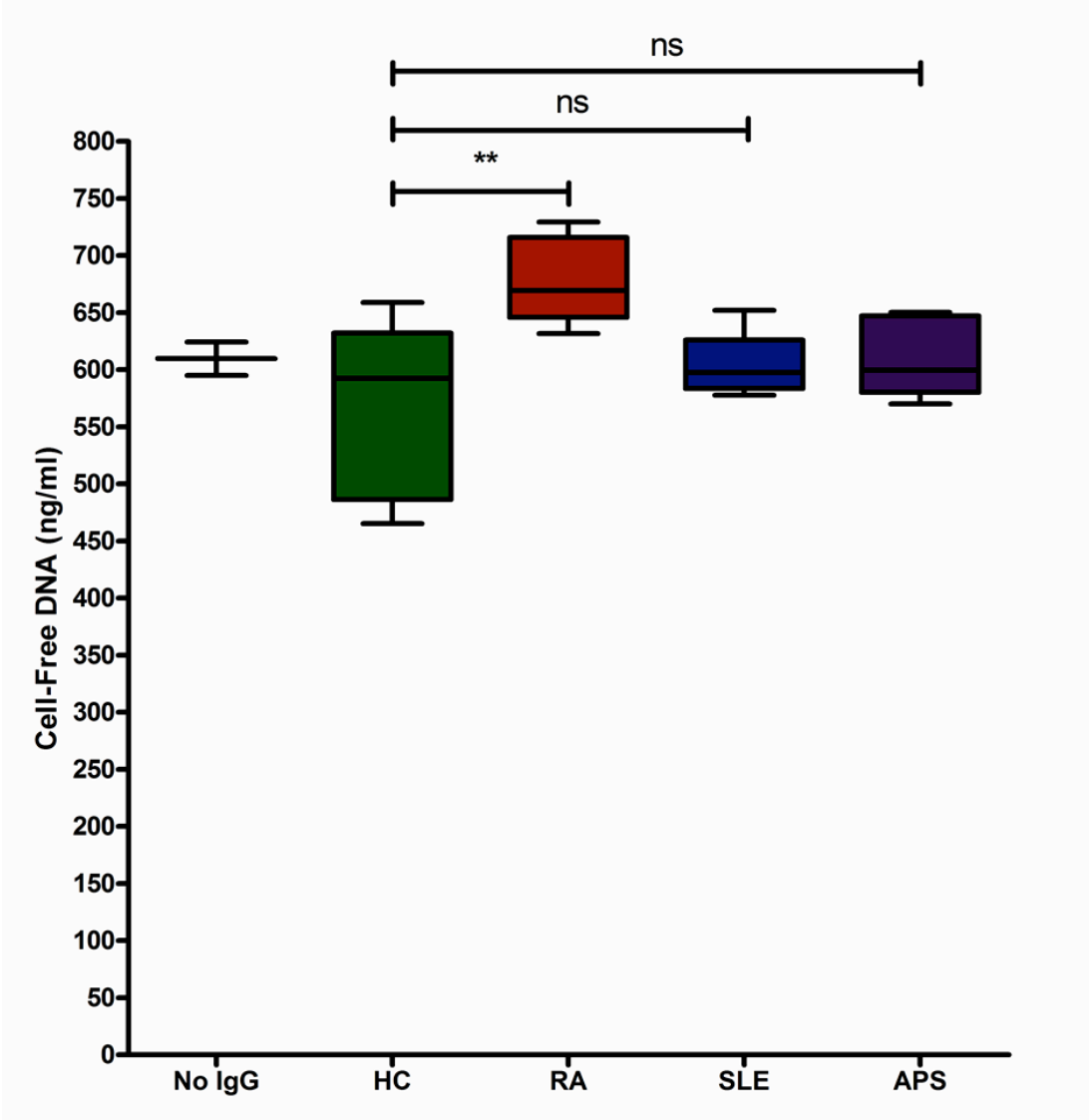


Figure 5.11: RA-IgG elevates PMA-induced NETosis compared to healthy control-IgG. Control neutrophils were isolated and treated with FcγR blockade. Cells were then incubated for 1 hour with 200µg/ml purified RA-IgG (n=5), SLE-IgG (n=5), APS-IgG (n=5) or HC-IgG (n=5). Following incubation, neutrophils were treated with 40nM PMA for a further 4 hours. Cell supernatants were assessed for the presence of NETs using the Quanti-iT™ PicoGreen® dsDNA kit. Significantly higher levels of cell-free DNA were found in supernatants from neutrophils incubated with RA-IgG compared to HC-IgG. Statistical significance was determined by one-way ANOVA with a Tukey post-test analysis. Data is presented as box and whisker plots from averaged observations from each IgG sample in two independent experiments. Abbreviations: HC, healthy control; RA, rheumatoid arthritis; SLE, systemic lupus erythematosus; APS, antiphospholipid syndrome. ns= no significance, **=$p<0.01$

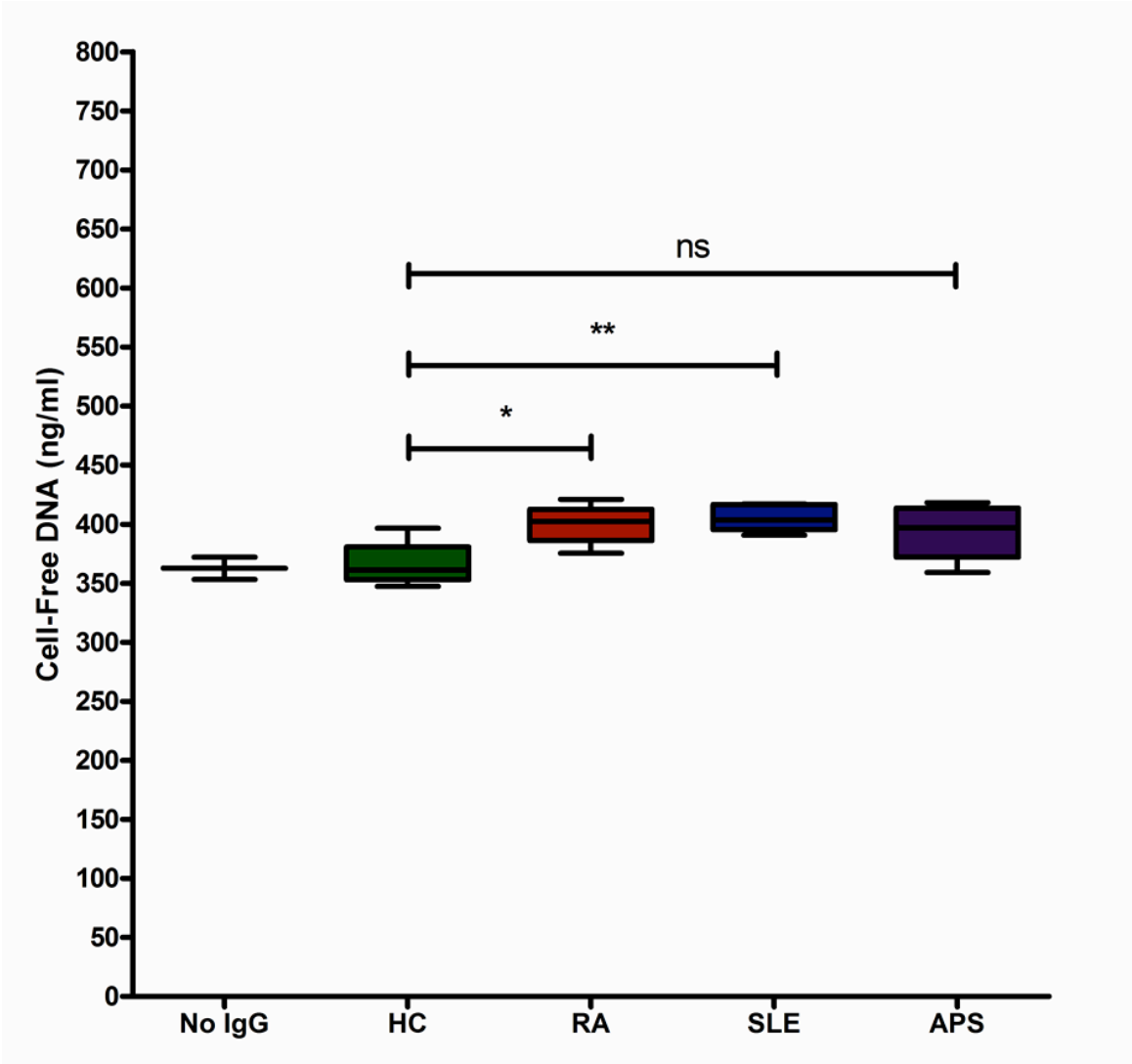


Figure 5.12: RA- and SLE-IgG enhance LPS-induced NETosis compared to healthy control-IgG. Control neutrophils were isolated and treated with FcγR blockade. Cells were then incubated for 1 hour with 200μg/ml purified RA-IgG (n=5), SLE-IgG (n=5), APS-IgG (n=5) or HC-IgG (n=5). Neutrophils were treated with 100ng/ml LPS for a further 4 hours. Cell supernatants were assessed for the presence of NETs using the Quanti-iT™ PicoGreen® dsDNA kit. Significantly higher levels of cell-free DNA were found in supernatants from neutrophils incubated with RA- or SLE-IgG compared to HC-IgG. Statistical significance was determined by a Kruskal-Wallis test with a Dunn's multiple comparison test. Data is presented as box and whisker plots from averaged observations from each IgG sample in two independent experiments. Abbreviations: HC, healthy control; RA, rheumatoid arthritis; SLE, systemic lupus erythematosus; APS, antiphospholipid syndrome. ns= no significance, *=$p<0.05$, **=$p<0.01$

5.9 Discussion

In this chapter, I have shown that RA-, SLE- and APS-IgG bind neutrophils. Moreover, I observed a differential effect of IgG upon neutrophil integrin activation, with APS-IgG enhancing PMA-stimulated neutrophil adhesion to fibronectin, whilst RA- and SLE-IgG augmented PMA-stimulated adhesion to fibrinogen. RA-, SLE- and APS-IgG were found to bind neutrophils however, determining the precise antigenic target was outside the scope of this PhD thesis. Given my data showing that RA- and APS-IgG binding was not affected by FcγR blockade, I hypothesise that these IgG samples are binding via antigen-specific interactions. In contrast, SLE-IgG binding was reduced following FcγR blockade, suggesting that SLE-IgG binds neutrophils via both Fab- and Fc-mediated interactions. *Ex vivo* neutrophils isolated from SLE patients had significantly lower rates of hydrogen peroxide production compared to RA, APS and HC neutrophils. Furthermore, RA- and SLE-IgG significantly increased hydrogen peroxide generation by HC neutrophils. Finally, RA- and SLE-IgG were also found to enhance spontaneous, PMA-induced and LPS-induced NETosis. This section will discuss my findings within the context of published reports, evaluate the limitations of my work and highlight areas in which my work could expand.

5.9.1 The effects of IgG upon neutrophil adhesion

The effects of purified IgG upon neutrophil integrin activation and adhesion have not been previously studied. Early reports found that SLE- and APS-IgG increased monocyte adhesion to ECs (Simantov et al., 1995, Del Papa et al., 1999). Both studies however, cultured HUVEC with 100μg/ml SLE- or APS-IgG before the adhesion of monocytic cell lines was assessed. Therefore, these reports examined the effects of IgG upon EC adhesion molecule expression and not leukocyte integrin activation.

My data show that ARD-IgG differentially regulate neutrophil integrin activation. APS-IgG enhanced PMA-stimulated neutrophil adhesion to fibronectin compared to HC-IgG. Conversely, RA- and SLE-IgG increased PMA-stimulated adhesion to fibrinogen. Insights to this differential response may be gained by consideration of the nature of the different ARDs.

RA and SLE are both characterised by chronic inflammation and endothelial dysfunction (Piper et al., 2007, Totoson et al., 2014). Although the biology of fibrinogen is complex, increased fibrinogen levels have been associated with inflammatory conditions (Kamath and Lip, 2003). It is therefore interesting that RA- and SLE-IgG promote Mac-1-mediated adhesion to fibrinogen. Further experiments would be required to determine the precise molecular interactions underlying my observations.

In contrast, APS can be characterised by cellular (including endothelial) activation (Pierangeli et al., 2008). Under resting physiological conditions, the endothelium displays polarised fibronectin expression, primarily forming a constituent of the sub-endothelial matrix within the basement membrane (Peters et al., 1990). Activated endothelial monolayers have been shown to deposit fibronectin at both apical and basal cell membranes (Kowalczyk et al., 1990). Therefore, persistent EC activation leads to increased fibronectin deposition within the vasculature. My observation that APS-IgG enhances neutrophil adhesion to immobilised fibronectin may indicate that APS-IgG promote neutrophil adhesion to ECM proteins expressed on the vessel wall by activated ECs. A potential limitation of this work however, is that adhesion assays were performed in the absence of FcγR blockade. Additional experiments would be required to conclusively determine whether my observations arise via antigen-specific or FcγR-mediated interactions.

5.9.2 The effects of IgG upon neutrophil ROS generation

Several groups have documented the dysregulation of NETosis in RA, SLE and APS (Garcia-Romo et al., 2011, Sur Chowdhury et al., 2014, Yalavarthi et al., 2015). These studies show that IgG-induced NETosis is dependent on ROS generation. Therefore, I tested the hypothesis that ARD-IgG can modulate ROS production. Through comparisons of neutrophil hydrogen peroxide generation, I found that SLE-derived neutrophils had significantly lower rates of hydrogen peroxide generation compared to neutrophils isolated from healthy volunteers and patients with RA and APS. Possible explanations for this observation are that SLE-derived neutrophils have defective *in vitro* hydrogen peroxide generation or due to persistent systemic

activation, *ex vivo* responses of SLE-derived neutrophils are blunted. The uptake of circulating nucleosomes has been reported to induce the systemic activation of neutrophils in SLE patients (Ronnefarth et al., 2006). Therefore, it is possible that the reduced hydrogen peroxide generation observed represents a form of neutrophil senescence arising from persistent cellular stimulation. Given these results, I examined the effects of purified IgG upon ROS generation of control neutrophils. Both RA- and SLE-IgG significantly increased hydrogen peroxide generation. The observation that SLE-IgG increases hydrogen peroxide generation of *ex vivo* control neutrophils adds to my hypothesis that SLE-derived neutrophils had lower rates of ROS generation due to the persistent activation by factors such as circulating nucleosomes and IgG. Given that SLE-IgG had reduced binding in the presence of FcγR blockade, it would be interesting in future work to test whether an enhanced response occurs in the absence of FcγR blockade.

A further limitation to these experiments is the significantly older RA cohorts. At the time of serum donation, my RA cohort had a mean age of 51 years whilst HC, SLE and APS cohorts had mean ages of 30, 32 and 39 respectively. This age difference was also seen in patients who donated whole blood. The RA cohort had a mean age of 62 years, whilst HC, SLE and APS cohorts had mean ages of 36, 40 and 44 years respectively. In the future, to acquire better age-matched controls for RA patients, patient relatives or non-ARD patients may have to be recruited. Whilst an older HC cohort would benefit comparisons with the RA cohort, both SLE and APS patients were significantly younger than RA patients and were similar in age to the HC cohort. This age difference between ARD patients complicates the recruitment of healthy volunteers. If healthy volunteers were recruited to satisfy RA, SLE and APS patients, large standard deviations in age would then exist within the HC cohort. Alternatively, two HC groups of different ages would be required to match either RA or SLE and APS patients.

5.9.3 The effects of IgG upon NETosis

Several reports have demonstrated increased NETosis following culture with RA-, SLE- or APS-IgG (Garcia-Romo et al., 2011, Khandpur et al., 2013, Knight et al., 2013, Sur

Chowdhury et al., 2014, Yalavarthi et al., 2015). I found incubation with RA- and SLE-IgG significantly increased spontaneous NETosis, which is consistent with published data (Carmona-Rivera and Kaplan, 2013, Khandpur et al., 2013). I also found that incubation with RA- or SLE-IgG enhanced LPS-stimulated NETosis. Given that LPS and TLR-4 signalling have been implicated in NETosis (Clark et al., 2007, Remijsen et al., 2011a), my data indirectly support involvement of TLR-4 in RA- and SLE-IgG induced NETosis. Only RA-IgG augmented PMA-induced NETosis, which may indicate differential signalling pathway induction by purified IgG. Published reports have shown APS-IgG induces spontaneous NETosis (Yalavarthi et al., 2015). There are differences between this study and my own work that may explain the conflicting results. I stimulated neutrophils with 200μg/ml whole IgG purified from APS serum, however in work by Yalavarthi and colleagues, neutrophils were stimulated with 10μg/ml of either whole IgG or affinity-purified anti-β_2GPI antibodies purified from APS serum. In addition, given that my work consisted of purified RA-, SLE and APS-IgG statistical analysis was performed using multiple comparison tests, whereas Yalavarthi et al. compared APS- and HC-IgG using unpaired t tests. These differences may account for the discrepant results of statistical significance between published reports and my own data. If unpaired t tests are performed on my data, the differences between APS-IgG and HC-IgG become significant however, given my experimental design comparing IgG purified from three patient cohorts, multiple comparison tests are the most appropriate statistical analysis to apply.

The identity of the antigenic targets bound by purified IgG remains unknown. For this thesis, FcγR blockade was achieved using an FcR blocking reagent (Miltenyi, UK), which is a human serum IgG preparation. A limitation of using this FcR blocking reagent is that whilst the high-affinity FcγRI is readily blocked, the lower affinity FcγRIIA and FcγRIIIB may not be as efficiently blocked. FcγRIIA and FcγRIIIB function synergistically to activate neutrophils (Zhou et al., 1995). FcγRIIA activation by soluble ICs has been shown to induce NETosis (Chen et al., 2012). Immobilised ICs can also activate FcγRIIIB and Mac-1, which induce NETosis (Behnen et al., 2014). Therefore, it is possible that whilst the FcR blocking reagent

efficiently blocks the high-affinity FcγRI, it is less efficient at FcγRIIA and FcγRIIIB blockade thus promoting neutrophil activation by IC engagement.

Many groups have tried to determine the antigenic targets that mediate IgG-induced NETosis, without a clear answer. Given the evidence demonstrating that aPL bind β_2GPI, APS-IgG may bind and crosslink β_2GPI with TLR-4 to induce intracellular signalling (Raschi et al., 2003, Mulla et al., 2009), which has been implicated in NETosis (Clark et al., 2007, Remijsen et al., 2011a). Evidence to support this hypothesis is provided by Yalavarthi and colleagues who demonstrate that NETosis induced by APS-IgG can be suppressed by depletion of anti-β_2GPI antibodies (Yalavarthi et al., 2015). This group also showed the induction of NETosis following incubation of affinity-purified anti-β_2GPI antibodies, which further supports this hypothesis. Multiple autoantigens have been identified in RA and SLE, such as citrullinated antigens in RA and nuclear contents in SLE, which make defining the precise antigenic targets on neutrophils difficult.

5.9.4 Future work

Whilst statistical significance was achieved in experiments using purified IgG, given more time I would have liked to expand on the number of samples in my SLE group. Furthermore, ANCA positivity was only characterised for the classical ANCA antigens PR3 and MPO. Additional non-classical ANCA antigens have been implicated in inflammatory conditions, including α-enolase, azurocidin, bactericidal permeability increasing protein, cathespin G, elastase, high mobility group box (HMGB)-1, HMGB-2, lactoferrin and lysozyme (Halbwachs-Mecarelli et al., 1992, Coremans et al., 1993, Peen et al., 1993, Skogh and Peen, 1993, Tervaert et al., 1993, Zhao et al., 1995, Zhao and Lockwood, 1996). Many of these additional antigens are also associated with NET formation and structure. Therefore, it would be interesting to examine ANCA positivity for non-classical ANCA antigens.

Several approaches could be applied to determine the mechanisms underlying IgG-mediated neutrophil activation. Fab or $F(ab')_2$ fragments could be generated by papain or pepsin digestion respectively, to determine whether neutrophil activation is mediated by IgG variable

regions rather than Fc-mediated. Purified IgG could also be subjected to ultracentrifugation to remove serum-induced complexes and functional responses assessed. ICs could be produced from HC-IgG by heat aggregation and the effects on neutrophil activation evaluated. Alternatively, specific blockade of FcγRI, FcγRIIA or FcγRIIIB would highlight whether my observations are Fc mediated and if so, which of the FcγRs mediate the response.

I would also like to explore the effects of IgG upon neutrophil adhesion to additional integrin ligands such as: ICAM-1, which is upregulated on cell activation and therefore physiologically relevant; VCAM-1, which is also upregulated by activated ECs; and collagen, which is expressed in arthritic joints.

Another model would be to examine the effects of purified IgG on neutrophil adhesion to ECs, fibroblasts, FLS and synoviocytes. These cell types would provide a better understanding of neutrophil interactions within tissues during and following extravasation, which may hold pathological relevance for RA, SLE and APS. Examination of the effects of IgG upon neutrophil adhesion to platelets may provide further insight to the mechanisms underlying thrombosis in APS patients. Adhesion assays could also be conducted under flow, which would also examine the effects of IgG upon neutrophil attachment and rolling. Transmigration assays could also be performed to examine whether IgG also affects neutrophil migration across the endothelium.

Various studies have shown that ROS are important in NETosis, however reactive nitrogen intermediates have also been implicated (Patel et al., 2010, Keshari et al., 2012). As RA- and SLE-IgG modulated neutrophil hydrogen peroxide generation, it would be interesting to examine reactive nitrogen intermediates production. The Griess test was not sensitive enough to detect nitrites produced by IFN-γ-stimulated neutrophils, however flow cytometry-based assays are able to examine reactive nitrogen intermediates. These experiments would give a broader understanding of the nitrogen radical species generated during neutrophil activation.

Chapter Six:
Overall Discussion and Future Directions

6.1 Key findings

Key findings obtained in this thesis can be found summarised in Table 6.1 below.

Chapter Three: The Effects of Hypoxia on Neutrophil Integrin Activation
• Hypoxia upregulates α_M and α_X integrin subunit expression
• Hypoxia enhances neutrophil adhesion to endothelial monolayers
• Hypoxia suppresses endothelial E-selectin expression, but increases ICAM-1 expression
• Hypoxia increases neutrophil transendothelial migration
Chapter Four: The Effects of Hypoxia on Neutrophil Function
• Rates of ROS generation are not modulated by hypoxia nor integrin blockade
• Hypoxia enhances NETosis
• NETosis can be inhibited with EDTA treatment
• Mac-1 ($\alpha_M\beta_2$) activation induces NETosis
• EC co-culture enhances NETosis
• Hypoxia increases PAD-4 expression in neutrophils
• Neutrophils stimulated under hypoxia have increased levels of MPO
• Inhibition of p38 MAPK reduces PMA-induced NETosis
Chapter Five: The Effects of Immunoglobulin on Neutrophil Function
• APS-IgG enhances β_1 integrin-mediated adhesion to fibronectin
• RA- and SLE-IgG promote Mac-1 ($\alpha_M\beta_2$)-mediated adhesion to fibrinogen
• RA-, SLE- and APS-IgG bind neutrophils, binding of SLE-IgG was reduced in the presence of FcγR blockade
• Neutrophils from SLE patients have lower rates of ROS generation
• RA- and SLE-IgG increase rates of hydrogen peroxide generation in control neutrophils
• RA-IgG increases spontaneous, PMA-induced and LPS-induced NETosis
• SLE-IgG increases spontaneous and LPS-induced NETosis

Table 6.1: Key findings obtained in this thesis. Listed above are the key observations made throughout my PhD.

6.2 Overall discussion

The underlying premise of all the experiments described in this thesis was that cellular environments can affect neutrophil function. The molecular mechanisms regulating neutrophil adhesion and ROS generation have been extensively studied. With regards to NETs, most published reports focus on elucidating NET structure and mechanisms regulating NETosis. My work adds to current literature by examining the effects of hypoxia upon integrin expression, integrin activation, ROS generation and NETosis. Many studies conduct experiments under atmospheric oxygen levels. Given that oxygen levels within the vasculature are far lower than atmospheric levels, examining neutrophil function under hypoxia is physiologically relevant.

In addition, the effects of purified RA-, SLE- and APS-IgG upon cellular function has also been examined in numerous cell types. The contribution of neutrophils however is less studied. Emerging work has begun to study the effects of ARD-IgG upon neutrophil activation. Data from this thesis has been consistent with published reports and builds upon current work. Differences in statistical significance between my own and published data can be attributed to my use of the more statistically stringent multiple comparison tests rather than the unpaired t tests, of other reports.

Overall, this thesis has shown that hypoxia does not affect surface expression of neutrophil β_1 integrins, but increases α_M and α_X expression. Therefore, under hypoxia there is increased expression of Mac-1 ($\alpha_M\beta_2$) and p150,95 ($\alpha_X\beta_2$). Given that Mac-1 and p150,95 are also complement receptors (CR3 and CR4 respectively), my data hints that CR-mediated adhesion and function may be enhanced under hypoxia. Neutrophil adhesion to ECs and trans-endothelial migration was also enhanced under hypoxia. Examination of EC adhesion molecule expression found increased ICAM-1 expression under hypoxia. Based on this observation and the elevated expression of Mac-1 and p150,95 on neutrophils, increased adhesion and transmigration under hypoxia may be mediated by interactions between Mac-1 and ICAM-1. NETosis was enhanced when neutrophils were stimulated in the presence of ECs, which may also be a result of increased cellular interactions between neutrophils and ECs. My work also

shows for the first time that hypoxia increases PAD-4 expression in neutrophils. Whilst this has not been previously described, published data demonstrates that hypoxia upregulates PAD-2 expression in astrocytes (Sambandam et al., 2004). HRE consensus sequences were also identified in within evolutionary conserved regions upstream of the PAD-4 coding gene, using the publically available software ECR browser (http://ecrbrowser.dcode.org). These conserved regions are likely to represent promoter regions, which indicate that PAD-4 may be regulated by HIF signalling. My experimental data confirms that PAD-4 is also upregulated under hypoxia. This observation may provide an insight to the mechanisms enhancing NETosis under hypoxia.

With regards to the effects of purified IgG, this thesis identified that ARD-IgG had differential effects upon neutrophil integrin activation and modulated both ROS production and NETosis. In particular, APS-IgG enhanced PMA-stimulated neutrophil adhesion to fibronectin via a β_1 integrin-dependent manner, whilst RA- and SLE-IgG increased PMA-stimulated adhesion to fibrinogen via Mac-1 activation. RA- and SLE-IgG increased rates of hydrogen peroxide generation in control neutrophils. NETosis was also modulated by the presence of purified IgG, with increased levels of spontaneous, PMA- and LPS-induced NETosis in neutrophils cultured with RA-IgG. SLE-IgG also increased spontaneous and LPS-induced NETosis. My data examining the effects of RA- and SLE-IgG upon NETosis was consistent with published data (Carmona-Rivera and Kaplan, 2013, Khandpur et al., 2013, Knight et al., 2013), however I did not find significant effects of APS-IgG upon ROS production or NETosis as described in published reports (Yalavarthi et al., 2015). This discrepancy can be explained by differences in statistical analysis. Whilst published reports demonstrated statistical significance using an unpaired t test, my experimental data failed to reproduce this significance using a multiple comparison test. Interestingly, performing unpaired t tests between HC- and APS-IgG groups in my data found statistical significance, demonstrating similar trends that were masked by the more stringent multiple comparison statistical analysis.

6.3 Future directions

In order to take these findings forward, there are several points I would consider. Having examined the effects of hypoxia and purified IgG upon neutrophil function separately, it would be important to study the combined effects of hypoxia and purified IgG to determine whether there is an additive effect upon neutrophil adhesion, ROS generation and NETosis.

Preliminary work in this thesis supports published reports and found that p38 MAPK inhibition significantly reduced PMA-stimulated NETosis (Riyapa et al., 2012). Further experiments to determine p38 MAPK expression and phosphorylation could be performed to dissect the signalling pathways involved in NETosis under hypoxia in the absence or presence of purified IgG and PMA. These experiments may highlight signalling pathways and molecules that could serve as novel therapeutic targets, which is interesting given that p38 MAPK inhibitors are currently in development for the treatment of RA. Pre-clinical studies in animal models demonstrated that p38 MAPK inhibition reduced both synovial inflammation and the destruction of bone and cartilage (Nishikawa et al., 2003, Zwerina et al., 2006). The benefits of p38 MAPK inhibition in RA have not translated into humans, with several p38 MAPK inhibitors having been trialled and discontinued due to adverse effects, such as infection or neurological effects (Weisman, 2002, Cohen et al., 2009, Damjanov et al., 2009, Alten et al., 2010). The lack of efficacy of p38 MAPK inhibition in RA patients may be due to inadequate dosing, which is limited by toxicity (Hammaker and Firestein, 2010) or by compensatory mechanism that appear to overcome the inhibition of p38 MAP kinase after several weeks (Genovese et al., 2008, Bonilla-Hernan et al., 2011). Losmapimod, a novel and potent p38 MAPK inhibitor, has also produced positive data in patients with non-ST-segment elevation myocardial infarction and was well tolerated by patients (Newby et al., 2014), which is now being trialled in RA patients.

Neutrophil activation was examined in this thesis by studying ROS generation and NETosis. An additional form of neutrophil activation is the generation of microparticles. Published reports have shown neutrophil microparticles to have a wide range of biological

effects, including causing endothelial damage (Pitanga et al., 2014) and promoting thrombosis (Kambas et al., 2014, Huang et al., 2015). A recent study found that a subpopulation of $CD66b^+$ neutrophil microparticles containing annexin A1 protected mice from inflammatory arthritis (Headland et al., 2015). This study also found elevated neutrophil-derived microparticles, as well as monocyte- and T cell-derived microparticles, in patients with RA. Given that oxygen levels in the vasculature are lower than atmospheric oxygen, studying the effects of hypoxia upon neutrophil microparticles populations would be relevant. In addition, examining the effects of purified IgG upon neutrophil microparticle generation would also be interesting. Given the evidence demonstrating the contributions of neutrophils to pathology, this field of work would be relevant to RA, SLE and APS.

All of the experiments conducted in this thesis used neutrophils isolated from peripheral blood. Given the evidence showing differential integrin expression in T cells isolated from peripheral blood and SF (Hemler et al., 1986, Laffon et al., 1991), and the importance of NETosis to mucosal immunology (Mohanty et al., 2015), examination of neutrophils isolated from BAL fluid or sputum may also be of interest. A limitation to these experiments however, is that whilst samples may be readily obtained from patient populations, examination of healthy control samples may prove difficult. These experiments would examine neutrophils within the context of mucosal immunity, as cells would have migrated out of the vasculature. Given its importance to mucosal immunology, it would become physiologically relevant to examine the effects of purified IgA, which is enriched within mucosal membranes. Similarly, examining the difference in neutrophil function in SF-derived neutrophils may provide an insight to the contribution of neutrophils to disease pathophysiology.

The contribution of neutrophils to ARD pathology is becoming increasing recognised, with studies beginning to explore the effects of purified IgG upon neutrophil activation. The effects of hypoxia have been studied within the context of cell biology, but few reports have examined hypoxia within the context of ARD. The work produced in this thesis has hopefully contributed to the study of neutrophil activation within the context of autoimmune disease.

References

ABRAHAMS, V. M. 2009. Mechanisms of antiphospholipid antibody-associated pregnancy complications. *Thromb Res,* 124**,** 521-5.

ABRAMSON, S. B., GIVEN, W. P., EDELSON, H. S. & WEISSMANN, G. 1983. Neutrophil aggregation induced by sera from patients with active systemic lupus erythematosus. *Arthritis Rheum,* 26**,** 630-6.

ADELMAN, D. M., GERTSENSTEIN, M., NAGY, A., SIMON, M. C. & MALTEPE, E. 2000. Placental cell fates are regulated in vivo by HIF-mediated hypoxia responses. *Genes Dev,* 14**,** 3191-203.

AFEK, A., SHOENFELD, Y., MANOR, R., GOLDBERG, I., ZIPOREN, L., GEORGE, J., POLAK-CHARCON, S., AMIGO, M. C., GARCIA-TORRES, R., SEGAL, R. & KOPOLOVIC, J. 1999. Increased endothelial cell expression of alpha3beta1 integrin in cardiac valvulopathy in the primary (Hughes) and secondary antiphospholipid syndrome. *Lupus,* 8**,** 502-7.

AFSHAR-KHARGHAN, V., DIZ-KUCUKKAYA, R., LUDWIG, E. H., MARIAN, A. J. & LOPEZ, J. A. 2001. Human polymorphism of P-selectin glycoprotein ligand 1 attributable to variable numbers of tandem decameric repeats in the mucinlike region. *Blood,* 97**,** 3306-7.

AGARWAL, A., SALEM, P. & ROBBINS, K. C. 1993. Involvement of p72syk, a protein-tyrosine kinase, in Fc gamma receptor signaling. *J Biol Chem,* 268**,** 15900-5.

AGGARWAL, A., SHARMA, A. & BHATNAGAR, A. 2014. Role of cytolytic impairment of natural killer and natural killer T-cell populations in rheumatoid arthritis. *Clin Rheumatol,* 33**,** 1067-78.

AGNELLO, V., DE BRACCO, M. M. & KUNKEL, H. G. 1972. Hereditary C2 deficiency with some manifestations of systemic lupus erythematosus. *J Immunol,* 108**,** 837-40.

AGOSTINIS, C., BIFFI, S., GARROVO, C., DURIGUTTO, P., LORENZON, A., BEK, A., BULLA, R., GROSSI, C., BORGHI, M. O., MERONI, P. & TEDESCO, F. 2011. In vivo distribution of beta2 glycoprotein I under various pathophysiologic conditions. *Blood,* 118**,** 4231-8.

AGOSTINIS, C., DURIGUTTO, P., SBLATTERO, D., BORGHI, M. O., GROSSI, C., GUIDA, F., BULLA, R., MACOR, P., PREGNOLATO, F., MERONI, P. L. & TEDESCO, F. 2014. A non-complement-fixing antibody to beta2 glycoprotein I as a novel therapy for antiphospholipid syndrome. *Blood,* 123**,** 3478-87.

AKHAVANI, M. A., MADDEN, L., BUYSSCHAERT, I., SIVAKUMAR, B., KANG, N. & PALEOLOG, E. M. 2009. Hypoxia upregulates angiogenesis and synovial cell migration in rheumatoid arthritis. *Arthritis Res Ther,* 11**,** R64.

ALARCON, G. S., MCGWIN, G., JR., PETRI, M., REVEILLE, J. D., RAMSEY-GOLDMAN, R. & KIMBERLY, R. P. 2002. Baseline characteristics of a multiethnic lupus cohort: PROFILE. *Lupus,* 11**,** 95-101.

ALETAHA, D., NEOGI, T., SILMAN, A. J., FUNOVITS, J., FELSON, D. T., BINGHAM, C. O., 3RD, BIRNBAUM, N. S., BURMESTER, G. R., BYKERK, V. P., COHEN, M. D., COMBE, B., COSTENBADER, K. H., DOUGADOS, M., EMERY, P., FERRACCIOLI, G., HAZES, J. M., HOBBS, K., HUIZINGA, T. W., KAVANAUGH, A., KAY, J., KVIEN, T. K., LAING, T., MEASE, P., MENARD, H. A., MORELAND, L. W., NADEN, R. L., PINCUS, T., SMOLEN, J. S., STANISLAWSKA-BIERNAT, E., SYMMONS, D., TAK, P. P., UPCHURCH, K. S., VENCOVSKY, J., WOLFE, F. & HAWKER, G. 2010. 2010 rheumatoid arthritis classification criteria: an American College of Rheumatology/European League Against Rheumatism collaborative initiative. *Ann Rheum Dis,* 69**,** 1580-8.

ALGECIRAS, M. E., TAKAHARA, H. & BHATTACHARYA, S. K. 2008. Mechanical stretching elevates peptidyl arginine deiminase 2 expression in astrocytes. *Curr Eye Res,* 33**,** 994-1001.

ALLEN, L. A. & ADEREM, A. 1996a. Mechanisms of phagocytosis. *Curr Opin Immunol,* 8**,** 36-40.

ALLEN, L. A. & ADEREM, A. 1996b. Molecular definition of distinct cytoskeletal structures involved in complement- and Fc receptor-mediated phagocytosis in macrophages. *J Exp Med,* 184**,** 627-37.

ALON, R., KASSNER, P. D., CARR, M. W., FINGER, E. B., HEMLER, M. E. & SPRINGER, T. A. 1995. The integrin VLA-4 supports tethering and rolling in flow on VCAM-1. *J Cell Biol,* 128**,** 1243-53.

ALTEN, R. E., ZERBINI, C., JEKA, S., IRAZOQUE, F., KHATIB, F., EMERY, P., BERTASSO, A., RABBIA, M. & CAULFIELD, J. P. 2010. Efficacy and safety of pamapimod in patients with active rheumatoid arthritis receiving stable methotrexate therapy. *Ann Rheum Dis,* 69**,** 364-7.

ALVARADO-SANCHEZ, B., HERNANDEZ-CASTRO, B., PORTALES-PEREZ, D., BARANDA, L., LAYSECA-ESPINOSA, E., ABUD-MENDOZA, C., CUBILLAS-TEJEDA, A. C. & GONZALEZ-AMARO, R. 2006. Regulatory T cells in patients with systemic lupus erythematosus. *J Autoimmun,* 27**,** 110-8.

ANDO, H., NATSUME, A., IWAMI, K., OHKA, F., KUCHIMARU, T., KIZAKA-KONDOH, S., ITO, K., SAITO, K., SUGITA, S., HOSHINO, T. & WAKABAYASHI, T. 2013. A hypoxia-inducible factor (HIF)-3alpha splicing variant, HIF-3alpha4 impairs angiogenesis in hypervascular malignant meningiomas with epigenetically silenced HIF-3alpha4. *Biochem Biophys Res Commun,* 433**,** 139-44.

ANDREOLI, L., CHIGHIZOLA, C. B., NALLI, C., GEROSA, M., BORGHI, M. O., PREGNOLATO, F., GROSSI, C., ZANOLA, A., ALLEGRI, F., NORMAN, G. L., MAHLER, M., MERONI, P. L. & TINCANI, A. 2015. Clinical characterization of antiphospholipid syndrome by detection of IgG antibodies against beta2 -glycoprotein i domain 1 and domain 4/5: ratio of anti-domain 1 to anti-domain 4/5 as a useful new biomarker for antiphospholipid syndrome. *Arthritis Rheumatol,* 67**,** 2196-204.

ANTHIS, N. J. & CAMPBELL, I. D. 2011. The tail of integrin activation. *Trends Biochem Sci,* 36**,** 191-8.

APLIN, J. D., CHARLTON, A. K. & AYAD, S. 1988. An immunohistochemical study of human endometrial extracellular matrix during the menstrual cycle and first trimester of pregnancy. *Cell Tissue Res,* 253**,** 231-40.

APLIN, J. D., HAIGH, T., JONES, C. J., CHURCH, H. J. & VICOVAC, L. 1999. Development of cytotrophoblast columns from explanted first-trimester human placental villi: role of fibronectin and integrin alpha5beta1. *Biol Reprod,* 60**,** 828-38.

ARATANI, Y., KOYAMA, H., NYUI, S., SUZUKI, K., KURA, F. & MAEDA, N. 1999. Severe impairment in early host defense against Candida albicans in mice deficient in myeloperoxidase. *Infect Immun,* 67**,** 1828-36.

ARBOUR, N. C., LORENZ, E., SCHUTTE, B. C., ZABNER, J., KLINE, J. N., JONES, M., FREES, K., WATT, J. L. & SCHWARTZ, D. A. 2000. TLR4 mutations are associated with endotoxin hyporesponsiveness in humans. *Nat Genet,* 25**,** 187-91.

ARMSTRONG, D. J., CROCKARD, A. D., WISDOM, B. G., WHITEHEAD, E. M. & BELL, A. L. 2006. Accelerated apoptosis in SLE neutrophils cultured with anti-dsDNA antibody isolated from SLE patient serum: a pilot study. *Rheumatol Int,* 27**,** 153-6.

ARNETT, F. C., THIAGARAJAN, P., AHN, C. & REVEILLE, J. D. 1999. Associations of anti-beta2-glycoprotein I autoantibodies with HLA class II alleles in three ethnic groups. *Arthritis Rheum,* 42**,** 268-74.

ARNOUT, J. 2001. Antiphospholipid syndrome: diagnostic aspects of lupus anticoagulants. *Thromb Haemost,* 86**,** 83-91.

ASHERSON, R. A., CERVERA, R., DE GROOT, P. G., ERKAN, D., BOFFA, M. C., PIETTE, J. C., KHAMASHTA, M. A. & SHOENFELD, Y. 2003. Catastrophic antiphospholipid syndrome: international consensus statement on classification criteria and treatment guidelines. *Lupus,* 12**,** 530-4.

AUSTIN, H. A., 3RD, MUENZ, L. R., JOYCE, K. M., ANTONOVYCH, T. T. & BALOW, J. E. 1984. Diffuse proliferative lupus nephritis: identification of specific pathologic features affecting renal outcome. *Kidney Int,* 25**,** 689-95.

AYHAN-ARDIC, F. F., OKEN, O., YORGANCIOGLU, Z. R., USTUN, N. & GOKHARMAN, F. D. 2006. Pulmonary involvement in lifelong non-smoking patients with rheumatoid arthritis and ankylosing spondylitis without respiratory symptoms. *Clin Rheumatol,* 25**,** 213-8.

BAKKALOGLU, A., TOPALOGLU, R., SAATCI, U., OZDEMIR, S., OZEN, S., BASSOY, Y. & BESBAS, N. 1998. Antineutrophil cytoplasmic antibodies in childhood systemic lupus erythematosus. *Clin Rheumatol,* 17**,** 265-7.

BALADA, E., CASTRO-MARRERO, J., FELIP, L., ORDI-ROS, J. & VILARDELL-TARRES, M. 2014. Clinical and serological findings associated with the expression of ITGAL, PRF1, and CD70 in systemic lupus erythematosus. *Clin Exp Rheumatol,* 32**,** 113-6.

BARGATZE, R. F., JUTILA, M. A. & BUTCHER, E. C. 1995. Distinct roles of L-selectin and integrins alpha 4 beta 7 and LFA-1 in lymphocyte homing to Peyer's patch-HEV in situ: the multistep model confirmed and refined. *Immunity,* 3**,** 99-108.

BARNHART, M. I., RIDDLE, J. M. & BLUHM, G. B. 1967. Immunocytology in arthritic joints. *Ann Rheum Dis,* 26**,** 281-96.

BARRAT, F. J., MEEKER, T., GREGORIO, J., CHAN, J. H., UEMATSU, S., AKIRA, S., CHANG, B., DURAMAD, O. & COFFMAN, R. L. 2005. Nucleic acids of mammalian origin can act as endogenous ligands for Toll-like receptors and may promote systemic lupus erythematosus. *J Exp Med,* 202**,** 1131-9.

BARREIRO, O., YANEZ-MO, M., SERRADOR, J. M., MONTOYA, M. C., VICENTE-MANZANARES, M., TEJEDOR, R., FURTHMAYR, H. & SANCHEZ-MADRID, F. 2002. Dynamic interaction of VCAM-1 and ICAM-1 with moesin and ezrin in a novel endothelial docking structure for adherent leukocytes. *J Cell Biol,* 157**,** 1233-45.

BARTON, A., BOWES, J., EYRE, S., SPRECKLEY, K., HINKS, A., JOHN, S. & WORTHINGTON, J. 2004. A functional haplotype of the PADI4 gene associated with rheumatoid arthritis in a Japanese population is not associated in a United Kingdom population. *Arthritis Rheum,* 50**,** 1117-21.

BAS, S., PERNEGER, T. V., SEITZ, M., TIERCY, J. M., ROUX-LOMBARD, P. & GUERNE, P. A. 2002. Diagnostic tests for rheumatoid arthritis: comparison of anti-cyclic citrullinated peptide antibodies, anti-keratin antibodies and IgM rheumatoid factors. *Rheumatology (Oxford),* 41**,** 809-14.

BATES, S. M., GREER, I. A., PABINGER, I., SOFAER, S. & HIRSH, J. 2008. Venous thromboembolism, thrombophilia, antithrombotic therapy, and pregnancy: American College of Chest Physicians Evidence-Based Clinical Practice Guidelines (8th Edition). *Chest,* 133**,** 844S-886S.

BECK-SCHIMMER, B., SCHIMMER, R. C., MADJDPOUR, C., BONVINI, J. M., PASCH, T. & WARD, P. A. 2001. Hypoxia mediates increased neutrophil and macrophage adhesiveness to alveolar epithelial cells. *Am J Respir Cell Mol Biol,* 25**,** 780-7.

BEHNEN, M., LESCHCZYK, C., MOLLER, S., BATEL, T., KLINGER, M., SOLBACH, W. & LASKAY, T. 2014. Immobilized immune complexes induce neutrophil extracellular trap release by human neutrophil granulocytes via FcgammaRIIIB and Mac-1. *J Immunol,* 193**,** 1954-65.

BEHRENS, F., HIMSEL, A., REHART, S., STANCZYK, J., BEUTEL, B., ZIMMERMANN, S. Y., KOEHL, U., MOLLER, B., GAY, S., KALTWASSER, J. P., PFEILSCHIFTER, J. M. & RADEKE, H. H. 2007. Imbalance in distribution of functional autologous regulatory T cells in rheumatoid arthritis. *Ann Rheum Dis,* 66**,** 1151-6.

BELAAOUAJ, A., MCCARTHY, R., BAUMANN, M., GAO, Z., LEY, T. J., ABRAHAM, S. N. & SHAPIRO, S. D. 1998. Mice lacking neutrophil elastase reveal impaired host defense against gram negative bacterial sepsis. *Nat Med,* 4**,** 615-8.

BELCHER, C., DOHERTY, M. & CROUCH, S. P. 2002. Synovial fluid neutrophil function in RA: the effect of pregnancy associated proteins. *Ann Rheum Dis,* 61**,** 379-80.

BENNETT, L., PALUCKA, A. K., ARCE, E., CANTRELL, V., BORVAK, J., BANCHEREAU, J. & PASCUAL, V. 2003. Interferon and granulopoiesis signatures in systemic lupus erythematosus blood. *J Exp Med,* 197**,** 711-23.

BERLIN-RUFENACH, C., OTTO, F., MATHIES, M., WESTERMANN, J., OWEN, M. J., HAMANN, A. & HOGG, N. 1999. Lymphocyte migration in lymphocyte function-associated antigen (LFA)-1-deficient mice. *J Exp Med,* 189**,** 1467-78.

BERLIN, C., BARGATZE, R. F., CAMPBELL, J. J., VON ANDRIAN, U. H., SZABO, M. C., HASSLEN, S. R., NELSON, R. D., BERG, E. L., ERLANDSEN, S. L. & BUTCHER, E. C. 1995. alpha 4 integrins mediate lymphocyte attachment and rolling under physiologic flow. *Cell,* 80**,** 413-22.

BERLIN, C., BERG, E. L., BRISKIN, M. J., ANDREW, D. P., KILSHAW, P. J., HOLZMANN, B., WEISSMAN, I. L., HAMANN, A. & BUTCHER, E. C. 1993. Alpha 4 beta 7 integrin mediates lymphocyte binding to the mucosal vascular addressin MAdCAM-1. *Cell,* 74**,** 185-95.

BERMAN, M. E. & MULLER, W. A. 1995. Ligation of platelet/endothelial cell adhesion molecule 1 (PECAM-1/CD31) on monocytes and neutrophils increases binding capacity of leukocyte CR3 (CD11b/CD18). *J Immunol,* 154**,** 299-307.

BERMAN, M. E., XIE, Y. & MULLER, W. A. 1996. Roles of platelet/endothelial cell adhesion molecule-1 (PECAM-1, CD31) in natural killer cell transendothelial migration and beta 2 integrin activation. *J Immunol,* 156**,** 1515-24.

BERTSIAS, G. & BOUMPAS, D. T. 2008. Update on the management of lupus nephritis: let the treatment fit the patient. *Nat Clin Pract Rheumatol,* 4**,** 464-72.

BEVILACQUA, M. P., STENGELIN, S., GIMBRONE, M. A., JR. & SEED, B. 1989. Endothelial leukocyte adhesion molecule 1: an inducible receptor for neutrophils related to complement regulatory proteins and lectins. *Science,* 243**,** 1160-5.

BILSLAND, C. A., DIAMOND, M. S. & SPRINGER, T. A. 1994. The leukocyte integrin p150,95 (CD11c/CD18) as a receptor for iC3b. Activation by a heterologous beta subunit and localization of a ligand recognition site to the I domain. *J Immunol,* 152**,** 4582-9.

BISCHOF, P., HAENGGELI, L. & CAMPANA, A. 1995. Gelatinase and oncofetal fibronectin secretion is dependent on integrin expression on human cytotrophoblasts. *Hum Reprod,* 10**,** 734-42.

BLAIR, P. A., NORENA, L. Y., FLORES-BORJA, F., RAWLINGS, D. J., ISENBERG, D. A., EHRENSTEIN, M. R. & MAURI, C. 2010. CD19(+)CD24(hi)CD38(hi) B cells exhibit regulatory capacity in healthy individuals but are functionally impaired in systemic Lupus Erythematosus patients. *Immunity,* 32**,** 129-40.

BLANK, M., KRAUSE, I., FRIDKIN, M., KELLER, N., KOPOLOVIC, J., GOLDBERG, I., TOBAR, A. & SHOENFELD, Y. 2002. Bacterial induction of autoantibodies to beta2-glycoprotein-I accounts for the infectious etiology of antiphospholipid syndrome. *J Clin Invest,* 109**,** 797-804.

BLASCHKE, F., STAWOWY, P., GOETZE, S., HINTZ, O., GRAFE, M., KINTSCHER, U., FLECK, E. & GRAF, K. 2002. Hypoxia activates beta(1)-integrin via ERK 1/2 and p38 MAP kinase in human vascular smooth muscle cells. *Biochem Biophys Res Commun,* 296**,** 890-6.

BLOMBERG, S., ELORANTA, M. L., CEDERBLAD, B., NORDLIN, K., ALM, G. V. & RONNBLOM, L. 2001. Presence of cutaneous interferon-alpha producing cells in patients with systemic lupus erythematosus. *Lupus,* 10**,** 484-90.

BODAMYALI, T., STEVENS, C. R., BILLINGHAM, M. E., OHTA, S. & BLAKE, D. R. 1998. Influence of hypoxia in inflammatory synovitis. *Ann Rheum Dis,* 57**,** 703-10.

BOGEN, S., PAK, J., GARIFALLOU, M., DENG, X. & MULLER, W. A. 1994. Monoclonal antibody to murine PECAM-1 (CD31) blocks acute inflammation in vivo. *J Exp Med,* 179**,** 1059-64.

BONELLI, M., SAVITSKAYA, A., VON DALWIGK, K., STEINER, C. W., ALETAHA, D., SMOLEN, J. S. & SCHEINECKER, C. 2008. Quantitative and qualitative deficiencies of

regulatory T cells in patients with systemic lupus erythematosus (SLE). *Int Immunol,* 20**,** 861-8.

BONFANTI, R., FURIE, B. C., FURIE, B. & WAGNER, D. D. 1989. PADGEM (GMP140) is a component of Weibel-Palade bodies of human endothelial cells. *Blood,* 73**,** 1109-12.

BONILLA-HERNAN, M. G., MIRANDA-CARUS, M. E. & MARTIN-MOLA, E. 2011. New drugs beyond biologics in rheumatoid arthritis: the kinase inhibitors. *Rheumatology (Oxford),* 50**,** 1542-50.

BORREGAARD, N., KJELDSEN, L., RYGAARD, K., BASTHOLM, L., NIELSEN, M. H., SENGELOV, H., BJERRUM, O. W. & JOHNSEN, A. H. 1992. Stimulus-dependent secretion of plasma proteins from human neutrophils. *J Clin Invest,* 90**,** 86-96.

BORREGAARD, N., KJELDSEN, L., SENGELOV, H., DIAMOND, M. S., SPRINGER, T. A., ANDERSON, H. C., KISHIMOTO, T. K. & BAINTON, D. F. 1994. Changes in subcellular localization and surface expression of L-selectin, alkaline phosphatase, and Mac-1 in human neutrophils during stimulation with inflammatory mediators. *J Leukoc Biol,* 56**,** 80-7.

BRACKEN, C. P., FEDELE, A. O., LINKE, S., BALRAK, W., LISY, K., WHITELAW, M. L. & PEET, D. J. 2006. Cell-specific regulation of hypoxia-inducible factor (HIF)-1alpha and HIF-2alpha stabilization and transactivation in a graded oxygen environment. *J Biol Chem,* 281**,** 22575-85.

BRANCH, D. W. 1994. Thoughts on the mechanism of pregnancy loss associated with the antiphospholipid syndrome. *Lupus,* 3**,** 275-80.

BRANDT, L. & HEDBERG, H. 1969. Impaired phagocytosis by peripheral blood granulocytes in systemic lupus erythematosus. *Scand J Haematol,* 6**,** 348-53.

BRANZK, N., LUBOJEMSKA, A., HARDISON, S. E., WANG, Q., GUTIERREZ, M. G., BROWN, G. D. & PAPAYANNOPOULOS, V. 2014. Neutrophils sense microbe size and selectively release neutrophil extracellular traps in response to large pathogens. *Nat Immunol,* 15**,** 1017-25.

BRAUN, R. D., LANZEN, J. L., SNYDER, S. A. & DEWHIRST, M. W. 2001. Comparison of tumor and normal tissue oxygen tension measurements using OxyLite or microelectrodes in rodents. *Am J Physiol Heart Circ Physiol,* 280**,** H2533-44.

BRENNAN, F. M., ZACHARIAE, C. O., CHANTRY, D., LARSEN, C. G., TURNER, M., MAINI, R. N., MATSUSHIMA, K. & FELDMANN, M. 1990. Detection of interleukin 8 biological activity in synovial fluids from patients with rheumatoid arthritis and production of interleukin 8 mRNA by isolated synovial cells. *Eur J Immunol,* 20**,** 2141-4.

BRENNAN, P. J., ZIGMOND, S. H., SCHREIBER, A. D., SMITH, E. R. & SOUTHWICK, F. S. 1991. Binding of IgG containing immune complexes to human neutrophil Fc gamma RII and Fc gamma RIII induces actin polymerization by a pertussis toxin-insensitive transduction pathway. *J Immunol,* 146**,** 4282-8.

BRILL, A., FUCHS, T. A., SAVCHENKO, A. S., THOMAS, G. M., MARTINOD, K., DE MEYER, S. F., BHANDARI, A. A. & WAGNER, D. D. 2012. Neutrophil extracellular traps promote deep vein thrombosis in mice. *J Thromb Haemost,* 10**,** 136-44.

BRINKMANN, V., REICHARD, U., GOOSMANN, C., FAULER, B., UHLEMANN, Y., WEISS, D. S., WEINRAUCH, Y. & ZYCHLINSKY, A. 2004. Neutrophil extracellular traps kill bacteria. *Science,* 303**,** 1532-1535.

BROSENS, I., ROBERTSON, W. B. & DIXON, H. G. 1967. The physiological response of the vessels of the placental bed to normal pregnancy. *J Pathol Bacteriol,* 93**,** 569-79.

BROWN, E. E., EDBERG, J. C. & KIMBERLY, R. P. 2007. Fc receptor genes and the systemic lupus erythematosus diathesis. *Autoimmunity,* 40**,** 567-81.

BROWN, E. J. 1991. Complement receptors and phagocytosis. *Curr Opin Immunol,* 3**,** 76-82.

BRUEHL, R. E., MOORE, K. L., LORANT, D. E., BORREGAARD, N., ZIMMERMAN, G. A., MCEVER, R. P. & BAINTON, D. F. 1997. Leukocyte activation induces surface redistribution of P-selectin glycoprotein ligand-1. *J Leukoc Biol,* 61**,** 489-99.

BRUHNS, P. 2012. Properties of mouse and human IgG receptors and their contribution to disease models. *Blood,* 119**,** 5640-9.

BRUSCA, S. B., ABRAMSON, S. B. & SCHER, J. U. 2014. Microbiome and mucosal inflammation as extra-articular triggers for rheumatoid arthritis and autoimmunity. *Curr Opin Rheumatol,* 26**,** 101-7.

BUCCIARELLI, S., ESPINOSA, G. & CERVERA, R. 2009. The CAPS Registry: morbidity and mortality of the catastrophic antiphospholipid syndrome. *Lupus,* 18**,** 905-12.

BUNN, H. F. & POYTON, R. O. 1996. Oxygen sensing and molecular adaptation to hypoxia. *Physiol Rev,* 76**,** 839-85.

BURR, M. L., NASEEM, H., HINKS, A., EYRE, S., GIBBONS, L. J., BOWES, J., WILSON, A. G., MAXWELL, J., MORGAN, A. W., EMERY, P., STEER, S., HOCKING, L., REID, D. M., WORDSWORTH, P., HARRISON, P., THOMSON, W., WORTHINGTON, J. & BARTON, A. 2010. PADI4 genotype is not associated with rheumatoid arthritis in a large UK Caucasian population. *Ann Rheum Dis,* 69**,** 666-70.

BURROWS, T. D., KING, A., SMITH, S. K. & LOKE, Y. W. 1995. Human trophoblast adhesion to matrix proteins: inhibition and signal transduction. *Hum Reprod,* 10**,** 2489-500.

BURTON, G. J., JAUNIAUX, E. & WATSON, A. L. 1999. Maternal arterial connections to the placental intervillous space during the first trimester of human pregnancy: the Boyd collection revisited. *Am J Obstet Gynecol,* 181**,** 718-24.

BYRD, A. S., O'BRIEN, X. M., JOHNSON, C. M., LAVIGNE, L. M. & REICHNER, J. S. 2013. An extracellular matrix-based mechanism of rapid neutrophil extracellular trap formation in response to Candida albicans. *J Immunol,* 190**,** 4136-48.

CALDWELL, C. C., KOJIMA, H., LUKASHEV, D., ARMSTRONG, J., FARBER, M., APASOV, S. G. & SITKOVSKY, M. V. 2001. Differential effects of physiologically relevant hypoxic conditions on T lymphocyte development and effector functions. *J Immunol,* 167**,** 6140-9.

CALIZ, R., ATSUMI, T., KONDEATIS, E., AMENGUAL, O., KHAMASHTA, M. A., VAUGHAN, R. W., LANCHBURY, J. S. & HUGHES, G. R. 2001. HLA class II gene polymorphisms in antiphospholipid syndrome: haplotype analysis in 83 Caucasoid patients. *Rheumatology (Oxford),* 40**,** 31-6.

CAPONI, L., PETIT-TEIXEIRA, E., SEBBAG, M., BONGIORNI, F., MOSCATO, S., PRATESI, F., PIERLOT, C., OSORIO, J., CHAPUY-REGAUD, S., GUERRIN, M., CORNELIS, F., SERRE, G. & MIGLIORINI, P. 2005. A family based study shows no association between rheumatoid arthritis and the PADI4 gene in a white French population. *Ann Rheum Dis,* 64**,** 587-93.

CARICCHIO, R., MCPHIE, L. & COHEN, P. L. 2003. Ultraviolet B radiation-induced cell death: critical role of ultraviolet dose in inflammation and lupus autoantigen redistribution. *J Immunol,* 171**,** 5778-86.

CARMAN, C. V. & SPRINGER, T. A. 2004. A transmigratory cup in leukocyte diapedesis both through individual vascular endothelial cells and between them. *J Cell Biol,* 167**,** 377-88.

CARMONA-RIVERA, C. & KAPLAN, M. J. 2013. Low-density granulocytes: a distinct class of neutrophils in systemic autoimmunity. *Semin Immunopathol,* 35**,** 455-63.

CARMONA-RIVERA, C., ZHAO, W., YALAVARTHI, S. & KAPLAN, M. J. 2015. Neutrophil extracellular traps induce endothelial dysfunction in systemic lupus erythematosus through the activation of matrix metalloproteinase-2. *Ann Rheum Dis,* 74**,** 1417-24.

CARMONA, F., FONT, J., AZULAY, M., CREUS, M., FABREGUES, F., CERVERA, R., PUERTO, B. & BALASCH, J. 2001. Risk factors associated with fetal losses in treated antiphospholipid syndrome pregnancies: a multivariate analysis. *Am J Reprod Immunol,* 46**,** 274-9.

CARON, E. & HALL, A. 1998. Identification of two distinct mechanisms of phagocytosis controlled by different Rho GTPases. *Science,* 282**,** 1717-21.

CARTER, T. D., HALLAM, T. J. & PEARSON, J. D. 1989. Protein kinase C activation alters the sensitivity of agonist-stimulated endothelial-cell prostacyclin production to intracellular Ca2+. *Biochem J,* 262**,** 431-7.

CEDENO, S., CIFARELLI, D. F., BLASINI, A. M., PARIS, M., PLACERES, F., ALONSO, G. & RODRIGUEZ, M. A. 2003. Defective activity of ERK-1 and ERK-2 mitogen-activated protein kinases in peripheral blood T lymphocytes from patients with systemic lupus erythematosus: potential role of altered coupling of Ras guanine nucleotide exchange factor hSos to adapter protein Grb2 in lupus T cells. *Clin Immunol,* 106**,** 41-9.

CELIK, E., FARIDI, M. H., KUMAR, V., DEEP, S., MOY, V. T. & GUPTA, V. 2013. Agonist leukadherin-1 increases CD11b/CD18-dependent adhesion via membrane tethers. *Biophys J,* 105**,** 2517-27.

CERF-BENSUSSAN, N., JARRY, A., BROUSSE, N., LISOWSKA-GROSPIERRE, B., GUY-GRAND, D. & GRISCELLI, C. 1987. A monoclonal antibody (HML-1) defining a novel membrane molecule present on human intestinal lymphocytes. *Eur J Immunol,* 17**,** 1279-85.

CERVERA, R., ASHERSON, R. A., ACEVEDO, M. L., GOMEZ-PUERTA, J. A., ESPINOSA, G., DE LA RED, G., GIL, V., RAMOS-CASALS, M., GARCIA-CARRASCO, M., INGELMO, M. & FONT, J. 2004. Antiphospholipid syndrome associated with infections: clinical and microbiological characteristics of 100 patients. *Ann Rheum Dis,* 63**,** 1312-7.

CERVERA, R., BOFFA, M. C., KHAMASHTA, M. A. & HUGHES, G. R. 2009a. The Euro-Phospholipid project: epidemiology of the antiphospholipid syndrome in Europe. *Lupus,* 18**,** 889-93.

CERVERA, R., BUCCIARELLI, S., PLASIN, M. A., GOMEZ-PUERTA, J. A., PLAZA, J., PONS-ESTEL, G., SHOENFELD, Y., INGELMO, M. & ESPINOS, G. 2009b. Catastrophic antiphospholipid syndrome (CAPS): descriptive analysis of a series of 280 patients from the "CAPS Registry". *J Autoimmun,* 32**,** 240-5.

CERVERA, R., FONT, J., GOMEZ-PUERTA, J. A., ESPINOSA, G., CUCHO, M., BUCCIARELLI, S., RAMOS-CASALS, M., INGELMO, M., PIETTE, J. C., SHOENFELD, Y. & ASHERSON, R. A. 2005. Validation of the preliminary criteria for the classification of catastrophic antiphospholipid syndrome. *Ann Rheum Dis,* 64**,** 1205-9.

CERVERA, R., KHAMASHTA, M. A. & HUGHES, G. R. 2009c. The Euro-lupus project: epidemiology of systemic lupus erythematosus in Europe. *Lupus,* 18**,** 869-74.

CERVERA, R., PIETTE, J. C., FONT, J., KHAMASHTA, M. A., SHOENFELD, Y., CAMPS, M. T., JACOBSEN, S., LAKOS, G., TINCANI, A., KONTOPOULOU-GRIVA, I., GALEAZZI, M., MERONI, P. L., DERKSEN, R. H., DE GROOT, P. G., GROMNICA-IHLE, E., BALEVA, M., MOSCA, M., BOMBARDIERI, S., HOUSSIAU, F., GRIS, J. C., QUERE, I., HACHULLA, E., VASCONCELOS, C., ROCH, B., FERNANDEZ-NEBRO, A., BOFFA, M. C., HUGHES, G. R. & INGELMO, M. 2002. Antiphospholipid syndrome: clinical and immunologic manifestations and patterns of disease expression in a cohort of 1,000 patients. *Arthritis Rheum,* 46**,** 1019-27.

CHABAUD, M., FOSSIEZ, F., TAUPIN, J. L. & MIOSSEC, P. 1998. Enhancing effect of IL-17 on IL-1-induced IL-6 and leukemia inhibitory factor production by rheumatoid arthritis synoviocytes and its regulation by Th2 cytokines. *J Immunol,* 161**,** 409-14.

CHAKRAVARTI, A., RAQUIL, M. A., TESSIER, P. & POUBELLE, P. E. 2009. Surface RANKL of Toll-like receptor 4-stimulated human neutrophils activates osteoclastic bone resorption. *Blood,* 114**,** 1633-44.

CHAN, B. M., ELICES, M. J., MURPHY, E. & HEMLER, M. E. 1992. Adhesion to vascular cell adhesion molecule 1 and fibronectin. Comparison of alpha 4 beta 1 (VLA-4) and alpha 4 beta 7 on the human B cell line JY. *J Biol Chem,* 267**,** 8366-70.

CHANDEL, N. S., TRZYNA, W. C., MCCLINTOCK, D. S. & SCHUMACKER, P. T. 2000. Role of oxidants in NF-kappa B activation and TNF-alpha gene transcription induced by hypoxia and endotoxin. *J Immunol,* 165**,** 1013-21.

CHATHAM, W. W., HECK, L. W. & BLACKBURN, W. D., JR. 1990. Lysis of fibrillar collagen by neutrophils in synovial fluid. A role for surface-bound immunoglobulins. *Arthritis Rheum,* 33**,** 1333-9.

CHAUDHRI, G., HUNT, N. H., CLARK, I. A. & CEREDIG, R. 1988. Antioxidants inhibit proliferation and cell surface expression of receptors for interleukin-2 and transferrin in T lymphocytes stimulated with phorbol myristate acetate and ionomycin. *Cell Immunol,* 115**,** 204-13.

CHAVAKIS, T., KEIPER, T., MATZ-WESTPHAL, R., HERSEMEYER, K., SACHS, U. J., NAWROTH, P. P., PREISSNER, K. T. & SANTOSO, S. 2004. The junctional adhesion molecule-C promotes neutrophil transendothelial migration in vitro and in vivo. *J Biol Chem,* 279**,** 55602-8.

CHEN, K., NISHI, H., TRAVERS, R., TSUBOI, N., MARTINOD, K., WAGNER, D. D., STAN, R., CROCE, K. & MAYADAS, T. N. 2012. Endocytosis of soluble immune complexes leads to their clearance by FcgammaRIIIB but induces neutrophil extracellular traps via FcgammaRIIA in vivo. *Blood,* 120**,** 4421-31.

CHENG, J., ZHANG, H., ZHUANG, C. & LIU, R. 2012. Peptidylarginine deiminase type 4 and methyl-CpG binding domain 4 polymorphisms in Chinese patients with rheumatoid arthritis. *J Rheumatol,* 39**,** 1159-65.

CHENG, M., LI, J., NEGRI, A. & COLLER, B. S. 2013. Swing-out of the beta3 hybrid domain is required for alphaIIbbeta3 priming and normal cytoskeletal reorganization, but not adhesion to immobilized fibrinogen. *PLoS One,* 8**,** e81609.

CHIBA, R., NAKAGAWA, N., KURASAWA, K., TANAKA, Y., SAITO, Y. & IWAMOTO, I. 1999. Ligation of CD31 (PECAM-1) on endothelial cells increases adhesive function of alphavbeta3 integrin and enhances beta1 integrin-mediated adhesion of eosinophils to endothelial cells. *Blood,* 94**,** 1319-29.

CHIU, J. J., LEE, P. L., CHEN, C. N., LEE, C. I., CHANG, S. F., CHEN, L. J., LIEN, S. C., KO, Y. C., USAMI, S. & CHIEN, S. 2004. Shear stress increases ICAM-1 and decreases VCAM-1 and E-selectin expressions induced by tumor necrosis factor-[alpha] in endothelial cells. *Arterioscler Thromb Vasc Biol,* 24**,** 73-9.

CICCO, N. A., LINDEMANN, A., CONTENT, J., VANDENBUSSCHE, P., LUBBERT, M., GAUSS, J., MERTELSMANN, R. & HERRMANN, F. 1990. Inducible production of interleukin-6 by human polymorphonuclear neutrophils: role of granulocyte-macrophage colony-stimulating factor and tumor necrosis factor-alpha. *Blood,* 75**,** 2049-52.

CLARK, S. R., MA, A. C., TAVENER, S. A., MCDONALD, B., GOODARZI, Z., KELLY, M. M., PATEL, K. D., CHAKRABARTI, S., MCAVOY, E., SINCLAIR, G. D., KEYS, E. M., ALLEN-VERCOE, E., DEVINNEY, R., DOIG, C. J., GREEN, F. H. & KUBES, P. 2007. Platelet TLR4 activates neutrophil extracellular traps to ensnare bacteria in septic blood. *Nat Med,* 13**,** 463-9.

COHEN, S. B., CHENG, T. T., CHINDALORE, V., DAMJANOV, N., BURGOS-VARGAS, R., DELORA, P., ZIMANY, K., TRAVERS, H. & CAULFIELD, J. P. 2009. Evaluation of the efficacy and safety of pamapimod, a p38 MAP kinase inhibitor, in a double-blind, methotrexate-controlled study of patients with active rheumatoid arthritis. *Arthritis Rheum,* 60**,** 335-44.

COJOCARU, M., COJOCARU, I. M., SILOSI, I., VRABIE, C. D. & TANASESCU, R. 2010. Extra-articular Manifestations in Rheumatoid Arthritis. *Maedica (Buchar),* 5**,** 286-91.

COLASANTI, T., MASELLI, A., CONTI, F., SANCHEZ, M., ALESSANDRI, C., BARBATI, C., VACIRCA, D., TINARI, A., CHIAROTTI, F., GIOVANNETTI, A., FRANCONI, F., VALESINI, G., MALORNI, W., PIERDOMINICI, M. & ORTONA, E. 2012. Autoantibodies to estrogen receptor alpha interfere with T lymphocyte homeostasis and are associated with disease activity in systemic lupus erythematosus. *Arthritis Rheum,* 64**,** 778-87.

COLGAN, S. P., DZUS, A. L. & PARKOS, C. A. 1996. Epithelial exposure to hypoxia modulates neutrophil transepithelial migration. *J Exp Med,* 184**,** 1003-15.

COREMANS, I. E., HAGEN, E. C., VAN DER VOORT, E. A., VAN DER WOUDE, F. J., DAHA, M. R. & BREEDVELD, F. C. 1993. Autoantibodies to neutrophil cytoplasmic enzymes in Felty's syndrome. *Clin Exp Rheumatol,* 11**,** 255-62.

CORTET, B., FLIPO, R. M., REMY-JARDIN, M., COQUERELLE, P., DUQUESNOY, B., REMY, J. & DELCAMBRE, B. 1995. Use of high resolution computed tomography of the lungs in patients with rheumatoid arthritis. *Ann Rheum Dis,* 54**,** 815-9.

COUTIFARIS, C., OMIGBODUN, A. & COUKOS, G. 2005. The fibronectin receptor alpha5 integrin subunit is upregulated by cell-cell adhesion via a cyclic AMP-dependent mechanism: implications for human trophoblast migration. *Am J Obstet Gynecol,* 192**,** 1240-53; discussion 1253-5.

COWDEN DAHL, K. D., FRYER, B. H., MACK, F. A., COMPERNOLLE, V., MALTEPE, E., ADELMAN, D. M., CARMELIET, P. & SIMON, M. C. 2005. Hypoxia-inducible factors 1alpha and 2alpha regulate trophoblast differentiation. *Mol Cell Biol,* 25**,** 10479-91.

COXON, A., CULLERE, X., KNIGHT, S., SETHI, S., WAKELIN, M. W., STAVRAKIS, G., LUSCINSKAS, F. W. & MAYADAS, T. N. 2001. Fc gamma RIII mediates neutrophil recruitment to immune complexes. a mechanism for neutrophil accumulation in immune-mediated inflammation. *Immunity,* 14**,** 693-704.

CRIBBS, A. P., KENNEDY, A., PENN, H., READ, J. E., AMJADI, P., GREEN, P., SYED, K., MANKA, S. W., BRENNAN, F. M., GREGORY, B. & WILLIAMS, R. O. 2014. Treg cell function in rheumatoid arthritis is compromised by ctla-4 promoter methylation resulting in a failure to activate the indoleamine 2,3-dioxygenase pathway. *Arthritis Rheumatol,* 66**,** 2344-54.

CROSS, A., BARNES, T., BUCKNALL, R. C., EDWARDS, S. W. & MOOTS, R. J. 2006. Neutrophil apoptosis in rheumatoid arthritis is regulated by local oxygen tensions within joints. *J Leukoc Biol,* 80**,** 521-8.

CROWLEY, M. T., COSTELLO, P. S., FITZER-ATTAS, C. J., TURNER, M., MENG, F., LOWELL, C., TYBULEWICZ, V. L. & DEFRANCO, A. L. 1997. A critical role for Syk in signal transduction and phagocytosis mediated by Fcgamma receptors on macrophages. *J Exp Med,* 186**,** 1027-39.

CUTOLO, M., BRIZZOLARA, R., ATZENI, F., CAPELLINO, S., STRAUB, R. H. & PUTTINI, P. C. 2010. The immunomodulatory effects of estrogens: clinical relevance in immune-mediated rheumatic diseases. *Ann N Y Acad Sci,* 1193**,** 36-42.

DAIEN, C. I., GAILHAC, S., MURA, T., AUDO, R., COMBE, B., HAHNE, M. & MOREL, J. 2014. Regulatory B10 cells are decreased in patients with rheumatoid arthritis and are inversely correlated with disease activity. *Arthritis Rheumatol,* 66**,** 2037-46.

DAMJANOV, N., KAUFFMAN, R. S. & SPENCER-GREEN, G. T. 2009. Efficacy, pharmacodynamics, and safety of VX-702, a novel p38 MAPK inhibitor, in rheumatoid arthritis: results of two randomized, double-blind, placebo-controlled clinical studies. *Arthritis Rheum,* 60**,** 1232-41.

DAMSKY, C. H., LIBRACH, C., LIM, K. H., FITZGERALD, M. L., MCMASTER, M. T., JANATPOUR, M., ZHOU, Y., LOGAN, S. K. & FISHER, S. J. 1994. Integrin switching regulates normal trophoblast invasion. *Development,* 120**,** 3657-66.

DARBY, C., GEAHLEN, R. L. & SCHREIBER, A. D. 1994. Stimulation of macrophage Fc gamma RIIIA activates the receptor-associated protein tyrosine kinase Syk and induces phosphorylation of multiple proteins including p95Vav and p62/GAP-associated protein. *J Immunol,* 152**,** 5429-37.

DASGUPTA, B., DUFOUR, E., MAMDOUH, Z. & MULLER, W. A. 2009. A novel and critical role for tyrosine 663 in platelet endothelial cell adhesion molecule-1 trafficking and transendothelial migration. *J Immunol,* 182**,** 5041-51.

DAYAN, F., ROUX, D., BRAHIMI-HORN, M. C., POUYSSEGUR, J. & MAZURE, N. M. 2006. The oxygen sensor factor-inhibiting hypoxia-inducible factor-1 controls expression of distinct genes through the bifunctional transcriptional character of hypoxia-inducible factor-1alpha. *Cancer Res,* 66**,** 3688-98.

DE FOUGEROLLES, A. R. & SPRINGER, T. A. 1992. Intercellular adhesion molecule 3, a third adhesion counter-receptor for lymphocyte function-associated molecule 1 on resting lymphocytes. *J Exp Med,* 175**,** 185-90.

DE FOUGEROLLES, A. R., STACKER, S. A., SCHWARTING, R. & SPRINGER, T. A. 1991. Characterization of ICAM-2 and evidence for a third counter-receptor for LFA-1. *J Exp Med,* 174**,** 253-67.

DE GROOT, P. G. & DERKSEN, R. H. 2005. Pathophysiology of the antiphospholipid syndrome. *J Thromb Haemost,* 3**,** 1854-60.

DE JESUS, G. R., AGMON-LEVIN, N., ANDRADE, C. A., ANDREOLI, L., CHIGHIZOLA, C. B., PORTER, T. F., SALMON, J., SILVER, R. M., TINCANI, A. & BRANCH, D. W. 2014. 14th International Congress on Antiphospholipid Antibodies Task Force report on obstetric antiphospholipid syndrome. *Autoimmun Rev,* 13**,** 795-813.

DE LAAT, B., DERKSEN, R. H., VAN LUMMEL, M., PENNINGS, M. T. & DE GROOT, P. G. 2006. Pathogenic anti-beta2-glycoprotein I antibodies recognize domain I of beta2-glycoprotein I only after a conformational change. *Blood,* 107**,** 1916-24.

DE LAAT, B., MERTENS, K. & DE GROOT, P. G. 2008. Mechanisms of disease: antiphospholipid antibodies-from clinical association to pathologic mechanism. *Nat Clin Pract Rheumatol,* 4**,** 192-9.

DECKER, B., MC, G. W., MC, K. B. & SLOCUMB, C. H. 1959. Concentration of hyaluronic acid in synovial fluid. *Clin Chem,* 5**,** 465-9.

DEL PAPA, N., RASCHI, E., MORONI, G., PANZERI, P., BORGHI, M. O., PONTICELLI, C., TINCANI, A., BALESTRIERI, G. & MERONI, P. L. 1999. Anti-endothelial cell IgG fractions from systemic lupus erythematosus patients bind to human endothelial cells and induce a pro-adhesive and a pro-inflammatory phenotype in vitro. *Lupus,* 8**,** 423-9.

DELVES, P. J. & ROITT, I. M. 2000. The immune system. First of two parts. *N Engl J Med,* 343**,** 37-49.

DEMIR, R., BODUR, H., TOKOGLU, F., OLCAY, I., UCAN, H. & BORMAN, P. 1999. High resolution computed tomography of the lungs in patients with rheumatoid arthritis. *Rheumatol Int,* 19**,** 19-22.

DEN BROEDER, A. A., WANTEN, G. J., OYEN, W. J., NABER, T., VAN RIEL, P. L. & BARRERA, P. 2003. Neutrophil migration and production of reactive oxygen species during treatment with a fully human anti-tumor necrosis factor-alpha monoclonal antibody in patients with rheumatoid arthritis. *J Rheumatol,* 30**,** 232-7.

DENG, W., REN, Y., FENG, X., YAO, G., CHEN, W., SUN, Y., WANG, H., GAO, X. & SUN, L. 2014. Hypoxia inducible factor-1 alpha promotes mesangial cell proliferation in lupus nephritis. *Am J Nephrol,* 40**,** 507-15.

DENNY, M. F., YALAVARTHI, S., ZHAO, W., THACKER, S. G., ANDERSON, M., SANDY, A. R., MCCUNE, W. J. & KAPLAN, M. J. 2010. A distinct subset of proinflammatory neutrophils isolated from patients with systemic lupus erythematosus induces vascular damage and synthesizes type I IFNs. *J Immunol,* 184**,** 3284-97.

DETMERS, P. A., ZHOU, D., POWELL, D., LICHENSTEIN, H., KELLEY, M. & PIRONKOVA, R. 1995. Endotoxin receptors (CD14) are found with CD16 (Fc gamma RIII) in an intracellular compartment of neutrophils that contains alkaline phosphatase. *J Immunol,* 155**,** 2085-95.

DEYOUNG, M. P., HORAK, P., SOFER, A., SGROI, D. & ELLISEN, L. W. 2008. Hypoxia regulates TSC1/2-mTOR signaling and tumor suppression through REDD1-mediated 14-3-3 shuttling. *Genes Dev,* 22**,** 239-51.

DI SIMONE, N., CASTELLANI, R., CALIANDRO, D. & CARUSO, A. 2002. Antiphospholid antibodies regulate the expression of trophoblast cell adhesion molecules. *Fertil Steril,* 77**,** 805-11.

DI SIMONE, N., MERONI, P. L., DE PAPA, N., RASCHI, E., CALIANDRO, D., DE CAROLIS, C. S., KHAMASHTA, M. A., ATSUMI, T., HUGHES, G. R., BALESTRIERI, G., TINCANI, A., CASALI, P. & CARUSO, A. 2000. Antiphospholipid antibodies affect trophoblast

gonadotropin secretion and invasiveness by binding directly and through adhered beta2-glycoprotein I. *Arthritis Rheum,* 43**,** 140-50.

DIACOVO, T. G., DEFOUGEROLLES, A. R., BAINTON, D. F. & SPRINGER, T. A. 1994. A functional integrin ligand on the surface of platelets: intercellular adhesion molecule-2. *J Clin Invest,* 94**,** 1243-51.

DIAMOND, M. S., STAUNTON, D. E., MARLIN, S. D. & SPRINGER, T. A. 1991. Binding of the integrin Mac-1 (CD11b/CD18) to the third immunoglobulin-like domain of ICAM-1 (CD54) and its regulation by glycosylation. *Cell,* 65**,** 961-71.

DING, Z. M., BABENSEE, J. E., SIMON, S. I., LU, H., PERRARD, J. L., BULLARD, D. C., DAI, X. Y., BROMLEY, S. K., DUSTIN, M. L., ENTMAN, M. L., SMITH, C. W. & BALLANTYNE, C. M. 1999. Relative contribution of LFA-1 and Mac-1 to neutrophil adhesion and migration. *J Immunol,* 163**,** 5029-38.

DIZ-KUCUKKAYA, R., INANC, M., AFSHAR-KHARGHAN, V., ZHANG, Q. E., LOPEZ, J. A. & PEKCELEN, Y. 2007. P-selectin glycoprotein ligand-1 VNTR polymorphisms and risk of thrombosis in the antiphospholipid syndrome. *Ann Rheum Dis,* 66**,** 1378-80.

DJEU, J. Y., SERBOUSEK, D. & BLANCHARD, D. K. 1990. Release of tumor necrosis factor by human polymorphonuclear leukocytes. *Blood,* 76**,** 1405-9.

DOMINICAL, V. M., BERTOLO, M. B., ALMEIDA, C. B., GARRIDO, V. T., MIGUEL, L. I., COSTA, F. F. & CONRAN, N. 2011. Neutrophils of rheumatoid arthritis patients on anti-TNF-alpha therapy and in disease remission present reduced adhesive functions in association with decreased circulating neutrophil-attractant chemokine levels. *Scand J Immunol,* 73**,** 309-18.

DONNELLY, S., ROAKE, W., BROWN, S., YOUNG, P., NAIK, H., WORDSWORTH, P., ISENBERG, D. A., REID, K. B. & EGGLETON, P. 2006. Impaired recognition of apoptotic neutrophils by the C1q/calreticulin and CD91 pathway in systemic lupus erythematosus. *Arthritis Rheum,* 54**,** 1543-56.

DORNER, T., GIESECKE, C. & LIPSKY, P. E. 2011. Mechanisms of B cell autoimmunity in SLE. *Arthritis Res Ther,* 13**,** 243.

DOUDA, D. N., YIP, L., KHAN, M. A., GRASEMANN, H. & PALANIYAR, N. 2014. Akt is essential to induce NADPH-dependent NETosis and to switch the neutrophil death to apoptosis. *Blood,* 123**,** 597-600.

DRANSFIELD, I., CABANAS, C., CRAIG, A. & HOGG, N. 1992. Divalent cation regulation of the function of the leukocyte integrin LFA-1. *J Cell Biol,* 116**,** 219-26.

DUBRAVEC, D. B., SPRIGGS, D. R., MANNICK, J. A. & RODRICK, M. L. 1990. Circulating human peripheral blood granulocytes synthesize and secrete tumor necrosis factor alpha. *Proc Natl Acad Sci U S A,* 87**,** 6758-61.

DULARAY, B., BADESHA, J. S., DIEPPE, P. A. & ELSON, C. J. 1990. Oxidative response of polymorphonuclear leucocytes to synovial fluids from patients with rheumatoid arthritis. *Ann Rheum Dis,* 49**,** 661-4.

DULARAY, B., ELSON, C. J. & DIEPPE, P. A. 1988. Enhanced oxidative response of polymorphonuclear leukocytes from synovial fluids of patients with rheumatoid arthritis. *Autoimmunity,* 1**,** 159-69.

DYETT, D. E., MALAWISTA, S. E., VAN BLARICOM, G., MELNICK, D. A. & MALECH, H. L. 1985. Functional integrity of cytokineplasts: specific chemotactic and capping responses. *J Immunol,* 135**,** 2090-4.

EDELMAN, G. M. 1973. Antibody structure and molecular immunology. *Science,* 180**,** 830-40.

EDWARDS, J. C., SZCZEPANSKI, L., SZECHINSKI, J., FILIPOWICZ-SOSNOWSKA, A., EMERY, P., CLOSE, D. R., STEVENS, R. M. & SHAW, T. 2004. Efficacy of B-cell-targeted therapy with rituximab in patients with rheumatoid arthritis. *N Engl J Med,* 350**,** 2572-81.

EDWARDS, L. J., MIZUI, M. & KYTTARIS, V. 2015. Signal transducer and activator of transcription (STAT) 3 inhibition delays the onset of lupus nephritis in MRL/lpr mice. *Clin Immunol,* 158**,** 221-30.

EDWARDS, S. W., HUGHES, V., BARLOW, J. & BUCKNALL, R. 1988. Immunological detection of myeloperoxidase in synovial fluid from patients with rheumatoid arthritis. *Biochem J,* 250**,** 81-5.

EGERER, K., FEIST, E., ROHR, U., PRUSS, A., BURMESTER, G. R. & DORNER, T. 2000. Increased serum soluble CD14, ICAM-1 and E-selectin correlate with disease activity and prognosis in systemic lupus erythematosus. *Lupus,* 9**,** 614-21.

EGESTEN, A., BRETON-GORIUS, J., GUICHARD, J., GULLBERG, U. & OLSSON, I. 1994. The heterogeneity of azurophil granules in neutrophil promyelocytes: immunogold localization of myeloperoxidase, cathepsin G, elastase, proteinase 3, and bactericidal/permeability increasing protein. *Blood,* 83**,** 2985-94.

EISENBERG, R. 2005. Update on rituximab. *Ann Rheum Dis,* 64 Suppl 4**,** iv55-7.

ELICES, M. J., OSBORN, L., TAKADA, Y., CROUSE, C., LUHOWSKYJ, S., HEMLER, M. E. & LOBB, R. R. 1990. VCAM-1 on activated endothelium interacts with the leukocyte integrin VLA-4 at a site distinct from the VLA-4/fibronectin binding site. *Cell,* 60**,** 577-84.

ELLMEIER, W., SAWADA, S. & LITTMAN, D. R. 1999. The regulation of CD4 and CD8 coreceptor gene expression during T cell development. *Annu Rev Immunol,* 17**,** 523-54.

EMA, M., TAYA, S., YOKOTANI, N., SOGAWA, K., MATSUDA, Y. & FUJII-KURIYAMA, Y. 1997. A novel bHLH-PAS factor with close sequence similarity to hypoxia-inducible factor 1alpha regulates the VEGF expression and is potentially involved in lung and vascular development. *Proc Natl Acad Sci U S A,* 94**,** 4273-8.

EMERY, P., LOPEZ, A. F., BURNS, G. F. & VADAS, M. A. 1988. Synovial fluid neutrophils of patients with rheumatoid arthritis have membrane antigen changes that reflect activation. *Ann Rheum Dis,* 47**,** 34-9.

EMSLEY, J., KNIGHT, C. G., FARNDALE, R. W., BARNES, M. J. & LIDDINGTON, R. C. 2000. Structural basis of collagen recognition by integrin alpha2beta1. *Cell,* 101**,** 47-56.

ENNIS, E., ISBERG, R. R. & SHIMIZU, Y. 1993. Very late antigen 4-dependent adhesion and costimulation of resting human T cells by the bacterial beta 1 integrin ligand invasin. *J Exp Med,* 177**,** 207-12.

ERKELLER-YUKSEL, F. M., LYDYARD, P. M. & ISENBERG, D. A. 1997. Lack of NK cells in lupus patients with renal involvement. *Lupus,* 6**,** 708-12.

ERRIDGE, C., STEWART, J. & POXTON, I. R. 2003. Monocytes heterozygous for the Asp299Gly and Thr399Ile mutations in the Toll-like receptor 4 gene show no deficit in lipopolysaccharide signalling. *J Exp Med,* 197**,** 1787-91.

ESAGUY, N., AGUAS, A. P., VILANOVA, M. & SILVA, M. T. 1991. Activation of human neutrophils by phorbol ester decreases the cytoplasm compactness and the lactoferrin content of the granulocytes. *J Leukoc Biol,* 50**,** 444-52.

ESPINOLA, R. G., LIU, X., COLDEN-STANFIELD, M., HALL, J., HARRIS, E. N. & PIERANGELI, S. S. 2003. E-Selectin mediates pathogenic effects of antiphospholipid antibodies. *J Thromb Haemost,* 1**,** 843-8.

ESTRADA-CAPETILLO, L., HERNANDEZ-CASTRO, B., MONSIVAIS-URENDA, A., ALVAREZ-QUIROGA, C., LAYSECA-ESPINOSA, E., ABUD-MENDOZA, C., BARANDA, L., URZAINQUI, A., SANCHEZ-MADRID, F. & GONZALEZ-AMARO, R. 2013. Induction of Th17 lymphocytes and Treg cells by monocyte-derived dendritic cells in patients with rheumatoid arthritis and systemic lupus erythematosus. *Clin Dev Immunol,* 2013**,** 584303.

ETULAIN, J., MARTINOD, K., WONG, S. L., CIFUNI, S. M., SCHATTNER, M. & WAGNER, D. D. 2015. P-selectin promotes neutrophil extracellular trap formation in mice. *Blood,* 126**,** 242-6.

EYLES, J. L., HICKEY, M. J., NORMAN, M. U., CROKER, B. A., ROBERTS, A. W., DRAKE, S. F., JAMES, W. G., METCALF, D., CAMPBELL, I. K. & WICKS, I. P. 2008. A key role for G-CSF-induced neutrophil production and trafficking during inflammatory arthritis. *Blood,* 112**,** 5193-201.

FAGERAS BOTTCHER, M., HMANI-AIFA, M., LINDSTROM, A., JENMALM, M. C., MAI, X. M., NILSSON, L., ZDOLSEK, H. A., BJORKSTEN, B., SODERKVIST, P. & VAARALA, O. 2004. A TLR4 polymorphism is associated with asthma and reduced lipopolysaccharide-induced interleukin-12(p70) responses in Swedish children. *J Allergy Clin Immunol,* 114**,** 561-7.

FALLMAN, M., ANDERSSON, R. & ANDERSSON, T. 1993. Signaling properties of CR3 (CD11b/CD18) and CR1 (CD35) in relation to phagocytosis of complement-opsonized particles. *J Immunol,* 151**,** 330-8.

FARKAS, L., BEISKE, K., LUND-JOHANSEN, F., BRANDTZAEG, P. & JAHNSEN, F. L. 2001. Plasmacytoid dendritic cells (natural interferon- alpha/beta-producing cells) accumulate in cutaneous lupus erythematosus lesions. *Am J Pathol,* 159**,** 237-43.

FARQUHARSON, D., BUTCHER, J. P. & CULSHAW, S. 2012. Periodontitis, Porphyromonas, and the pathogenesis of rheumatoid arthritis. *Mucosal Immunol,* 5**,** 112-20.

FAURSCHOU, M. & BORREGAARD, N. 2003. Neutrophil granules and secretory vesicles in inflammation. *Microbes Infect,* 5**,** 1317-27.

FERNANDO, M. M., STEVENS, C. R., SABETI, P. C., WALSH, E. C., MCWHINNIE, A. J., SHAH, A., GREEN, T., RIOUX, J. D. & VYSE, T. J. 2007. Identification of two independent risk factors for lupus within the MHC in United Kingdom families. *PLoS Genet,* 3**,** e192.

FIGUEROA-VEGA, N., GALINDO-RODRIGUEZ, G., BAJANA, S., PORTALES-PEREZ, D., ABUD-MENDOZA, C., SANCHEZ-TORRES, C. & GONZALEZ-AMARO, R. 2006. Phenotypic analysis of IL-10-treated, monocyte-derived dendritic cells in patients with systemic lupus erythematosus. *Scand J Immunol,* 64**,** 668-76.

FILLATREAU, S., SWEENIE, C. H., MCGEACHY, M. J., GRAY, D. & ANDERTON, S. M. 2002. B cells regulate autoimmunity by provision of IL-10. *Nat Immunol,* 3**,** 944-50.

FIORE, N., CASTELLANO, G., BLASI, A., CAPOBIANCO, C., LOVERRE, A., MONTINARO, V., NETTI, S., TORRES, D., MANNO, C., GRANDALIANO, G., RANIERI, E., SCHENA, F. P. & GESUALDO, L. 2008. Immature myeloid and plasmacytoid dendritic cells infiltrate renal tubulointerstitium in patients with lupus nephritis. *Mol Immunol,* 45**,** 259-65.

FISCHETTI, F., DURIGUTTO, P., PELLIS, V., DEBEUS, A., MACOR, P., BULLA, R., BOSSI, F., ZILLER, F., SBLATTERO, D., MERONI, P. & TEDESCO, F. 2005. Thrombus formation induced by antibodies to beta2-glycoprotein I is complement dependent and requires a priming factor. *Blood,* 106**,** 2340-6.

FITZER-ATTAS, C. J., LOWRY, M., CROWLEY, M. T., FINN, A. J., MENG, F., DEFRANCO, A. L. & LOWELL, C. A. 2000. Fcgamma receptor-mediated phagocytosis in macrophages lacking the Src family tyrosine kinases Hck, Fgr, and Lyn. *J Exp Med,* 191**,** 669-82.

FLAMME, I., FROHLICH, T., VON REUTERN, M., KAPPEL, A., DAMERT, A. & RISAU, W. 1997. HRF, a putative basic helix-loop-helix-PAS-domain transcription factor is closely related to hypoxia-inducible factor-1 alpha and developmentally expressed in blood vessels. *Mech Dev,* 63**,** 51-60.

FLEMING, S. D., EGAN, R. P., CHAI, C., GIRARDI, G., HOLERS, V. M., SALMON, J., MONESTIER, M. & TSOKOS, G. C. 2004. Anti-phospholipid antibodies restore mesenteric ischemia/reperfusion-induced injury in complement receptor 2/complement receptor 1-deficient mice. *J Immunol,* 173**,** 7055-61.

FOSSATI-JIMACK, L., LING, G. S., CORTINI, A., SZAJNA, M., MALIK, T. H., MCDONALD, J. U., PICKERING, M. C., COOK, H. T., TAYLOR, P. R. & BOTTO, M. 2013. Phagocytosis is the main CR3-mediated function affected by the lupus-associated variant of CD11b in human myeloid cells. *PLoS One,* 8**,** e57082.

FREITAS, M., PORTO, G., LIMA, J. L. & FERNANDES, E. 2008. Isolation and activation of human neutrophils in vitro. The importance of the anticoagulant used during blood collection. *Clin Biochem,* 41**,** 570-5.

FROSCH, M., VOGL, T., WALDHERR, R., SORG, C., SUNDERKOTTER, C. & ROTH, J. 2004. Expression of MRP8 and MRP14 by macrophages is a marker for severe forms of glomerulonephritis. *J Leukoc Biol,* 75**,** 198-206.

FUCHS, T. A., ABED, U., GOOSMANN, C., HURWITZ, R., SCHULZE, I., WAHN, V., WEINRAUCH, Y., BRINKMANN, V. & ZYCHLINSKY, A. 2007. Novel cell death program leads to neutrophil extracellular traps. *J Cell Biol,* 176**,** 231-41.

FUCHS, T. A., BRILL, A., DUERSCHMIED, D., SCHATZBERG, D., MONESTIER, M., MYERS, D. D., JR., WROBLESKI, S. K., WAKEFIELD, T. W., HARTWIG, J. H. & WAGNER, D. D. 2010. Extracellular DNA traps promote thrombosis. *Proc Natl Acad Sci U S A,* 107**,** 15880-5.

FUJISHIMA, S., HOFFMAN, A. R., VU, T., KIM, K. J., ZHENG, H., DANIEL, D., KIM, Y., WALLACE, E. F., LARRICK, J. W. & RAFFIN, T. A. 1993. Regulation of neutrophil interleukin 8 gene expression and protein secretion by LPS, TNF-alpha, and IL-1 beta. *J Cell Physiol,* 154**,** 478-85.

FUNAUCHI, M., OHNO, M., MINODA, M. & HORIUCHI, A. 1993. Abnormal expression of intercellular adhesion molecule-1 on peripheral blood mononuclear cells from patients with systemic lupus erythematosus. *J Clin Lab Immunol,* 40**,** 115-24.

GABBAY, E., TARALA, R., WILL, R., CARROLL, G., ADLER, B., CAMERON, D. & LAKE, F. R. 1997. Interstitial lung disease in recent onset rheumatoid arthritis. *Am J Respir Crit Care Med,* 156**,** 528-35.

GAL, I., BAJNOK, E., SZANTO, S., SARRAJ, B., GLANT, T. T. & MIKECZ, K. 2005. Visualization and in situ analysis of leukocyte trafficking into the ankle joint in a systemic murine model of rheumatoid arthritis. *Arthritis Rheum,* 52**,** 3269-78.

GALEAZZI, M., SEBASTIANI, G. D., TINCANI, A., PIETTE, J. C., ALLEGRI, F., MOROZZI, G., BELLISAI, F., SCORZA, R., FERRARA, G. B., CARCASSI, C., FONT, J., PASSIU, G., SMOLEN, J., PAPASTERIADES, C., HOUSSIAU, F., NEBRO, A. F., RAMON GARRIDO, E. D., JEDRYKA-GORAL, A. & MARCOLONGO, R. 2000. HLA class II alleles associations of anticardiolipin and anti-beta2GPI antibodies in a large series of European patients with systemic lupus erythematosus. *Lupus,* 9**,** 47-55.

GALLATIN, W. M., WEISSMAN, I. L. & BUTCHER, E. C. 1983. A cell-surface molecule involved in organ-specific homing of lymphocytes. *Nature,* 304**,** 30-4.

GALLI, M. & BARBUI, T. 2003. Antiphospholipid syndrome: definition and treatment. *Semin Thromb Hemost,* 29**,** 195-204.

GALLI, M., COMFURIUS, P., MAASSEN, C., HEMKER, H. C., DE BAETS, M. H., VAN BREDA-VRIESMAN, P. J., BARBUI, T., ZWAAL, R. F. & BEVERS, E. M. 1990. Anticardiolipin antibodies (ACA) directed not to cardiolipin but to a plasma protein cofactor. *Lancet,* 335**,** 1544-7.

GARCIA-ROMO, G. S., CAIELLI, S., VEGA, B., CONNOLLY, J., ALLANTAZ, F., XU, Z., PUNARO, M., BAISCH, J., GUIDUCCI, C., COFFMAN, R. L., BARRAT, F. J., BANCHEREAU, J. & PASCUAL, V. 2011. Netting neutrophils are major inducers of type I IFN production in pediatric systemic lupus erythematosus. *Sci Transl Med,* 3**,** 73ra20.

GARCIA, R. C., PETERSON, C. G., SEGAL, A. W. & VENGE, P. 1985. Elastase in the different primary granules of the human neutrophil. *Biochem Biophys Res Commun,* 132**,** 1130-6.

GARRETT, I. R., BOYCE, B. F., OREFFO, R. O., BONEWALD, L., POSER, J. & MUNDY, G. R. 1990. Oxygen-derived free radicals stimulate osteoclastic bone resorption in rodent bone in vitro and in vivo. *J Clin Invest,* 85**,** 632-9.

GEBOREK, P., SAXNE, T., PETTERSSON, H. & WOLLHEIM, F. A. 1989. Synovial fluid acidosis correlates with radiological joint destruction in rheumatoid arthritis knee joints. *J Rheumatol,* 16**,** 468-72.

GENOVESE, M. C., COHEN, S. B., WOFSY, D., WEINBLATT, M. E., FIRESTEIN, G. S., BRAHN, E., STRAND, V., BAKER, D. G. & TONG, S. E. 2008. A randomized, double-blind, placebo-controlled phase 2 study of an oral p38 alpha MAPK inhibitor, SCIO-469, in patients with active rheumatoid arthritis. *Arthritis Rheum,* 58**,** S431-S432.

GEORGE, J., GILBURD, B., HOJNIK, M., LEVY, Y., LANGEVITZ, P., MATSUURA, E., KOIKE, T. & SHOENFELD, Y. 1998. Target recognition of beta2-glycoprotein I (beta2GPI)-

dependent anticardiolipin antibodies: evidence for involvement of the fourth domain of beta2GPI in antibody binding. *J Immunol,* 160**,** 3917-23.

GEORGIADIS, A. N., METAFRATZI, Z. M. & DROSOS, A. A. 2009. Pulmonary abnormalities in patients with early and longstanding rheumatoid arthritis. *J Rheumatol,* 36**,** 444-5; author reply 445-6.

GHAZIZADEH, S., BOLEN, J. B. & FLEIT, H. B. 1995. Tyrosine phosphorylation and association of Syk with Fc gamma RII in monocytic THP-1 cells. *Biochem J,* 305 (Pt 2)**,** 669-74.

GHEZZI, P., DINARELLO, C. A., BIANCHI, M., ROSANDICH, M. E., REPINE, J. E. & WHITE, C. W. 1991. Hypoxia increases production of interleukin-1 and tumor necrosis factor by human mononuclear cells. *Cytokine,* 3**,** 189-94.

GIANNAKOPOULOS, B. & KRILIS, S. A. 2009. How I treat the antiphospholipid syndrome. *Blood,* 114**,** 2020-30.

GILLENIUS, E. & URBAN, C. F. 2015. The adhesive protein invasin of Yersinia pseudotuberculosis induces neutrophil extracellular traps via beta1 integrins. *Microbes Infect,* 17**,** 327-36.

GINSBERG, M. H., LIGHTSEY, A., KUNICKI, T. J., KAUFMANN, A., MARGUERIE, G. & PLOW, E. F. 1986. Divalent cation regulation of the surface orientation of platelet membrane glycoprotein IIb. Correlation with fibrinogen binding function and definition of a novel variant of Glanzmann's thrombasthenia. *J Clin Invest,* 78**,** 1103-11.

GINSBURG, K. S., LIANG, M. H., NEWCOMER, L., GOLDHABER, S. Z., SCHUR, P. H., HENNEKENS, C. H. & STAMPFER, M. J. 1992. Anticardiolipin antibodies and the risk for ischemic stroke and venous thrombosis. *Ann Intern Med,* 117**,** 997-1002.

GIRARDI, G., BERMAN, J., REDECHA, P., SPRUCE, L., THURMAN, J. M., KRAUS, D., HOLLMANN, T. J., CASALI, P., CAROLL, M. C., WETSEL, R. A., LAMBRIS, J. D., HOLERS, V. M. & SALMON, J. E. 2003. Complement C5a receptors and neutrophils mediate fetal injury in the antiphospholipid syndrome. *J Clin Invest,* 112**,** 1644-54.

GOETZL, E. J., FALCHUK, K. H., ZEIGER, L. S., SULLIVAN, A. L., HEBERT, C. L., ADAMS, J. P. & DECKER, J. L. 1971. A physiological approach to the assessment of disease activity in rheumatoid arthritis. *J Clin Invest,* 50**,** 1167-80.

GOLDBERG, S. N., CONTI-KELLY, A. M. & GRECO, T. P. 1995. A family study of anticardiolipin antibodies and associated clinical conditions. *Am J Med,* 99**,** 473-9.

GOLDSBY, R. A., KINDT, T. J., OSBORNE, B. A. & KUBY, J. 2003. *Immunology.* 5th ed. New York: W.H. Freeman and Company.

GONG, H., SHEN, B., FLEVARIS, P., CHOW, C., LAM, S. C., VOYNO-YASENETSKAYA, T. A., KOZASA, T. & DU, X. 2010. G protein subunit Galpha13 binds to integrin alphaIIbbeta3 and mediates integrin "outside-in" signaling. *Science,* 327**,** 340-3.

GONZALEZ-QUINTELA, A., ALENDE, R., GUDE, F., CAMPOS, J., REY, J., MEIJIDE, L. M., FERNANDEZ-MERINO, C. & VIDAL, C. 2008. Serum levels of immunoglobulins (IgG, IgA, IgM) in a general adult population and their relationship with alcohol consumption, smoking and common metabolic abnormalities. *Clin Exp Immunol,* 151**,** 42-50.

GOOD, D. W., GEORGE, T. & WATTS, B. A., 3RD 2012. Toll-like receptor 2 is required for LPS-induced Toll-like receptor 4 signaling and inhibition of ion transport in renal thick ascending limb. *J Biol Chem,* 287**,** 20208-20.

GOODSON, N. & SYMMONS, D. 2002. Rheumatoid arthritis in women: still associated with an increased mortality. *Ann Rheum Dis,* 61**,** 955-6.

GORDON, D. L., JOHNSON, G. M. & HOSTETTER, M. K. 1987. Characteristics of iC3b binding to human polymorphonuclear leucocytes. *Immunology,* 60**,** 553-8.

GORMAN, J. D., LUM, R. F., CHEN, J. J., SUAREZ-ALMAZOR, M. E., THOMSON, G. & CRISWELL, L. A. 2004. Impact of shared epitope genotype and ethnicity on erosive disease: a meta-analysis of 3,240 rheumatoid arthritis patients. *Arthritis Rheum,* 50**,** 400-12.

GRAHAM, R. R., ORTMANN, W., RODINE, P., ESPE, K., LANGEFELD, C., LANGE, E., WILLIAMS, A., BECK, S., KYOGOKU, C., MOSER, K., GAFFNEY, P., GREGERSEN, P. K.,

CRISWELL, L. A., HARLEY, J. B. & BEHRENS, T. W. 2007. Specific combinations of HLA-DR2 and DR3 class II haplotypes contribute graded risk for disease susceptibility and autoantibodies in human SLE. *Eur J Hum Genet,* 15**,** 823-30.

GRANADOS, J., VARGAS-ALARCON, G., DRENKARD, C., ANDRADE, F., MELIN-ALDANA, H., ALCOCER-VARELA, J. & ALARCON-SEGOVIA, D. 1997. Relationship of anticardiolipin antibodies and antiphospholipid syndrome to HLA-DR7 in Mexican patients with systemic lupus erythematosus (SLE). *Lupus,* 6**,** 57-62.

GREENBERG, S., CHANG, P. & SILVERSTEIN, S. C. 1994. Tyrosine phosphorylation of the gamma subunit of Fc gamma receptors, p72syk, and paxillin during Fc receptor-mediated phagocytosis in macrophages. *J Biol Chem,* 269**,** 3897-902.

GRIFFITHS, R. J., PETTIPHER, E. R., KOCH, K., FARRELL, C. A., BRESLOW, R., CONKLYN, M. J., SMITH, M. A., HACKMAN, B. C., WIMBERLY, D. J., MILICI, A. J. & ET AL. 1995. Leukotriene B4 plays a critical role in the progression of collagen-induced arthritis. *Proc Natl Acad Sci U S A,* 92**,** 517-21.

GRILLO-LOPEZ, A. J., WHITE, C. A., VARNS, C., SHEN, D., WEI, A., MCCLURE, A. & DALLAIRE, B. K. 1999. Overview of the clinical development of rituximab: first monoclonal antibody approved for the treatment of lymphoma. *Semin Oncol,* 26**,** 66-73.

GROOTVELD, M., HENDERSON, E. B., FARRELL, A., BLAKE, D. R., PARKES, H. G. & HAYCOCK, P. 1991. Oxidative damage to hyaluronate and glucose in synovial fluid during exercise of the inflamed rheumatoid joint. Detection of abnormal low-molecular-mass metabolites by proton-n.m.r. spectroscopy. *Biochem J,* 273(Pt 2)**,** 459-67.

GROTENDORST, G. R., SMALE, G. & PENCEV, D. 1989. Production of transforming growth factor beta by human peripheral blood monocytes and neutrophils. *J Cell Physiol,* 140**,** 396-402.

GRUMET, F. C., COUKELL, A., BODMER, J. G., BODMER, W. F. & MCDEVITT, H. O. 1971. Histocompatibility (HL-A) antigens associated with systemic lupus erythematosus. A possible genetic predisposition to disease. *N Engl J Med,* 285**,** 193-6.

GU, Y. Z., MORAN, S. M., HOGENESCH, J. B., WARTMAN, L. & BRADFIELD, C. A. 1998. Molecular characterization and chromosomal localization of a third alpha-class hypoxia inducible factor subunit, HIF3alpha. *Gene Expr,* 7**,** 205-13.

GUPTA, A. K., JOSHI, M. B., PHILIPPOVA, M., ERNE, P., HASLER, P., HAHN, S. & RESINK, T. J. 2010. Activated endothelial cells induce neutrophil extracellular traps and are susceptible to NETosis-mediated cell death. *FEBS Lett,* 584**,** 3193-7.

GUTOWSKA-OWSIAK, D., BIRCHALL, M. A., MOOTS, R. J., CHRISTMAS, S. E. & PAZMANY, L. 2014. Proliferatory defect of invariant population and accumulation of non-invariant CD1d-restricted natural killer T cells in the joints of RA patients. *Mod Rheumatol,* 24**,** 434-42.

GUTTMAN-YASSKY, E., VUGMEYSTER, Y., LOWES, M. A., CHAMIAN, F., KIKUCHI, T., KAGEN, M., GILLEAUDEAU, P., LEE, E., HUNTE, B., HOWELL, K., DUMMER, W., BODARY, S. C. & KRUEGER, J. G. 2008. Blockade of CD11a by efalizumab in psoriasis patients induces a unique state of T-cell hyporesponsiveness. *J Invest Dermatol,* 128**,** 1182-91.

GUYRE, P. M., CAMPBELL, A. S., KNIFFIN, W. D. & FANGER, M. W. 1990. Monocytes and polymorphonuclear neutrophils of patients with streptococcal pharyngitis express increased numbers of type I IgG Fc receptors. *J Clin Invest,* 86**,** 1892-6.

HACBARTH, E. & KAJDACSY-BALLA, A. 1986. Low density neutrophils in patients with systemic lupus erythematosus, rheumatoid arthritis, and acute rheumatic fever. *Arthritis Rheum,* 29**,** 1334-42.

HAGBERG, N., THEORELL, J., HJORTON, K., SPEE, P., ELORANTA, M. L., BRYCESON, Y. T. & RONNBLOM, L. 2015. Functional anti-CD94/NKG2A and anti-CD94/NKG2C autoantibodies in patients with systemic lupus erythematosus. *Arthritis Rheumatol,* 67**,** 1000-11.

HAKKIM, A., FUCHS, T. A., MARTINEZ, N. E., HESS, S., PRINZ, H., ZYCHLINSKY, A. & WALDMANN, H. 2011. Activation of the Raf-MEK-ERK pathway is required for neutrophil extracellular trap formation. *Nat Chem Biol,* 7**,** 75-7.

HAKKIM, A., FURNROHR, B. G., AMANN, K., LAUBE, B., ABED, U. A., BRINKMANN, V., HERRMANN, M., VOLL, R. E. & ZYCHLINSKY, A. 2010. Impairment of neutrophil extracellular trap degradation is associated with lupus nephritis. *Proc Natl Acad Sci U S A,* 107**,** 9813-8.

HALBWACHS-MECARELLI, L., NUSBAUM, P., NOEL, L. H., REUMAUX, D., ERLINGER, S., GRUNFELD, J. P. & LESAVRE, P. 1992. Antineutrophil cytoplasmic antibodies (ANCA) directed against cathepsin G in ulcerative colitis, Crohn's disease and primary sclerosing cholangitis. *Clin Exp Immunol,* 90**,** 79-84.

HAMMAKER, D. & FIRESTEIN, G. S. 2010. "Go upstream, young man": lessons learned from the p38 saga. *Ann Rheum Dis,* 69 Suppl 1**,** i77-82.

HAN, S., KIM-HOWARD, X., DESHMUKH, H., KAMATANI, Y., VISWANATHAN, P., GUTHRIDGE, J. M., THOMAS, K., KAUFMAN, K. M., OJWANG, J., ROJAS-VILLARRAGA, A., BACA, V., OROZCO, L., RHODES, B., CHOI, C. B., GREGERSEN, P. K., MERRILL, J. T., JAMES, J. A., GAFFNEY, P. M., MOSER, K. L., JACOB, C. O., KIMBERLY, R. P., HARLEY, J. B., BAE, S. C., ANAYA, J. M., ALARCON-RIQUELME, M. E., MATSUDA, K., VYSE, T. J. & NATH, S. K. 2009. Evaluation of imputation-based association in and around the integrin-alpha-M (ITGAM) gene and replication of robust association between a non-synonymous functional variant within ITGAM and systemic lupus erythematosus (SLE). *Hum Mol Genet,* 18**,** 1171-80.

HARA, S., HAMADA, J., KOBAYASHI, C., KONDO, Y. & IMURA, N. 2001. Expression and characterization of hypoxia-inducible factor (HIF)-3alpha in human kidney: suppression of HIF-mediated gene expression by HIF-3alpha. *Biochem Biophys Res Commun,* 287**,** 808-13.

HARADA, T., KYTTARIS, V., LI, Y., JUANG, Y. T., WANG, Y. & TSOKOS, G. C. 2007. Increased expression of STAT3 in SLE T cells contributes to enhanced chemokine-mediated cell migration. *Autoimmunity,* 40**,** 1-8.

HARINGMAN, J. J., GERLAG, D. M., ZWINDERMAN, A. H., SMEETS, T. J., KRAAN, M. C., BAETEN, D., MCINNES, I. B., BRESNIHAN, B. & TAK, P. P. 2005. Synovial tissue macrophages: a sensitive biomarker for response to treatment in patients with rheumatoid arthritis. *Ann Rheum Dis,* 64**,** 834-8.

HARLEY, J. B., ALARCON-RIQUELME, M. E., CRISWELL, L. A., JACOB, C. O., KIMBERLY, R. P., MOSER, K. L., TSAO, B. P., VYSE, T. J., LANGEFELD, C. D., NATH, S. K., GUTHRIDGE, J. M., COBB, B. L., MIREL, D. B., MARION, M. C., WILLIAMS, A. H., DIVERS, J., WANG, W., FRANK, S. G., NAMJOU, B., GABRIEL, S. B., LEE, A. T., GREGERSEN, P. K., BEHRENS, T. W., TAYLOR, K. E., FERNANDO, M., ZIDOVETZKI, R., GAFFNEY, P. M., EDBERG, J. C., RIOUX, J. D., OJWANG, J. O., JAMES, J. A., MERRILL, J. T., GILKESON, G. S., SELDIN, M. F., YIN, H., BAECHLER, E. C., LI, Q. Z., WAKELAND, E. K., BRUNER, G. R., KAUFMAN, K. M. & KELLY, J. A. 2008. Genome-wide association scan in women with systemic lupus erythematosus identifies susceptibility variants in ITGAM, PXK, KIAA1542 and other loci. *Nat Genet,* 40**,** 204-10.

HARTUNG, K., COLDEWEY, R., CORVETTA, A., DEICHER, H., KALDEN, J. R., KRAPF, F., LANG, B., LAKOMEK, H. J., LIEDVOGEL, B., PETER, H. H. & ET AL. 1992. MHC gene products and anticardiolipin antibodies in systemic lupus erythematosus results of a multicenter study. SLE Study Group. *Autoimmunity,* 13**,** 95-9.

HASLETT, C., GUTHRIE, L. A., KOPANIAK, M. M., JOHNSTON, R. B., JR. & HENSON, P. M. 1985. Modulation of multiple neutrophil functions by preparative methods or trace concentrations of bacterial lipopolysaccharide. *Am J Pathol,* 119**,** 101-10.

HEADLAND, S. E., JONES, H. R., NORLING, L. V., KIM, A., SOUZA, P. R., CORSIERO, E., GIL, C. D., NERVIANI, A., DELL'ACCIO, F., PITZALIS, C., OLIANI, S. M., JAN, L. Y. & PERRETTI, M. 2015. Neutrophil-derived microvesicles enter cartilage and protect the joint in inflammatory arthritis. *Sci Transl Med,* 7**,** 315ra190.

HEMLER, M. E., GLASS, D., COBLYN, J. S. & JACOBSON, J. G. 1986. Very late activation antigens on rheumatoid synovial fluid T lymphocytes. Association with stages of T cell activation. *J Clin Invest,* 78**,** 696-702.

HERMAND, P., GANE, P., HUET, M., JALLU, V., KAPLAN, C., SONNEBORN, H. H., CARTRON, J. P. & BAILLY, P. 2003. Red cell ICAM-4 is a novel ligand for platelet-activated alpha IIbbeta 3 integrin. *J Biol Chem,* 278**,** 4892-8.

HERVIER, B., BEZIAT, V., HAROCHE, J., MATHIAN, A., LEBON, P., GHILLANI-DALBIN, P., MUSSET, L., DEBRE, P., AMOURA, Z. & VIEILLARD, V. 2011. Phenotype and function of natural killer cells in systemic lupus erythematosus: excess interferon-gamma production in patients with active disease. *Arthritis Rheum,* 63**,** 1698-706.

HEWITSON, K. S., MCNEILL, L. A., RIORDAN, M. V., TIAN, Y. M., BULLOCK, A. N., WELFORD, R. W., ELKINS, J. M., OLDHAM, N. J., BHATTACHARYA, S., GLEADLE, J. M., RATCLIFFE, P. J., PUGH, C. W. & SCHOFIELD, C. J. 2002. Hypoxia-inducible factor (HIF) asparagine hydroxylase is identical to factor inhibiting HIF (FIH) and is related to the cupin structural family. *J Biol Chem,* 277**,** 26351-5.

HINKS, A., BARTON, A., JOHN, S., BRUCE, I., HAWKINS, C., GRIFFITHS, C. E., DONN, R., THOMSON, W., SILMAN, A. & WORTHINGTON, J. 2005. Association between the PTPN22 gene and rheumatoid arthritis and juvenile idiopathic arthritis in a UK population: further support that PTPN22 is an autoimmunity gene. *Arthritis Rheum,* 52**,** 1694-9.

HIRAOKA, N., PETRYNIAK, B., NAKAYAMA, J., TSUBOI, S., SUZUKI, M., YEH, J. C., IZAWA, D., TANAKA, T., MIYASAKA, M., LOWE, J. B. & FUKUDA, M. 1999. A novel, high endothelial venule-specific sulfotransferase expresses 6-sulfo sialyl Lewis(x), an L-selectin ligand displayed by CD34. *Immunity,* 11**,** 79-89.

HIROSE, N., WILLIAMS, R., ALBERTS, A. R., FURIE, R. A., CHARTASH, E. K., JAIN, R. I., SISON, C., LAHITA, R. G., MERRILL, J. T., CUCURULL, E., GHARAVI, A. E., SAMMARITANO, L. R., SALMON, J. E., HASHIMOTO, S., SAWADA, T., CHU, C. C., GREGERSEN, P. K. & CHIORAZZI, N. 1999. A role for the polymorphism at position 247 of the beta2-glycoprotein I gene in the generation of anti-beta2-glycoprotein I antibodies in the antiphospholipid syndrome. *Arthritis Rheum,* 42**,** 1655-61.

HOGENESCH, J. B., CHAN, W. K., JACKIW, V. H., BROWN, R. C., GU, Y. Z., PRAY-GRANT, M., PERDEW, G. H. & BRADFIELD, C. A. 1997. Characterization of a subset of the basic-helix-loop-helix-PAS superfamily that interacts with components of the dioxin signaling pathway. *J Biol Chem,* 272**,** 8581-93.

HOLERS, V. M., GIRARDI, G., MO, L., GUTHRIDGE, J. M., MOLINA, H., PIERANGELI, S. S., ESPINOLA, R., XIAOWEI, L. E., MAO, D., VIALPANDO, C. G. & SALMON, J. E. 2002. Complement C3 activation is required for antiphospholipid antibody-induced fetal loss. *J Exp Med,* 195**,** 211-20.

HOM, G., GRAHAM, R. R., MODREK, B., TAYLOR, K. E., ORTMANN, W., GARNIER, S., LEE, A. T., CHUNG, S. A., FERREIRA, R. C., PANT, P. V., BALLINGER, D. G., KOSOY, R., DEMIRCI, F. Y., KAMBOH, M. I., KAO, A. H., TIAN, C., GUNNARSSON, I., BENGTSSON, A. A., RANTAPAA-DAHLQVIST, S., PETRI, M., MANZI, S., SELDIN, M. F., RONNBLOM, L., SYVANEN, A. C., CRISWELL, L. A., GREGERSEN, P. K. & BEHRENS, T. W. 2008. Association of systemic lupus erythematosus with C8orf13-BLK and ITGAM-ITGAX. *N Engl J Med,* 358**,** 900-9.

HOPPE, B., HAUPL, T., GRUBER, R., KIESEWETTER, H., BURMESTER, G. R., SALAMA, A. & DORNER, T. 2006. Detailed analysis of the variability of peptidylarginine deiminase type 4 in German patients with rheumatoid arthritis: a case-control study. *Arthritis Res Ther,* 8**,** R34.

HORTELANO, S., LOPEZ-FONTAL, R., TRAVES, P. G., VILLA, N., GRASHOFF, C., BOSCA, L. & LUQUE, A. 2010. ILK mediates LPS-induced vascular adhesion receptor expression and subsequent leucocyte trans-endothelial migration. *Cardiovasc Res,* 86**,** 283-92.

HSIEH, S. C., WU, T. H., TSAI, C. Y., LI, K. J., LU, M. C., WU, C. H. & YU, C. L. 2008. Abnormal in vitro CXCR2 modulation and defective cationic ion transporter expression on

polymorphonuclear neutrophils responsible for hyporesponsiveness to IL-8 stimulation in patients with active systemic lupus erythematosus. *Rheumatology (Oxford),* 47**,** 150-7.

HSU-LIN, S., BERMAN, C. L., FURIE, B. C., AUGUST, D. & FURIE, B. 1984. A platelet membrane protein expressed during platelet activation and secretion. Studies using a monoclonal antibody specific for thrombin-activated platelets. *J Biol Chem,* 259**,** 9121-6.

HSU, H. C., YANG, P., WANG, J., WU, Q., MYERS, R., CHEN, J., YI, J., GUENTERT, T., TOUSSON, A., STANUS, A. L., LE, T. V., LORENZ, R. G., XU, H., KOLLS, J. K., CARTER, R. H., CHAPLIN, D. D., WILLIAMS, R. W. & MOUNTZ, J. D. 2008. Interleukin 17-producing T helper cells and interleukin 17 orchestrate autoreactive germinal center development in autoimmune BXD2 mice. *Nat Immunol,* 9**,** 166-75.

HU, D. D., HOYER, J. R. & SMITH, J. W. 1995. Ca2+ suppresses cell adhesion to osteopontin by attenuating binding affinity for integrin alpha v beta 3. *J Biol Chem,* 270**,** 9917-25.

HU, F., MU, R., ZHU, J., SHI, L., LI, Y., LIU, X., SHAO, W., LI, G., LI, M., SU, Y., COHEN, P. L., QIU, X. & LI, Z. 2014. Hypoxia and hypoxia-inducible factor-1alpha provoke toll-like receptor signalling-induced inflammation in rheumatoid arthritis. *Ann Rheum Dis,* 73**,** 928-36.

HUANG, A. J., SILVERSTEIN, S. C. & MALAWISTA, S. E. 1991. Cryopreserved cytoplasts from human neutrophils migrate across monolayers of human endothelial cells in response to a chemoattractant gradient. *J Leukoc Biol,* 50**,** 624-7.

HUANG, C., ZANG, Q., TAKAGI, J. & SPRINGER, T. A. 2000. Structural and functional studies with antibodies to the integrin beta 2 subunit. A model for the I-like domain. *J Biol Chem,* 275**,** 21514-24.

HUANG, J., DIBBLE, C. C., MATSUZAKI, M. & MANNING, B. D. 2008. The TSC1-TSC2 complex is required for proper activation of mTOR complex 2. *Mol Cell Biol,* 28**,** 4104-15.

HUANG, M. T., LARBI, K. Y., SCHEIERMANN, C., WOODFIN, A., GERWIN, N., HASKARD, D. O. & NOURSHARGH, S. 2006. ICAM-2 mediates neutrophil transmigration in vivo: evidence for stimulus specificity and a role in PECAM-1-independent transmigration. *Blood,* 107**,** 4721-7.

HUANG, Y. M., WANG, H., WANG, C., CHEN, M. & ZHAO, M. H. 2015. Promotion of hypercoagulability in antineutrophil cytoplasmic antibody-associated vasculitis by C5a-induced tissue factor-expressing microparticles and neutrophil extracellular traps. *Arthritis Rheumatol,* 67**,** 2780-90.

HUANG, Z., FU, B., ZHENG, S. G., LI, X., SUN, R., TIAN, Z. & WEI, H. 2011a. Involvement of CD226+ NK cells in immunopathogenesis of systemic lupus erythematosus. *J Immunol,* 186**,** 3421-31.

HUANG, Z. Y., HUNTER, S., CHIEN, P., KIM, M. K., HAN-KIM, T. H., INDIK, Z. K. & SCHREIBER, A. D. 2011b. Interaction of two phagocytic host defense systems: Fcgamma receptors and complement receptor 3. *J Biol Chem,* 286**,** 160-8.

HUGHES, G. R. 1983. Thrombosis, abortion, cerebral disease, and the lupus anticoagulant. *Br Med J (Clin Res Ed),* 287**,** 1088-9.

HUGHES, G. R. 1993. The antiphospholipid syndrome: ten years on. *Lancet,* 342**,** 341-4.

HUGHES, J. R., ERHARDT, C. C. & CLEMENT, M. 1995. Neutrophilic dermatosis in association with rheumatoid arthritis. *Clin Exp Dermatol,* 20**,** 168-70.

HUMPHREYS, J. H., VERSTAPPEN, S. M., HYRICH, K. L., CHIPPING, J. R., MARSHALL, T. & SYMMONS, D. P. 2013. The incidence of rheumatoid arthritis in the UK: comparisons using the 2010 ACR/EULAR classification criteria and the 1987 ACR classification criteria. Results from the Norfolk Arthritis Register. *Ann Rheum Dis,* 72**,** 1315-20.

HYNES, R. O. 2002. Integrins: bidirectional, allosteric signaling machines. *Cell,* 110**,** 673-87.

ICHIKAWA, K., KHAMASHTA, M. A., KOIKE, T., MATSUURA, E. & HUGHES, G. R. 1994. beta 2-Glycoprotein I reactivity of monoclonal anticardiolipin antibodies from patients with the antiphospholipid syndrome. *Arthritis Rheum,* 37**,** 1453-61.

IHANUS, E., UOTILA, L. M., TOIVANEN, A., VARIS, M. & GAHMBERG, C. G. 2007. Red-cell ICAM-4 is a ligand for the monocyte/macrophage integrin CD11c/CD18: characterization of the binding sites on ICAM-4. *Blood,* 109**,** 802-10.

IKEMATSU, W., LUAN, F. L., LA ROSA, L., BELTRAMI, B., NICOLETTI, F., BUYON, J. P., MERONI, P. L., BALESTRIERI, G. & CASALI, P. 1998. Human anticardiolipin monoclonal autoantibodies cause placental necrosis and fetal loss in BALB/c mice. *Arthritis Rheum,* 41**,** 1026-39.

IKEZUMI, Y., SUZUKI, T., HAYAFUJI, S., OKUBO, S., NIKOLIC-PATERSON, D. J., KAWACHI, H., SHIMIZU, F. & UCHIYAMA, M. 2005. The sialoadhesin (CD169) expressing a macrophage subset in human proliferative glomerulonephritis. *Nephrol Dial Transplant,* 20**,** 2704-13.

IMAI, Y., SINGER, M. S., FENNIE, C., LASKY, L. A. & ROSEN, S. D. 1991. Identification of a carbohydrate-based endothelial ligand for a lymphocyte homing receptor. *J Cell Biol,* 113**,** 1213-21.

INOUE, O., SUZUKI-INOUE, K., DEAN, W. L., FRAMPTON, J. & WATSON, S. P. 2003. Integrin alpha2beta1 mediates outside-in regulation of platelet spreading on collagen through activation of Src kinases and PLCgamma2. *J Cell Biol,* 160**,** 769-80.

IOANNOU, Y., PERICLEOUS, C., GILES, I., LATCHMAN, D. S., ISENBERG, D. A. & RAHMAN, A. 2007. Binding of antiphospholipid antibodies to discontinuous epitopes on domain I of human beta(2)-glycoprotein I: mutation studies including residues R39 to R43. *Arthritis Rheum,* 56**,** 280-90.

IRVING, J. A., LYSIAK, J. J., GRAHAM, C. H., HEARN, S., HAN, V. K. & LALA, P. K. 1995. Characteristics of trophoblast cells migrating from first trimester chorionic villus explants and propagated in culture. *Placenta,* 16**,** 413-33.

ITAKURA, A. & MCCARTY, O. J. 2013. Pivotal role for the mTOR pathway in the formation of neutrophil extracellular traps via regulation of autophagy. *Am J Physiol Cell Physiol,* 305**,** C348-54.

IVAN, M., KONDO, K., YANG, H., KIM, W., VALIANDO, J., OHH, M., SALIC, A., ASARA, J. M., LANE, W. S. & KAELIN, W. G., JR. 2001. HIFalpha targeted for VHL-mediated destruction by proline hydroxylation: implications for O2 sensing. *Science,* 292**,** 464-8.

IVERSON, G. M., VICTORIA, E. J. & MARQUIS, D. M. 1998. Anti-beta2 glycoprotein I (beta2GPI) autoantibodies recognize an epitope on the first domain of beta2GPI. *Proc Natl Acad Sci U S A,* 95**,** 15542-6.

IWAMOTO, T., IKARI, K., NAKAMURA, T., KUWAHARA, M., TOYAMA, Y., TOMATSU, T., MOMOHARA, S. & KAMATANI, N. 2006. Association between PADI4 and rheumatoid arthritis: a meta-analysis. *Rheumatology (Oxford),* 45**,** 804-7.

JAAKKOLA, P., MOLE, D. R., TIAN, Y. M., WILSON, M. I., GIELBERT, J., GASKELL, S. J., VON KRIEGSHEIM, A., HEBESTREIT, H. F., MUKHERJI, M., SCHOFIELD, C. J., MAXWELL, P. H., PUGH, C. W. & RATCLIFFE, P. J. 2001. Targeting of HIF-alpha to the von Hippel-Lindau ubiquitylation complex by O2-regulated prolyl hydroxylation. *Science,* 292**,** 468-72.

JAFFE, R., JAUNIAUX, E. & HUSTIN, J. 1997. Maternal circulation in the first-trimester human placenta--myth or reality? *Am J Obstet Gynecol,* 176**,** 695-705.

JAKUS, Z., NEMETH, T., VERBEEK, J. S. & MOCSAI, A. 2008. Critical but overlapping role of FcgammaRIII and FcgammaRIV in activation of murine neutrophils by immobilized immune complexes. *J Immunol,* 180**,** 618-29.

JAMES, M. J., CLELAND, L. G., ROFE, A. M. & LESLIE, A. L. 1990. Intraarticular pressure and the relationship between synovial perfusion and metabolic demand. *J Rheumatol,* 17**,** 521-7.

JANEWAY, C. A., JR. 1993. How the immune system recognizes invaders. *Sci Am,* 269**,** 72-9.

JASIN, H. E., LIGHTFOOT, E., DAVIS, L. S., ROTHLEIN, R., FAANES, R. B. & LIPSKY, P. E. 1992. Amelioration of antigen-induced arthritis in rabbits treated with monoclonal antibodies to leukocyte adhesion molecules. *Arthritis Rheum,* 35**,** 541-9.

JIANG, B. H., SEMENZA, G. L., BAUER, C. & MARTI, H. H. 1996. Hypoxia-inducible factor 1 levels vary exponentially over a physiologically relevant range of O2 tension. *Am J Physiol,* 271**,** C1172-80.

JIMENEZ, S., TASSIES, D., ESPINOSA, G., GARCIA-CRIADO, A., PLAZA, J., MONTEAGUDO, J., CERVERA, R. & REVERTER, J. C. 2008. Double heterozygosity polymorphisms for platelet glycoproteins Ia/IIa and IIb/IIIa increases arterial thrombosis and arteriosclerosis in patients with the antiphospholipid syndrome or with systemic lupus erythematosus. *Ann Rheum Dis,* 67**,** 835-40.

JIN, O., KAVIKONDALA, S., MOK, M. Y., SUN, L., GU, J., FU, R., CHAN, A., YEUNG, J., NIE, Y. & LAU, C. S. 2010. Abnormalities in circulating plasmacytoid dendritic cells in patients with systemic lupus erythematosus. *Arthritis Res Ther,* 12**,** R137.

JIN, O., SUN, L. Y., ZHOU, K. X., ZHANG, X. S., FENG, X. B., MOK, M. Y. & LAU, C. S. 2005. Lymphocyte apoptosis and macrophage function: correlation with disease activity in systemic lupus erythematosus. *Clin Rheumatol,* 24**,** 107-10.

JOHNSON-LEGER, C. A., AURRAND-LIONS, M., BELTRAMINELLI, N., FASEL, N. & IMHOF, B. A. 2002. Junctional adhesion molecule-2 (JAM-2) promotes lymphocyte transendothelial migration. *Blood,* 100**,** 2479-86.

JURY, E. C., FLORES-BORJA, F., KALSI, H. S., LAZARUS, M., ISENBERG, D. A., MAURI, C. & EHRENSTEIN, M. R. 2010. Abnormal CTLA-4 function in T cells from patients with systemic lupus erythematosus. *Eur J Immunol,* 40**,** 569-78.

KAELIN, W. G., JR. & RATCLIFFE, P. J. 2008. Oxygen sensing by metazoans: the central role of the HIF hydroxylase pathway. *Mol Cell,* 30**,** 393-402.

KAHLENBERG, J. M., CARMONA-RIVERA, C., SMITH, C. K. & KAPLAN, M. J. 2013. Neutrophil extracellular trap-associated protein activation of the NLRP3 inflammasome is enhanced in lupus macrophages. *J Immunol,* 190**,** 1217-26.

KAKIMOTO, K., NAKAMURA, T., ISHII, K., TAKASHI, T., IIGOU, H., YAGITA, H., OKUMURA, K. & ONOUE, K. 1992. The effect of anti-adhesion molecule antibody on the development of collagen-induced arthritis. *Cell Immunol,* 142**,** 326-37.

KALLIO, P. J., PONGRATZ, I., GRADIN, K., MCGUIRE, J. & POELLINGER, L. 1997. Activation of hypoxia-inducible factor 1alpha: posttranscriptional regulation and conformational change by recruitment of the Arnt transcription factor. *Proc Natl Acad Sci U S A,* 94**,** 5667-72.

KAMATA, T. & TAKADA, Y. 1994. Direct binding of collagen to the I domain of integrin alpha 2 beta 1 (VLA-2, CD49b/CD29) in a divalent cation-independent manner. *J Biol Chem,* 269**,** 26006-10.

KAMATH, S. & LIP, G. Y. 2003. Fibrinogen: biochemistry, epidemiology and determinants. *QJM,* 96**,** 711-29.

KAMBAS, K., CHRYSANTHOPOULOU, A., VASSILOPOULOS, D., APOSTOLIDOU, E., SKENDROS, P., GIROD, A., ARELAKI, S., FROUDARAKIS, M., NAKOPOULOU, L., GIATROMANOLAKI, A., SIDIROPOULOS, P., KOFFA, M., BOUMPAS, D. T., RITIS, K. & MITROULIS, I. 2014. Tissue factor expression in neutrophil extracellular traps and neutrophil derived microparticles in antineutrophil cytoplasmic antibody associated vasculitis may promote thromboinflammation and the thrombophilic state associated with the disease. *Ann Rheum Dis,* 73**,** 1854-63.

KAMIGUCHI, K., TACHIBANA, K., IWATA, S., OHASHI, Y. & MORIMOTO, C. 1999. Cas-L is required for beta 1 integrin-mediated costimulation in human Tcells. *J Immunol,* 163**,** 563-8.

KANDA, N., TSUCHIDA, T. & TAMAKI, K. 1999. Estrogen enhancement of anti-double-stranded DNA antibody and immunoglobulin G production in peripheral blood mononuclear cells from patients with systemic lupus erythematosus. *Arthritis Rheum,* 42**,** 328-37.

KANG, C. P., LEE, H. S., JU, H., CHO, H., KANG, C. & BAE, S. C. 2006. A functional haplotype of the PADI4 gene associated with increased rheumatoid arthritis susceptibility in Koreans. *Arthritis Rheum,* 54**,** 90-6.

KAO, L. C., CALTABIANO, S., WU, S., STRAUSS, J. F., 3RD & KLIMAN, H. J. 1988. The human villous cytotrophoblast: interactions with extracellular matrix proteins, endocrine function, and cytoplasmic differentiation in the absence of syncytium formation. *Dev Biol,* 130**,** 693-702.

KARASSA, F. B., TRIKALINOS, T. A. & IOANNIDIS, J. P. 2002. Role of the Fcgamma receptor IIa polymorphism in susceptibility to systemic lupus erythematosus and lupus nephritis: a meta-analysis. *Arthritis Rheum,* 46**,** 1563-71.

KARASSA, F. B., TRIKALINOS, T. A. & IOANNIDIS, J. P. 2003. The Fc gamma RIIIA-F158 allele is a risk factor for the development of lupus nephritis: a meta-analysis. *Kidney Int,* 63**,** 1475-82.

KEELY, S., GLOVER, L. E., MACMANUS, C. F., CAMPBELL, E. L., SCULLY, M. M., FURUTA, G. T. & COLGAN, S. P. 2009. Selective induction of integrin beta1 by hypoxia-inducible factor: implications for wound healing. *FASEB J,* 23**,** 1338-46.

KESHARI, R. S., JYOTI, A., DUBEY, M., KOTHARI, N., KOHLI, M., BOGRA, J., BARTHWAL, M. K. & DIKSHIT, M. 2012. Cytokines induced neutrophil extracellular traps formation: implication for the inflammatory disease condition. *PLoS One,* 7**,** e48111.

KESHARI, R. S., VERMA, A., BARTHWAL, M. K. & DIKSHIT, M. 2013. Reactive oxygen species-induced activation of ERK and p38 MAPK mediates PMA-induced NETs release from human neutrophils. *J Cell Biochem,* 114**,** 532-40.

KHANDPUR, R., CARMONA-RIVERA, C., VIVEKANANDAN-GIRI, A., GIZINSKI, A., YALAVARTHI, S., KNIGHT, J. S., FRIDAY, S., LI, S., PATEL, R. M., SUBRAMANIAN, V., THOMPSON, P., CHEN, P., FOX, D. A., PENNATHUR, S. & KAPLAN, M. J. 2013. NETs are a source of citrullinated autoantigens and stimulate inflammatory responses in rheumatoid arthritis. *Sci Transl Med,* 5**,** 178ra40.

KIEFER, F., BRUMELL, J., AL-ALAWI, N., LATOUR, S., CHENG, A., VEILLETTE, A., GRINSTEIN, S. & PAWSON, T. 1998. The Syk protein tyrosine kinase is essential for Fcgamma receptor signaling in macrophages and neutrophils. *Mol Cell Biol,* 18**,** 4209-20.

KILSHAW, P. J. 1999. Alpha E beta 7. *Mol Pathol,* 52**,** 203-7.

KIM-HOWARD, X., MAITI, A. K., ANAYA, J. M., BRUNER, G. R., BROWN, E., MERRILL, J. T., EDBERG, J. C., PETRI, M. A., REVEILLE, J. D., RAMSEY-GOLDMAN, R., ALARCON, G. S., VYSE, T. J., GILKESON, G., KIMBERLY, R. P., JAMES, J. A., GUTHRIDGE, J. M., HARLEY, J. B. & NATH, S. K. 2010. ITGAM coding variant (rs1143679) influences the risk of renal disease, discoid rash and immunological manifestations in patients with systemic lupus erythematosus with European ancestry. *Ann Rheum Dis,* 69**,** 1329-32.

KIM, E. J., ELICKER, B. M., MALDONADO, F., WEBB, W. R., RYU, J. H., VAN UDEN, J. H., LEE, J. S., KING, T. E., JR. & COLLARD, H. R. 2010. Usual interstitial pneumonia in rheumatoid arthritis-associated interstitial lung disease. *Eur Respir J,* 35**,** 1322-8.

KIM, M., CARMAN, C. V. & SPRINGER, T. A. 2003. Bidirectional transmembrane signaling by cytoplasmic domain separation in integrins. *Science,* 301**,** 1720-5.

KITZING, T. M., WANG, Y., PERTZ, O., COPELAND, J. W. & GROSSE, R. 2010. Formin-like 2 drives amoeboid invasive cell motility downstream of RhoC. *Oncogene,* 29**,** 2441-8.

KLEIN-SCHNEEGANS, A. S., KUNTZ, L., FONTENEAU, P. & LOOR, F. 1989. Serum concentrations of IgM, IgG1, IgG2b, IgG3 and IgA in C57BL/6 mice and their congenics at the lpr (lymphoproliferation) locus. *J Autoimmun,* 2**,** 869-75.

KLIMOVA, T. & CHANDEL, N. S. 2008. Mitochondrial complex III regulates hypoxic activation of HIF. *Cell Death Differ,* 15**,** 660-6.

KLINGHOFFER, R. A., SACHSENMAIER, C., COOPER, J. A. & SORIANO, P. 1999. Src family kinases are required for integrin but not PDGFR signal transduction. *EMBO J,* 18**,** 2459-71.

KLINMAN, D. M., SHIRAI, A., ISHIGATSUBO, Y., CONOVER, J. & STEINBERG, A. D. 1991. Quantitation of IgM- and IgG-secreting B cells in the peripheral blood of patients with systemic lupus erythematosus. *Arthritis Rheum,* 34**,** 1404-10.

KNIGHT, J. S., ZHAO, W., LUO, W., SUBRAMANIAN, V., O'DELL, A. A., YALAVARTHI, S., HODGIN, J. B., EITZMAN, D. T., THOMPSON, P. R. & KAPLAN, M. J. 2013. Peptidylarginine deiminase inhibition is immunomodulatory and vasculoprotective in murine lupus. *J Clin Invest,* 123**,** 2981-93.

KONG, T., ELTZSCHIG, H. K., KARHAUSEN, J., COLGAN, S. P. & SHELLEY, C. S. 2004. Leukocyte adhesion during hypoxia is mediated by HIF-1-dependent induction of beta2 integrin gene expression. *Proc Natl Acad Sci U S A,* 101**,** 10440-5.

KORMAN, B. D., HUANG, C. C., SKAMRA, C., WU, P., KOESSLER, R., YAO, D., HUANG, Q. Q., PEARCE, W., SUTTON-TYRRELL, K., KONDOS, G., EDMUNDOWICZ, D., POPE, R. & RAMSEY-GOLDMAN, R. 2014. Inflammatory expression profiles in monocyte-to-macrophage differentiation in patients with systemic lupus erythematosus and relationship with atherosclerosis. *Arthritis Res Ther,* 16**,** R147.

KOWALCZYK, A. P., TULLOH, R. H. & MCKEOWN-LONGO, P. J. 1990. Polarized fibronectin secretion and localized matrix assembly sites correlate with subendothelial matrix formation. *Blood,* 75**,** 2335-42.

KRISHNAN, S., JUANG, Y. T., CHOWDHURY, B., MAGILAVY, A., FISHER, C. U., NGUYEN, H., NAMBIAR, M. P., KYTTARIS, V., WEINSTEIN, A., BAHJAT, R., PINE, P., RUS, V. & TSOKOS, G. C. 2008. Differential expression and molecular associations of Syk in systemic lupus erythematosus T cells. *J Immunol,* 181**,** 8145-52.

KRUISBEEK, A. M. 1999. Introduction: regulation of T cell development by the thymic microenvironment. *Semin Immunol,* 11**,** 1-2.

KUNICKI, T. J., PIDARD, D., ROSA, J. P. & NURDEN, A. T. 1981. The formation of Ca++-dependent complexes of platelet membrane glycoproteins IIb and IIIa in solution as determined by crossed immunoelectrophoresis. *Blood,* 58**,** 268-78.

KUZNIK, A., BENCINA, M., SVAJGER, U., JERAS, M., ROZMAN, B. & JERALA, R. 2011. Mechanism of endosomal TLR inhibition by antimalarial drugs and imidazoquinolines. *J Immunol,* 186**,** 4794-804.

KWAK-KIM, J., AGCAOILI, M. S., ALETA, L., LIAO, A., OTA, K., DAMBAEVA, S., BEAMAN, K., KIM, J. W. & GILMAN-SACHS, A. 2013. Management of women with recurrent pregnancy losses and antiphospholipid antibody syndrome. *Am J Reprod Immunol,* 69**,** 596-607.

KWOK, S. K., LEE, J. Y., PARK, S. H., CHO, M. L., MIN, S. Y., KIM, H. Y. & CHO, Y. G. 2008. Dysfunctional interferon-alpha production by peripheral plasmacytoid dendritic cells upon Toll-like receptor-9 stimulation in patients with systemic lupus erythematosus. *Arthritis Res Ther,* 10**,** R29.

KYOGOKU, C., DIJSTELBLOEM, H. M., TSUCHIYA, N., HATTA, Y., KATO, H., YAMAGUCHI, A., FUKAZAWA, T., JANSEN, M. D., HASHIMOTO, H., VAN DE WINKEL, J. G., KALLENBERG, C. G. & TOKUNAGA, K. 2002. Fcgamma receptor gene polymorphisms in Japanese patients with systemic lupus erythematosus: contribution of FCGR2B to genetic susceptibility. *Arthritis Rheum,* 46**,** 1242-54.

KYTTARIS, V. C. & TSOKOS, G. C. 2011. Targeting lymphocyte signaling pathways as a therapeutic approach to systemic lupus erythematosus. *Curr Opin Rheumatol,* 23**,** 449-53.

LAFFON, A., GARCIA-VICUNA, R., HUMBRIA, A., POSTIGO, A. A., CORBI, A. L., DE LANDAZURI, M. O. & SANCHEZ-MADRID, F. 1991. Upregulated expression and function of VLA-4 fibronectin receptors on human activated T cells in rheumatoid arthritis. *J Clin Invest,* 88**,** 546-52.

LAHITA, R. G., KUNKEL, H. G. & BRADLOW, H. L. 1983. Increased oxidation of testosterone in systemic lupus erythematosus. *Arthritis Rheum,* 26**,** 1517-21.

LAKI, J., LUNDSTROM, E., SNIR, O., RONNELID, J., GANJI, I., CATRINA, A. I., BENGTSSON, C., SAEVARSDOTTIR, S., WICK, M. C., ALFREDSSON, L., KLARESKOG, L. & PADYUKOV,

L. 2012. Very high levels of anti-citrullinated protein antibodies are associated with HLA-DRB1*15 non-shared epitope allele in patients with rheumatoid arthritis. *Arthritis Rheum,* 64**,** 2078-84.

LANDE, R., GANGULY, D., FACCHINETTI, V., FRASCA, L., CONRAD, C., GREGORIO, J., MELLER, S., CHAMILOS, G., SEBASIGARI, R., RICCIERI, V., BASSETT, R., AMURO, H., FUKUHARA, S., ITO, T., LIU, Y. J. & GILLIET, M. 2011. Neutrophils activate plasmacytoid dendritic cells by releasing self-DNA-peptide complexes in systemic lupus erythematosus. *Sci Transl Med,* 3**,** 73ra19.

LANDO, D., PEET, D. J., WHELAN, D. A., GORMAN, J. J. & WHITELAW, M. L. 2002. Asparagine hydroxylation of the HIF transactivation domain a hypoxic switch. *Science,* 295**,** 858-61.

LAVIGNE, L. M., O'BRIEN, X. M., KIM, M., JANOWSKI, J. W., ALBINA, J. E. & REICHNER, J. S. 2007. Integrin engagement mediates the human polymorphonuclear leukocyte response to a fungal pathogen-associated molecular pattern. *J Immunol,* 178**,** 7276-82.

LEAVESLEY, D. I., OLIVER, J. M., SWART, B. W., BERNDT, M. C., HAYLOCK, D. N. & SIMMONS, P. J. 1994. Signals from platelet/endothelial cell adhesion molecule enhance the adhesive activity of the very late antigen-4 integrin of human CD34+ hemopoietic progenitor cells. *J Immunol,* 153**,** 4673-83.

LEE, J. O., RIEU, P., ARNAOUT, M. A. & LIDDINGTON, R. 1995. Crystal structure of the A domain from the alpha subunit of integrin CR3 (CD11b/CD18). *Cell,* 80**,** 631-8.

LEE, K. M., CHUANG, E., GRIFFIN, M., KHATTRI, R., HONG, D. K., ZHANG, W., STRAUS, D., SAMELSON, L. E., THOMPSON, C. B. & BLUESTONE, J. A. 1998. Molecular basis of T cell inactivation by CTLA-4. *Science,* 282**,** 2263-6.

LEE, S. H., LEE, Y. J. & HAN, H. J. 2011. Role of hypoxia-induced fibronectin-integrin beta1 expression in embryonic stem cell proliferation and migration: Involvement of PI3K/Akt and FAK. *J Cell Physiol,* 226**,** 484-93.

LEE, Y. H., JI, J. D. & SONG, G. G. 2009. Fcgamma receptor IIB and IIIB polymorphisms and susceptibility to systemic lupus erythematosus and lupus nephritis: a meta-analysis. *Lupus,* 18**,** 727-34.

LEE, Y. W., KUHN, H., HENNIG, B., NEISH, A. S. & TOBOREK, M. 2001. IL-4-induced oxidative stress upregulates VCAM-1 gene expression in human endothelial cells. *J Mol Cell Cardiol,* 33**,** 83-94.

LEFFLER, J., MARTIN, M., GULLSTRAND, B., TYDEN, H., LOOD, C., TRUEDSSON, L., BENGTSSON, A. A. & BLOM, A. M. 2012. Neutrophil extracellular traps that are not degraded in systemic lupus erythematosus activate complement exacerbating the disease. *J Immunol,* 188**,** 3522-31.

LEFORT, C. T., ROSSAINT, J., MOSER, M., PETRICH, B. G., ZARBOCK, A., MONKLEY, S. J., CRITCHLEY, D. R., GINSBERG, M. H., FASSLER, R. & LEY, K. 2012. Distinct roles for talin-1 and kindlin-3 in LFA-1 extension and affinity regulation. *Blood,* 119**,** 4275-82.

LEVINE, J. S., BRANCH, D. W. & RAUCH, J. 2002. The antiphospholipid syndrome. *N Engl J Med,* 346**,** 752-63.

LI, B., YUE, Y., DONG, C., SHI, Y. & XIONG, S. 2014. Blockade of macrophage autophagy ameliorates activated lymphocytes-derived DNA induced murine lupus possibly via inhibition of proinflammatory cytokine production. *Clin Exp Rheumatol,* 32**,** 705-14.

LI, P., LI, M., LINDBERG, M. R., KENNETT, M. J., XIONG, N. & WANG, Y. 2010a. PAD4 is essential for antibacterial innate immunity mediated by neutrophil extracellular traps. *J Exp Med,* 207**,** 1853-62.

LI, W. X., PAN, H. F., HU, J. L., WANG, C. Z., ZHANG, N., LI, J., LI, X. P., XU, J. H. & YE, D. Q. 2010b. Assay of T- and NK-cell subsets and the expression of NKG2A and NKG2D in patients with new-onset systemic lupus erythematosus. *Clin Rheumatol,* 29**,** 315-23.

LINDSLEY, H. B., SMITH, D. D., COHICK, C. B., KOCH, A. E. & DAVIS, L. S. 1993. Proinflammatory cytokines enhance human synoviocyte expression of functional intercellular adhesion molecule-1 (ICAM-1). *Clin Immunol Immunopathol,* 68**,** 311-20.

LINKER-ISRAELI, M., DEANS, R. J., WALLACE, D. J., PREHN, J., OZERI-CHEN, T. & KLINENBERG, J. R. 1991. Elevated levels of endogenous IL-6 in systemic lupus erythematosus. A putative role in pathogenesis. *J Immunol,* 147**,** 117-23.

LIOSSIS, S. N., KOVACS, B., DENNIS, G., KAMMER, G. M. & TSOKOS, G. C. 1996. B cells from patients with systemic lupus erythematosus display abnormal antigen receptor-mediated early signal transduction events. *J Clin Invest,* 98**,** 2549-57.

LISNEVSKAIA, L., MURPHY, G. & ISENBERG, D. 2014. Systemic lupus erythematosus. *Lancet,* 384**,** 1878-88.

LIU, Y., NUSRAT, A., SCHNELL, F. J., REAVES, T. A., WALSH, S., POCHET, M. & PARKOS, C. A. 2000. Human junction adhesion molecule regulates tight junction resealing in epithelia. *J Cell Sci,* 113 (Pt 13)**,** 2363-74.

LLORENTE, L., RICHAUD-PATIN, Y., FIOR, R., ALCOCER-VARELA, J., WIJDENES, J., FOURRIER, B. M., GALANAUD, P. & EMILIE, D. 1994. In vivo production of interleukin-10 by non-T cells in rheumatoid arthritis, Sjogren's syndrome, and systemic lupus erythematosus. A potential mechanism of B lymphocyte hyperactivity and autoimmunity. *Arthritis Rheum,* 37**,** 1647-55.

LLORENTE, L., RICHAUD-PATIN, Y., WIJDENES, J., ALCOCER-VARELA, J., MAILLOT, M. C., DURAND-GASSELIN, I., FOURRIER, B. M., GALANAUD, P. & EMILIE, D. 1993. Spontaneous production of interleukin-10 by B lymphocytes and monocytes in systemic lupus erythematosus. *Eur Cytokine Netw,* 4**,** 421-7.

LLORENTE, L., ZOU, W., LEVY, Y., RICHAUD-PATIN, Y., WIJDENES, J., ALCOCER-VARELA, J., MOREL-FOURRIER, B., BROUET, J. C., ALARCON-SEGOVIA, D., GALANAUD, P. & EMILIE, D. 1995. Role of interleukin 10 in the B lymphocyte hyperactivity and autoantibody production of human systemic lupus erythematosus. *J Exp Med,* 181**,** 839-44.

LOPEZ-PEDRERA, C., CUADRADO, M. J., HERANDEZ, V., BUENDIA, P., AGUIRRE, M. A., BARBARROJA, N., TORRES, L. A., VILLALBA, J. M., VELASCO, F. & KHAMASHTA, M. 2008. Proteomic analysis in monocytes of antiphospholipid syndrome patients: deregulation of proteins related to the development of thrombosis. *Arthritis Rheum,* 58**,** 2835-44.

LORD, P. C., WILMOTH, L. M., MIZEL, S. B. & MCCALL, C. E. 1991. Expression of interleukin-1 alpha and beta genes by human blood polymorphonuclear leukocytes. *J Clin Invest,* 87**,** 1312-21.

LOU, O., ALCAIDE, P., LUSCINSKAS, F. W. & MULLER, W. A. 2007. CD99 is a key mediator of the transendothelial migration of neutrophils. *J Immunol,* 178**,** 1136-43.

LOVGREN, T., ELORANTA, M. L., KASTNER, B., WAHREN-HERLENIUS, M., ALM, G. V. & RONNBLOM, L. 2006. Induction of interferon-alpha by immune complexes or liposomes containing systemic lupus erythematosus autoantigen- and Sjogren's syndrome autoantigen-associated RNA. *Arthritis Rheum,* 54**,** 1917-27.

LUDWIG, R. J., HARDT, K., HATTING, M., BISTRIAN, R., DIEHL, S., RADEKE, H. H., PODDA, M., SCHON, M. P., KAUFMANN, R., HENSCHLER, R., PFEILSCHIFTER, J. M., SANTOSO, S. & BOEHNCKE, W. H. 2009. Junctional adhesion molecule (JAM)-B supports lymphocyte rolling and adhesion through interaction with alpha4beta1 integrin. *Immunology,* 128**,** 196-205.

LUND-OLESEN, K. 1970. Oxygen tension in synovial fluids. *Arthritis Rheum,* 13**,** 769-76.

LUSCINSKAS, F. W., KANSAS, G. S., DING, H., PIZCUETA, P., SCHLEIFFENBAUM, B. E., TEDDER, T. F. & GIMBRONE, M. A., JR. 1994. Monocyte rolling, arrest and spreading on IL-4-activated vascular endothelium under flow is mediated via sequential action of L-selectin, beta 1-integrins, and beta 2-integrins. *J Cell Biol,* 125**,** 1417-27.

MA, C. Y., JIAO, Y. L., ZHANG, J., YANG, Q. R., ZHANG, Z. F., SHEN, Y. J., CHEN, Z. J. & ZHAO, Y. R. 2012. Elevated plasma level of HMGB1 is associated with disease activity and combined alterations with IFN-alpha and TNF-alpha in systemic lupus erythematosus. *Rheumatol Int,* 32**,** 395-402.

MA, L., LIU, B., JIANG, Z. & JIANG, Y. 2014. Reduced numbers of regulatory B cells are negatively correlated with disease activity in patients with new-onset rheumatoid arthritis. *Clin Rheumatol,* 33**,** 187-95.

MACKWORTH-YOUNG, C., CHAN, J., HARRIS, N., WALPORT, M., BERNSTEIN, R., BATCHELOR, R., HUGHES, G. & GHARAVI, A. 1987. High incidence of anticardiolipin antibodies in relatives of patients with systemic lupus erythematosus. *J Rheumatol,* 14**,** 723-6.

MACPHERSON, M., LEK, H. S., PRESCOTT, A. & FAGERHOLM, S. C. 2011. A systemic lupus erythematosus-associated R77H substitution in the CD11b chain of the Mac-1 integrin compromises leukocyte adhesion and phagocytosis. *J Biol Chem,* 286**,** 17303-10.

MAIGUEL, D., FARIDI, M. H., WEI, C., KUWANO, Y., BALLA, K. M., HERNANDEZ, D., BARTH, C. J., LUGO, G., DONNELLY, M., NAYER, A., MOITA, L. F., SCHURER, S., TRAVER, D., RUIZ, P., VAZQUEZ-PADRON, R. I., LEY, K., REISER, J. & GUPTA, V. 2011. Small molecule-mediated activation of the integrin CD11b/CD18 reduces inflammatory disease. *Sci Signal,* 4**,** ra57.

MAJMUNDAR, A. J., WONG, W. J. & SIMON, M. C. 2010. Hypoxia-inducible factors and the response to hypoxic stress. *Mol Cell,* 40**,** 294-309.

MAJOR, E. O. 2010. Progressive multifocal leukoencephalopathy in patients on immunomodulatory therapies. *Annu Rev Med,* 61**,** 35-47.

MAKINO, Y., CAO, R., SVENSSON, K., BERTILSSON, G., ASMAN, M., TANAKA, H., CAO, Y., BERKENSTAM, A. & POELLINGER, L. 2001. Inhibitory PAS domain protein is a negative regulator of hypoxia-inducible gene expression. *Nature,* 414**,** 550-4.

MAKRYGIANNAKIS, D., HERMANSSON, M., ULFGREN, A. K., NICHOLAS, A. P., ZENDMAN, A. J., EKLUND, A., GRUNEWALD, J., SKOLD, C. M., KLARESKOG, L. & CATRINA, A. I. 2008. Smoking increases peptidylarginine deiminase 2 enzyme expression in human lungs and increases citrullination in BAL cells. *Annals of the rheumatic diseases,* 67**,** 1488-92.

MALAWISTA, S. E. & DE BOISFLEURY CHEVANCE, A. 1982. The cytokineplast: purified, stable, and functional motile machinery from human blood polymorphonuclear leukocytes. *J Cell Biol,* 95**,** 960-73.

MALAWISTA, S. E., SMITH, E. O. & SEIBYL, J. P. 2006. Cryopreservable neutrophil surrogates: granule-poor, motile cytoplasts from polymorphonuclear leukocytes home to inflammatory lesions in vivo. *Cell Motil Cytoskeleton,* 63**,** 254-7.

MALYAK, M., SMITH, M. F., JR., ABEL, A. A. & AREND, W. P. 1994. Peripheral blood neutrophil production of interleukin-1 receptor antagonist and interleukin-1 beta. *J Clin Immunol,* 14**,** 20-30.

MAMDOUH, Z., CHEN, X., PIERINI, L. M., MAXFIELD, F. R. & MULLER, W. A. 2003. Targeted recycling of PECAM from endothelial surface-connected compartments during diapedesis. *Nature,* 421**,** 748-53.

MANDEVILLE, J. T. & MAXFIELD, F. R. 1997. Effects of buffering intracellular free calcium on neutrophil migration through three-dimensional matrices. *J Cell Physiol,* 171**,** 168-78.

MARTIN-PADURA, I., LOSTAGLIO, S., SCHNEEMANN, M., WILLIAMS, L., ROMANO, M., FRUSCELLA, P., PANZERI, C., STOPPACCIARO, A., RUCO, L., VILLA, A., SIMMONS, D. & DEJANA, E. 1998. Junctional adhesion molecule, a novel member of the immunoglobulin superfamily that distributes at intercellular junctions and modulates monocyte transmigration. *J Cell Biol,* 142**,** 117-27.

MARTINEZ, A., VALDIVIA, A., PASCUAL-SALCEDO, D., LAMAS, J. R., FERNANDEZ-ARQUERO, M., BALSA, A., FERNANDEZ-GUTIERREZ, B., DE LA CONCHA, E. G. & URCELAY, E.

2005. PADI4 polymorphisms are not associated with rheumatoid arthritis in the Spanish population. *Rheumatology (Oxford),* 44**,** 1263-6.

MARUCHA, P. T., ZEFF, R. A. & KREUTZER, D. L. 1990. Cytokine regulation of IL-1 beta gene expression in the human polymorphonuclear leukocyte. *J Immunol,* 145**,** 2932-7.

MARUI, N., OFFERMANN, M. K., SWERLICK, R., KUNSCH, C., ROSEN, C. A., AHMAD, M., ALEXANDER, R. W. & MEDFORD, R. M. 1993. Vascular cell adhesion molecule-1 (VCAM-1) gene transcription and expression are regulated through an antioxidant-sensitive mechanism in human vascular endothelial cells. *J Clin Invest,* 92**,** 1866-74.

MASSON, N., WILLAM, C., MAXWELL, P. H., PUGH, C. W. & RATCLIFFE, P. J. 2001. Independent function of two destruction domains in hypoxia-inducible factor-alpha chains activated by prolyl hydroxylation. *EMBO J,* 20**,** 5197-206.

MATSUDA, M., PARK, J. G., WANG, D. C., HUNTER, S., CHIEN, P. & SCHREIBER, A. D. 1996. Abrogation of the Fc gamma receptor IIA-mediated phagocytic signal by stem-loop Syk antisense oligonucleotides. *Mol Biol Cell,* 7**,** 1095-106.

MATSUURA, E., IGARASHI, Y., FUJIMOTO, M., ICHIKAWA, K. & KOIKE, T. 1990. Anticardiolipin cofactor(s) and differential diagnosis of autoimmune disease. *Lancet,* 336**,** 177-8.

MAURUS, C. F., SCHMIDT, D., SCHNEIDER, M. K., TURINA, M. I., SEEBACH, J. D. & ZUND, G. 2003. Hypoxia and reoxygenation do not upregulate adhesion molecules and natural killer cell adhesion on human endothelial cells in vitro. *Eur J Cardiothorac Surg,* 23**,** 976-83; discussion 983.

MAXWELL, P. H., PUGH, C. W. & RATCLIFFE, P. J. 1993. Inducible operation of the erythropoietin 3' enhancer in multiple cell lines: evidence for a widespread oxygen-sensing mechanism. *Proc Natl Acad Sci U S A,* 90**,** 2423-7.

MAYNARD, M. A., EVANS, A. J., HOSOMI, T., HARA, S., JEWETT, M. A. & OHH, M. 2005. Human HIF-3alpha4 is a dominant-negative regulator of HIF-1 and is down-regulated in renal cell carcinoma. *FASEB J,* 19**,** 1396-406.

MAYNARD, M. A., QI, H., CHUNG, J., LEE, E. H., KONDO, Y., HARA, S., CONAWAY, R. C., CONAWAY, J. W. & OHH, M. 2003. Multiple splice variants of the human HIF-3 alpha locus are targets of the von Hippel-Lindau E3 ubiquitin ligase complex. *J Biol Chem,* 278**,** 11032-40.

MCEVER, R. P. & CUMMINGS, R. D. 1997. Perspectives series: cell adhesion in vascular biology. Role of PSGL-1 binding to selectins in leukocyte recruitment. *J Clin Invest,* 100**,** 485-91.

MCGOVERN, N. N., COWBURN, A. S., PORTER, L., WALMSLEY, S. R., SUMMERS, C., THOMPSON, A. A., ANWAR, S., WILLCOCKS, L. C., WHYTE, M. K., CONDLIFFE, A. M. & CHILVERS, E. R. 2011. Hypoxia selectively inhibits respiratory burst activity and killing of Staphylococcus aureus in human neutrophils. *J Immunol,* 186**,** 453-63.

MCINTURFF, A. M., CODY, M. J., ELLIOTT, E. A., GLENN, J. W., ROWLEY, J. W., RONDINA, M. T. & YOST, C. C. 2012. Mammalian target of rapamycin regulates neutrophil extracellular trap formation via induction of hypoxia-inducible factor 1 alpha. *Blood,* 120**,** 3118-25.

MCKENZIE, S. E. & SCHREIBER, A. D. 1998. Fc gamma receptors in phagocytes. *Curr Opin Hematol,* 5**,** 16-21.

MCNEIL, H. P., SIMPSON, R. J., CHESTERMAN, C. N. & KRILIS, S. A. 1990. Anti-phospholipid antibodies are directed against a complex antigen that includes a lipid-binding inhibitor of coagulation: beta 2-glycoprotein I (apolipoprotein H). *Proc Natl Acad Sci U S A,* 87**,** 4120-4.

MCNEILL, L. A., HEWITSON, K. S., CLARIDGE, T. D., SEIBEL, J. F., HORSFALL, L. E. & SCHOFIELD, C. J. 2002. Hypoxia-inducible factor asparaginyl hydroxylase (FIH-1) catalyses hydroxylation at the beta-carbon of asparagine-803. *Biochem J,* 367**,** 571-5.

MELANI, C., MATTIA, G. F., SILVANI, A., CARE, A., RIVOLTINI, L., PARMIANI, G. & COLOMBO, M. P. 1993. Interleukin-6 expression in human neutrophil and eosinophil peripheral blood granulocytes. *Blood,* 81**,** 2744-9.

MENON, M., BLAIR, P. A., ISENBERG, D. A. & MAURI, C. 2016. A Regulatory Feedback between Plasmacytoid Dendritic Cells and Regulatory B Cells Is Aberrant in Systemic Lupus Erythematosus. *Immunity*.

MERONI, P. L., BORGHI, M. O., RASCHI, E., VENTURA, D., SARZI PUTTINI, P. C., ATZENI, F., LONATI, L., PARATI, G., TINCANI, A., MARI, D. & TEDESCO, F. 2004. Inflammatory response and the endothelium. *Thromb Res,* 114**,** 329-34.

MERONI, P. L. & RIBOLDI, P. 2001. Pathogenic mechanisms mediating antiphospholipid syndrome. *Curr Opin Rheumatol,* 13**,** 377-82.

METZLER, K. D., FUCHS, T. A., NAUSEEF, W. M., REUMAUX, D., ROESLER, J., SCHULZE, I., WAHN, V., PAPAYANNOPOULOS, V. & ZYCHLINSKY, A. 2011. Myeloperoxidase is required for neutrophil extracellular trap formation: implications for innate immunity. *Blood,* 117**,** 953-9.

MIAO, J., GENG, J., ZHANG, K., LI, X., LI, Q., LI, C. & ZHU, P. 2014. Frequencies of circulating IL-17-producing CD4+CD161+ T cells and CD4+CD161+ T cells correlate with disease activity in rheumatoid arthritis. *Mod Rheumatol,* 24**,** 265-570.

MIGITA, K., MIYASHITA, T., MAEDA, Y., KIMURA, H., NAKAMURA, M., YATSUHASHI, H., ISHIBASHI, H. & EGUCHI, K. 2005. Reduced blood BDCA-2+ (lymphoid) and CD11c+ (myeloid) dendritic cells in systemic lupus erythematosus. *Clin Exp Immunol,* 142**,** 84-91.

MIN, J. K., KIM, Y. M., KIM, S. W., KWON, M. C., KONG, Y. Y., HWANG, I. K., WON, M. H., RHO, J. & KWON, Y. G. 2005. TNF-related activation-induced cytokine enhances leukocyte adhesiveness: induction of ICAM-1 and VCAM-1 via TNF receptor-associated factor and protein kinase C-dependent NF-kappaB activation in endothelial cells. *J Immunol,* 175**,** 531-40.

MINE, S., TABATA, T., WADA, Y., FUJISAKI, T., IIDA, T., NOGUCHI, N., NIKI, E., KODAMA, T. & TANAKA, Y. 2002. Oxidized low density lipoprotein-induced LFA-1-dependent adhesion and transendothelial migration of monocytes via the protein kinase C pathway. *Atherosclerosis,* 160**,** 281-8.

MIOSSEC, P., KORN, T. & KUCHROO, V. K. 2009. Interleukin-17 and type 17 helper T cells. *N Engl J Med,* 361**,** 888-98.

MITROULIS, I., KAMBAS, K., CHRYSANTHOPOULOU, A., SKENDROS, P., APOSTOLIDOU, E., KOURTZELIS, I., DROSOS, G. I., BOUMPAS, D. T. & RITIS, K. 2011. Neutrophil extracellular trap formation is associated with IL-1beta and autophagy-related signaling in gout. *PLoS One,* 6**,** e29318.

MITROULIS, I., KOURTZELIS, I., KAMBAS, K., RAFAIL, S., CHRYSANTHOPOULOU, A., SPELETAS, M. & RITIS, K. 2010. Regulation of the autophagic machinery in human neutrophils. *Eur J Immunol,* 40**,** 1461-72.

MIYAKE, S., YAGITA, H., MARUYAMA, T., HASHIMOTO, H., MIYASAKA, N. & OKUMURA, K. 1993. Beta 1 integrin-mediated interaction with extracellular matrix proteins regulates cytokine gene expression in synovial fluid cells of rheumatoid arthritis patients. *J Exp Med,* 177**,** 863-8.

MIYAKIS, S., LOCKSHIN, M. D., ATSUMI, T., BRANCH, D. W., BREY, R. L., CERVERA, R., DERKSEN, R. H., PG, D. E. G., KOIKE, T., MERONI, P. L., REBER, G., SHOENFELD, Y., TINCANI, A., VLACHOYIANNOPOULOS, P. G. & KRILIS, S. A. 2006. International consensus statement on an update of the classification criteria for definite antiphospholipid syndrome (APS). *J Thromb Haemost,* 4**,** 295-306.

MIYARA, M., AMOURA, Z., PARIZOT, C., BADOUAL, C., DORGHAM, K., TRAD, S., NOCHY, D., DEBRE, P., PIETTE, J. C. & GOROCHOV, G. 2005. Global natural regulatory T cell depletion in active systemic lupus erythematosus. *J Immunol,* 175**,** 8392-400.

MO, L. & SALMON, J. E. 2001. Intercellular adhesion molecule 1 expression is required for antiphospholipid antibody-induced pregnancy loss. *Arthritis Rheum,* 44**,** 1225-6.

MOHAN, C. & PUTTERMAN, C. 2015. Genetics and pathogenesis of systemic lupus erythematosus and lupus nephritis. *Nat Rev Nephrol,* 11**,** 329-41.

MOHANTY, T., SJOGREN, J., KAHN, F., ABU-HUMAIDAN, A. H., FISKER, N., ASSING, K., MORGELIN, M., BENGTSSON, A. A., BORREGAARD, N. & SORENSEN, O. E. 2015. A novel mechanism for NETosis provides antimicrobial defense at the oral mucosa. *Blood,* 126**,** 2128-37.

MOISAN, J., GRENNINGLOH, R., BETTELLI, E., OUKKA, M. & HO, I. C. 2007. Ets-1 is a negative regulator of Th17 differentiation. *J Exp Med,* 204**,** 2825-35.

MOK, C. C. & LAU, C. S. 2000. Profile of sex hormones in male patients with systemic lupus erythematosus. *Lupus,* 9**,** 252-7.

MOLE, D. R., BLANCHER, C., COPLEY, R. R., POLLARD, P. J., GLEADLE, J. M., RAGOUSSIS, J. & RATCLIFFE, P. J. 2009. Genome-wide association of hypoxia-inducible factor (HIF)-1alpha and HIF-2alpha DNA binding with expression profiling of hypoxia-inducible transcripts. *J Biol Chem,* 284**,** 16767-75.

MOLLINEDO, F., NAKAJIMA, M., LLORENS, A., BARBOSA, E., CALLEJO, S., GAJATE, C. & FABRA, A. 1997. Major co-localization of the extracellular-matrix degradative enzymes heparanase and gelatinase in tertiary granules of human neutrophils. *Biochem J,* 327 (Pt 3)**,** 917-23.

MOLLINEDO, F., PULIDO, R., LACAL, P. M. & SANCHEZ-MADRID, F. 1991. Mobilization of gelatinase-rich granules as a regulatory mechanism of early functional responses in human neutrophils. *Scand J Immunol,* 34**,** 33-43.

MONNEAUX, F. & MULLER, S. 2002. Epitope spreading in systemic lupus erythematosus: identification of triggering peptide sequences. *Arthritis Rheum,* 46**,** 1430-8.

MONTIEL-MANZANO, G., ROMAY-PENABAD, Z., PAPALARDO DE MARTINEZ, E., MEILLON-GARCIA, L. A., GARCIA-LATORRE, E., REYES-MALDONADO, E. & PIERANGELI, S. S. 2007. In vivo effects of an inhibitor of nuclear factor-kappa B on thrombogenic properties of antiphospholipid antibodies. *Ann N Y Acad Sci,* 1108**,** 540-53.

MORET, F. M., HACK, C. E., VAN DER WURFF-JACOBS, K. M., DE JAGER, W., RADSTAKE, T. R., LAFEBER, F. P. & VAN ROON, J. A. 2013. Intra-articular CD1c-expressing myeloid dendritic cells from rheumatoid arthritis patients express a unique set of T cell-attracting chemokines and spontaneously induce Th1, Th17 and Th2 cell activity. *Arthritis Res Ther,* 15**,** R155.

MORI, S., CHO, I., KOGA, Y. & SUGIMOTO, M. 2008. Comparison of pulmonary abnormalities on high-resolution computed tomography in patients with early versus longstanding rheumatoid arthritis. *J Rheumatol,* 35**,** 1513-21.

MOULTON, V. R. & TSOKOS, G. C. 2011. Abnormalities of T cell signaling in systemic lupus erythematosus. *Arthritis Res Ther,* 13**,** 207.

MUKAI, K., MATSUOKA, K., TAYA, C., SUZUKI, H., YOKOZEKI, H., NISHIOKA, K., HIROKAWA, K., ETORI, M., YAMASHITA, M., KUBOTA, T., MINEGISHI, Y., YONEKAWA, H. & KARASUYAMA, H. 2005. Basophils play a critical role in the development of IgE-mediated chronic allergic inflammation independently of T cells and mast cells. *Immunity,* 23**,** 191-202.

MULLA, M. J., BROSENS, J. J., CHAMLEY, L. W., GILES, I., PERICLEOUS, C., RAHMAN, A., JOYCE, S. K., PANDA, B., PAIDAS, M. J. & ABRAHAMS, V. M. 2009. Antiphospholipid antibodies induce a pro-inflammatory response in first trimester trophoblast via the TLR4/MyD88 pathway. *Am J Reprod Immunol,* 62**,** 96-111.

MULLER, W. A., WEIGL, S. A., DENG, X. & PHILLIPS, D. M. 1993. PECAM-1 is required for transendothelial migration of leukocytes. *J Exp Med,* 178**,** 449-60.

MYONES, B. L., DALZELL, J. G., HOGG, N. & ROSS, G. D. 1988. Neutrophil and monocyte cell surface p150,95 has iC3b-receptor (CR4) activity resembling CR3. *J Clin Invest,* 82**,** 640-51.

NAKASHIMA, K., HAGIWARA, T. & YAMADA, M. 2002. Nuclear localization of peptidylarginine deiminase V and histone deimination in granulocytes. *J Biol Chem,* 277**,** 49562-8.

NAKAYAMADA, S., SAITO, K., FUJII, K., YASUDA, M., TAMURA, M. & TANAKA, Y. 2003. beta1 integrin-mediated signaling induces intercellular adhesion molecule 1 and Fas on rheumatoid synovial cells and Fas-mediated apoptosis. *Arthritis Rheum,* 48**,** 1239-48.

NAKAYAMADA, S., SAITO, K., NAKANO, K. & TANAKA, Y. 2007. Activation signal transduction by beta1 integrin in T cells from patients with systemic lupus erythematosus. *Arthritis Rheum,* 56**,** 1559-68.

NAKOU, M., KNOWLTON, N., FRANK, M. B., BERTSIAS, G., OSBAN, J., SANDEL, C. E., PAPADAKI, H., RAPTOPOULOU, A., SIDIROPOULOS, P., KRITIKOS, I., TASSIULAS, I., CENTOLA, M. & BOUMPAS, D. T. 2008. Gene expression in systemic lupus erythematosus: bone marrow analysis differentiates active from inactive disease and reveals apoptosis and granulopoiesis signatures. *Arthritis Rheum,* 58**,** 3541-9.

NATH, S. K., HAN, S., KIM-HOWARD, X., KELLY, J. A., VISWANATHAN, P., GILKESON, G. S., CHEN, W., ZHU, C., MCEVER, R. P., KIMBERLY, R. P., ALARCON-RIQUELME, M. E., VYSE, T. J., LI, Q. Z., WAKELAND, E. K., MERRILL, J. T., JAMES, J. A., KAUFMAN, K. M., GUTHRIDGE, J. M. & HARLEY, J. B. 2008. A nonsynonymous functional variant in integrin-alpha(M) (encoded by ITGAM) is associated with systemic lupus erythematosus. *Nat Genet,* 40**,** 152-4.

NAZIRUDDIN, B., DUFFY, B. F., TUCKER, J. & MOHANAKUMAR, T. 1992. Evidence for cross-regulation of Fc gamma RIIIB (CD16) receptor-mediated signaling by Fc gamma RII (CD32) expressed on polymorphonuclear neutrophils. *J Immunol,* 149**,** 3702-9.

NEELI, I., DWIVEDI, N., KHAN, S. & RADIC, M. 2009. Regulation of extracellular chromatin release from neutrophils. *J Innate Immun,* 1**,** 194-201.

NERMUT, M. V., GREEN, N. M., EASON, P., YAMADA, S. S. & YAMADA, K. M. 1988. Electron microscopy and structural model of human fibronectin receptor. *EMBO J,* 7**,** 4093-9.

NEWBY, L. K., MARBER, M. S., MELLONI, C., SAROV-BLAT, L., ABERLE, L. H., AYLWARD, P. E., CAI, G., DE WINTER, R. J., HAMM, C. W., HEITNER, J. F., KIM, R., LERMAN, A., PATEL, M. R., TANGUAY, J. F., LEPORE, J. J., AL-KHALIDI, H. R., SPRECHER, D. L. & GRANGER, C. B. 2014. Losmapimod, a novel p38 mitogen-activated protein kinase inhibitor, in non-ST-segment elevation myocardial infarction: a randomised phase 2 trial. *Lancet,* 384**,** 1187-95.

NICOLA, P. J., CROWSON, C. S., MARADIT-KREMERS, H., BALLMAN, K. V., ROGER, V. L., JACOBSEN, S. J. & GABRIEL, S. E. 2006. Contribution of congestive heart failure and ischemic heart disease to excess mortality in rheumatoid arthritis. *Arthritis Rheum,* 54**,** 60-7.

NIE, Y. J., MOK, M. Y., CHAN, G. C., CHAN, A. W., JIN, O. U., KAVIKONDALA, S., LIE, A. K. & LAU, C. S. 2010. Phenotypic and functional abnormalities of bone marrow-derived dendritic cells in systemic lupus erythematosus. *Arthritis Res Ther,* 12**,** R91.

NING, L., TIAN, L., SMIRNOV, S., VIHINEN, H., LLANO, O., VICK, K., DAVIS, R. L., RIVERA, C. & GAHMBERG, C. G. 2013. Interactions between ICAM-5 and beta1 integrins regulate neuronal synapse formation. *J Cell Sci,* 126**,** 77-89.

NISHIDA, N., XIE, C., SHIMAOKA, M., CHENG, Y., WALZ, T. & SPRINGER, T. A. 2006. Activation of leukocyte beta2 integrins by conversion from bent to extended conformations. *Immunity,* 25**,** 583-94.

NISHIKAWA, M., MYOUI, A., TOMITA, T., TAKAHI, K., NAMPEI, A. & YOSHIKAWA, H. 2003. Prevention of the onset and progression of collagen-induced arthritis in rats by the potent p38 mitogen-activated protein kinase inhibitor FR167653. *Arthritis Rheum,* 48**,** 2670-81.

NISHINO, H., SHIBUYA, K., NISHIDA, Y. & MUSHIMOTO, M. 1981. Lupus erythematosus-like syndrome with selective complete deficiency of C1q. *Ann Intern Med,* 95**,** 322-4.

NOCKHER, W. A., WIGAND, R., SCHOEPPE, W. & SCHERBERICH, J. E. 1994. Elevated levels of soluble CD14 in serum of patients with systemic lupus erythematosus. *Clin Exp Immunol,* 96**,** 15-9.

NOJIMA, J., MASUDA, Y., IWATANI, Y., SUEHISA, E., FUTSUKAICHI, Y., KURATSUNE, H., WATANABE, Y., TAKANO, T., HIDAKA, Y. & KANAKURA, Y. 2008. Tissue factor expression on monocytes induced by anti-phospholipid antibodies as a strong risk factor for thromboembolic complications in SLE patients. *Biochem Biophys Res Commun,* 365**,** 195-200.

NORDENFELT, P. & TAPPER, H. 2011. Phagosome dynamics during phagocytosis by neutrophils. *J Leukoc Biol,* 90**,** 271-84.

NURCOMBE, H. L., BUCKNALL, R. C. & EDWARDS, S. W. 1991a. Activation of the neutrophil myeloperoxidase-H2O2 system by synovial fluid isolated from patients with rheumatoid arthritis. *Ann Rheum Dis,* 50**,** 237-42.

NURCOMBE, H. L., BUCKNALL, R. C. & EDWARDS, S. W. 1991b. Neutrophils isolated from the synovial fluid of patients with rheumatoid arthritis: priming and activation in vivo. *Ann Rheum Dis,* 50**,** 147-53.

NUTTALL, A. & ISENBERG, D. A. 2013. Assessment of disease activity, damage and quality of life in systemic lupus erythematosus: new aspects. *Best Pract Res Clin Rheumatol,* 27**,** 309-18.

O'FLAHERTY, J. T., JACOBSON, D. P., REDMAN, J. F. & ROSSI, A. G. 1990. Translocation of protein kinase C in human polymorphonuclear neutrophils. Regulation by cytosolic Ca2(+)-independent and Ca2(+)-dependent mechanisms. *J Biol Chem,* 265**,** 9146-52.

O'SHEA, J. J., BROWN, E. J., SELIGMANN, B. E., METCALF, J. A., FRANK, M. M. & GALLIN, J. I. 1985. Evidence for distinct intracellular pools of receptors for C3b and C3bi in human neutrophils. *J Immunol,* 134**,** 2580-7.

OBATA, K., MUKAI, K., TSUJIMURA, Y., ISHIWATA, K., KAWANO, Y., MINEGISHI, Y., WATANABE, N. & KARASUYAMA, H. 2007. Basophils are essential initiators of a novel type of chronic allergic inflammation. *Blood,* 110**,** 913-20.

OBERGFELL, A., ETO, K., MOCSAI, A., BUENSUCESO, C., MOORES, S. L., BRUGGE, J. S., LOWELL, C. A. & SHATTIL, S. J. 2002. Coordinate interactions of Csk, Src, and Syk kinases with [alpha]IIb[beta]3 initiate integrin signaling to the cytoskeleton. *J Cell Biol,* 157**,** 265-75.

OBERMOSER, G. & PASCUAL, V. 2010. The interferon-alpha signature of systemic lupus erythematosus. *Lupus,* 19**,** 1012-9.

OFOSU-APPIAH, W., WARRINGTON, R. J., MORGAN, K. & WILKINS, J. A. 1989a. Lymphocyte extracellular matrix interactions. Induction of interferon by connective tissue components. *Scand J Immunol,* 29**,** 517-25.

OFOSU-APPIAH, W. A., WARRINGTON, R. J. & WILKINS, J. A. 1989b. Interleukin 2 responsive T cell clones from rheumatoid and normal subjects: proliferative responses to connective tissue elements. *Clin Immunol Immunopathol,* 50**,** 264-71.

OGSTON, A. G. & STANIER, J. E. 1953. The physiological function of hyaluronic acid in synovial fluid; viscous, elastic and lubricant properties. *J Physiol,* 119**,** 244-52.

OLSON, A. L., SWIGRIS, J. J., SPRUNGER, D. B., FISCHER, A., FERNANDEZ-PEREZ, E. R., SOLOMON, J., MURPHY, J., COHEN, M., RAGHU, G. & BROWN, K. K. 2011. Rheumatoid arthritis-interstitial lung disease-associated mortality. *American journal of respiratory and critical care medicine,* 183**,** 372-8.

ONG, C. K., LIRK, P., TAN, C. H. & SEYMOUR, R. A. 2007. An evidence-based update on nonsteroidal anti-inflammatory drugs. *Clin Med Res,* 5**,** 19-34.

OSTERMANN, G., WEBER, K. S., ZERNECKE, A., SCHRODER, A. & WEBER, C. 2002. JAM-1 is a ligand of the beta(2) integrin LFA-1 involved in transendothelial migration of leukocytes. *Nat Immunol,* 3**,** 151-8.

PALLUY, O., MORLIERE, L., GRIS, J. C., BONNE, C. & MODAT, G. 1992. Hypoxia/reoxygenation stimulates endothelium to promote neutrophil adhesion. *Free Radic Biol Med,* 13**,** 21-30.

PALMA, C., CASSONE, A., SERBOUSEK, D., PEARSON, C. A. & DJEU, J. Y. 1992. Lactoferrin release and interleukin-1, interleukin-6, and tumor necrosis factor production by

human polymorphonuclear cells stimulated by various lipopolysaccharides: relationship to growth inhibition of Candida albicans. *Infect Immun,* 60**,** 4604-11.

PANAYI, G. S. 2006. Even though T-cell-directed trials have been of limited success, is there reason for optimism? *Nat Clin Pract Rheumatol,* 2**,** 58-9.

PANAYI, G. S., LANCHBURY, J. S. & KINGSLEY, G. H. 1992. The importance of the T cell in initiating and maintaining the chronic synovitis of rheumatoid arthritis. *Arthritis Rheum,* 35**,** 729-35.

PANG, M., ABE, T., FUJIHARA, T., MORI, S., TSUZAKA, K., AMANO, K., KOIDE, J. & TAKEUCHI, T. 1998. Up-regulation of alphaEbeta7, a novel integrin adhesion molecule, on T cells from systemic lupus erythematosus patients with specific epithelial involvement. *Arthritis Rheum,* 41**,** 1456-63.

PAPAYANNOPOULOS, V., METZLER, K. D., HAKKIM, A. & ZYCHLINSKY, A. 2010. Neutrophil elastase and myeloperoxidase regulate the formation of neutrophil extracellular traps. *J Cell Biol,* 191**,** 677-91.

PARDOS-GEA, J., CASTRO-MARRERO, J., CORTES-HERNANDEZ, J., BALADA, E., PEDROSA, A., VILARDELL-TARRES, M. & ORDI-ROS, J. 2012. Beta2-glycoprotein I gene polymorphisms Val247Leu and Trp316Ser in Spanish patients with primary antiphospholipid syndrome. *Rheumatol Int,* 32**,** 927-32.

PARK, Y. W., KEE, S. J., CHO, Y. N., LEE, E. H., LEE, H. Y., KIM, E. M., SHIN, M. H., PARK, J. J., KIM, T. J., LEE, S. S., YOO, D. H. & KANG, H. S. 2009. Impaired differentiation and cytotoxicity of natural killer cells in systemic lupus erythematosus. *Arthritis Rheum,* 60**,** 1753-63.

PARKER, C. M., CEPEK, K. L., RUSSELL, G. J., SHAW, S. K., POSNETT, D. N., SCHWARTING, R. & BRENNER, M. B. 1992. A family of beta 7 integrins on human mucosal lymphocytes. *Proc Natl Acad Sci U S A,* 89**,** 1924-8.

PARKES, H. G., GROOTVELD, M. C., HENDERSON, E. B., FARRELL, A. & BLAKE, D. R. 1991. Oxidative damage to synovial fluid from the inflamed rheumatoid joint detected by 1H NMR spectroscopy. *J Pharm Biomed Anal,* 9**,** 75-82.

PASANEN, A., HEIKKILA, M., RAUTAVUOMA, K., HIRSILA, M., KIVIRIKKO, K. I. & MYLLYHARJU, J. 2010. Hypoxia-inducible factor (HIF)-3alpha is subject to extensive alternative splicing in human tissues and cancer cells and is regulated by HIF-1 but not HIF-2. *Int J Biochem Cell Biol,* 42**,** 1189-200.

PATEL, S., KUMAR, S., JYOTI, A., SRINAG, B. S., KESHARI, R. S., SALUJA, R., VERMA, A., MITRA, K., BARTHWAL, M. K., KRISHNAMURTHY, H., BAJPAI, V. K. & DIKSHIT, M. 2010. Nitric oxide donors release extracellular traps from human neutrophils by augmenting free radical generation. *Nitric Oxide,* 22**,** 226-34.

PEEN, E., ALMER, S., BODEMAR, G., RYDEN, B. O., SJOLIN, C., TEJLE, K. & SKOGH, T. 1993. Anti-lactoferrin antibodies and other types of ANCA in ulcerative colitis, primary sclerosing cholangitis, and Crohn's disease. *Gut,* 34**,** 56-62.

PEREZ-SANCHEZ, C., RUIZ-LIMON, P., AGUIRRE, M. A., BERTOLACCINI, M. L., KHAMASHTA, M. A., RODRIGUEZ-ARIZA, A., SEGUI, P., COLLANTES-ESTEVEZ, E., BARBARROJA, N., KHRAIWESH, H., GONZALEZ-REYES, J. A., VILLALBA, J. M., VELASCO, F., CUADRADO, M. J. & LOPEZ-PEDRERA, C. 2012. Mitochondrial dysfunction in antiphospholipid syndrome: implications in the pathogenesis of the disease and effects of coenzyme Q(10) treatment. *Blood,* 119**,** 5859-5870.

PERICLEOUS, C., TAYLOR, V., BOURKE, L., STUCKEY, D., WINGROVE, J., LYTHGOE, M., PIERANGELI, S. S., RAHMAN, A., GILES, I. & IOANNOU, Y. 2014. IgG Antiphospholipid Antibodies Enhance Stroke Damage: An in Vivo Ischemia/Reperfusion Study. *Arthritis & Rheumatology,* 66**,** S1251-S1251.

PETERS, J. H., SPORN, L. A., GINSBERG, M. H. & WAGNER, D. D. 1990. Human endothelial cells synthesize, process, and secrete fibronectin molecules bearing an alternatively spliced type III homology (ED1). *Blood,* 75**,** 1801-8.

PETERS, M. A., WENDHOLT, D., STRIETHOLT, S., FRANK, S., PUNDT, N., KORB-PAP, A., JOOSTEN, L. A., VAN DEN BERG, W. B., KOLLIAS, G., ECKES, B. & PAP, T. 2012. The

loss of alpha2beta1 integrin suppresses joint inflammation and cartilage destruction in mouse models of rheumatoid arthritis. *Arthritis Rheum,* 64**,** 1359-68.

PETREQUIN, P. R., TODD, R. F., 3RD, DEVALL, L. J., BOXER, L. A. & CURNUTTE, J. T., 3RD 1987. Association between gelatinase release and increased plasma membrane expression of the Mo1 glycoprotein. *Blood,* 69**,** 605-10.

PIALI, L., ALBELDA, S. M., BALDWIN, H. S., HAMMEL, P., GISLER, R. H. & IMHOF, B. A. 1993. Murine platelet endothelial cell adhesion molecule (PECAM-1)/CD31 modulates beta 2 integrins on lymphokine-activated killer cells. *Eur J Immunol,* 23**,** 2464-71.

PIERANGELI, S. S., CHEN, P. P., RASCHI, E., SCURATI, S., GROSSI, C., BORGHI, M. O., PALOMO, I., HARRIS, E. N. & MERONI, P. L. 2008. Antiphospholipid antibodies and the antiphospholipid syndrome: pathogenic mechanisms. *Semin Thromb Hemost,* 34**,** 236-50.

PIERANGELI, S. S., COLDEN-STANFIELD, M., LIU, X., BARKER, J. H., ANDERSON, G. L. & HARRIS, E. N. 1999. Antiphospholipid antibodies from antiphospholipid syndrome patients activate endothelial cells in vitro and in vivo. *Circulation,* 99**,** 1997-2002.

PIERANGELI, S. S., ESPINOLA, R. G., LIU, X. & HARRIS, E. N. 2001. Thrombogenic effects of antiphospholipid antibodies are mediated by intercellular cell adhesion molecule-1, vascular cell adhesion molecule-1, and P-selectin. *Circ Res,* 88**,** 245-50.

PIERANGELI, S. S., GIRARDI, G., VEGA-OSTERTAG, M., LIU, X., ESPINOLA, R. G. & SALMON, J. 2005. Requirement of activation of complement C3 and C5 for antiphospholipid antibody-mediated thrombophilia. *Arthritis Rheum,* 52**,** 2120-4.

PIERANGELI, S. S., LIU, X., ESPINOLA, R., OLEE, T., ZHU, M., HARRIS, N. E. & CHEN, P. P. 2000. Functional analyses of patient-derived IgG monoclonal anticardiolipin antibodies using in vivo thrombosis and in vivo microcirculation models. *Thromb Haemost,* 84**,** 388-95.

PIERANGELI, S. S., VEGA-OSTERTAG, M. E., RASCHI, E., LIU, X., ROMAY-PENABAD, Z., DE MICHELI, V., GALLI, M., MOIA, M., TINCANI, A., BORGHI, M. O., NGUYEN-OGHALAI, T. & MERONI, P. L. 2007. Toll-like receptor and antiphospholipid mediated thrombosis: in vivo studies. *Ann Rheum Dis,* 66**,** 1327-33.

PILCHER, B. K., DUMIN, J. A., SUDBECK, B. D., KRANE, S. M., WELGUS, H. G. & PARKS, W. C. 1997. The activity of collagenase-1 is required for keratinocyte migration on a type I collagen matrix. *J Cell Biol,* 137**,** 1445-57.

PILSCZEK, F. H., SALINA, D., POON, K. K., FAHEY, C., YIPP, B. G., SIBLEY, C. D., ROBBINS, S. M., GREEN, F. H., SURETTE, M. G., SUGAI, M., BOWDEN, M. G., HUSSAIN, M., ZHANG, K. & KUBES, P. 2010. A novel mechanism of rapid nuclear neutrophil extracellular trap formation in response to Staphylococcus aureus. *J Immunol,* 185**,** 7413-25.

PIPER, M. K., RAZA, K., NUTTALL, S. L., STEVENS, R., TOESCU, V., HEATON, S., GARDNER-MEDWIN, J., HILLER, L., MARTIN, U., TOWNEND, J., BACON, P. A. & GORDON, C. 2007. Impaired endothelial function in systemic lupus erythematosus. *Lupus,* 16**,** 84-8.

PITANGA, T. N., DE ARAGAO FRANCA, L., ROCHA, V. C., MEIRELLES, T., BORGES, V. M., GONCALVES, M. S., PONTES-DE-CARVALHO, L. C., NORONHA-DUTRA, A. A. & DOS-SANTOS, W. L. 2014. Neutrophil-derived microparticles induce myeloperoxidase-mediated damage of vascular endothelial cells. *BMC Cell Biol,* 15**,** 21.

PLENGE, R. M., PADYUKOV, L., REMMERS, E. F., PURCELL, S., LEE, A. T., KARLSON, E. W., WOLFE, F., KASTNER, D. L., ALFREDSSON, L., ALTSHULER, D., GREGERSEN, P. K., KLARESKOG, L. & RIOUX, J. D. 2005. Replication of putative candidate-gene associations with rheumatoid arthritis in >4,000 samples from North America and Sweden: association of susceptibility with PTPN22, CTLA4, and PADI4. *Am J Hum Genet,* 77**,** 1044-60.

PORTER, J. C. & HALL, A. 2009. Epithelial ICAM-1 and ICAM-2 regulate the egression of human T cells across the bronchial epithelium. *FASEB J,* 23**,** 492-502.

POUBELLE, P. E., CHAKRAVARTI, A., FERNANDES, M. J., DOIRON, K. & MARCEAU, A. A. 2007. Differential expression of RANK, RANK-L, and osteoprotegerin by synovial

fluid neutrophils from patients with rheumatoid arthritis and by healthy human blood neutrophils. *Arthritis Res Ther,* 9**,** R25.

POULTON, K., RIPOLL, V. M., PERICLEOUS, C., MERONI, P. L., GEROSA, M., IOANNOU, Y., RAHMAN, A. & GILES, I. P. 2015. Purified IgG from Patients with Obstetric but not IgG from Non-obstetric Antiphospholipid Syndrome Inhibit Trophoblast Invasion. *Am J Reprod Immunol,* 73**,** 390-401.

PRATESI, F., DIONI, I., TOMMASI, C., ALCARO, M. C., PAOLINI, I., BARBETTI, F., BOSCARO, F., PANZA, F., PUXEDDU, I., ROVERO, P. & MIGLIORINI, P. 2013. Antibodies from patients with rheumatoid arthritis target citrullinated histone 4 contained in neutrophils extracellular traps. *Ann Rheum Dis*.

PRUYNE, D., EVANGELISTA, M., YANG, C., BI, E., ZIGMOND, S., BRETSCHER, A. & BOONE, C. 2002. Role of formins in actin assembly: nucleation and barbed-end association. *Science,* 297**,** 612-5.

PUXEDDU, I., BONGIORNI, F., CHIMENTI, D., BOMBARDIERI, S., MORETTA, A., BOTTINO, C. & MIGLIORINI, P. 2012. Cell surface expression of activating receptors and co-receptors on peripheral blood NK cells in systemic autoimmune diseases. *Scand J Rheumatol,* 41**,** 298-304.

QUANDT, D., ROTHE, K., SCHOLZ, R., BAERWALD, C. W. & WAGNER, U. 2014. Peripheral CD4CD8 double positive T cells with a distinct helper cytokine profile are increased in rheumatoid arthritis. *PLoS One,* 9**,** e93293.

QUAYLE, J. A., ADAMS, S., BUCKNALL, R. C. & EDWARDS, S. W. 1995. Interleukin-1 expression by neutrophils in rheumatoid arthritis. *Ann Rheum Dis,* 54**,** 930-3.

RAFTERY, M. J., LALWANI, P., KRAUTKRMER, E., PETERS, T., SCHARFFETTER-KOCHANEK, K., KRUGER, R., HOFMANN, J., SEEGER, K., KRUGER, D. H. & SCHONRICH, G. 2014. beta2 integrin mediates hantavirus-induced release of neutrophil extracellular traps. *J Exp Med,* 211**,** 1485-97.

RAHMAN, A. & ISENBERG, D. A. 2008. Systemic lupus erythematosus. *N Engl J Med,* 358**,** 929-39.

RAINGER, G. E., FISHER, A., SHEARMAN, C. & NASH, G. B. 1995. Adhesion of flowing neutrophils to cultured endothelial cells after hypoxia and reoxygenation in vitro. *Am J Physiol,* 269**,** H1398-406.

RAMESH, S., MORRELL, C. N., TARANGO, C., THOMAS, G. D., YUHANNA, I. S., GIRARDI, G., HERZ, J., URBANUS, R. T., DE GROOT, P. G., THORPE, P. E., SALMON, J. E., SHAUL, P. W. & MINEO, C. 2011. Antiphospholipid antibodies promote leukocyte-endothelial cell adhesion and thrombosis in mice by antagonizing eNOS via beta2GPI and apoER2. *J Clin Invest,* 121**,** 120-31.

RAMOS, P. S., BROWN, E. E., KIMBERLY, R. P. & LANGEFELD, C. D. 2010. Genetic factors predisposing to systemic lupus erythematosus and lupus nephritis. *Semin Nephrol,* 30**,** 164-76.

RAND, J. H., WU, X. X., ANDREE, H. A., LOCKWOOD, C. J., GULLER, S., SCHER, J. & HARPEL, P. C. 1997. Pregnancy loss in the antiphospholipid-antibody syndrome--a possible thrombogenic mechanism. *N Engl J Med,* 337**,** 154-60.

RAND, J. H., WU, X. X., GULLER, S., GIL, J., GUHA, A., SCHER, J. & LOCKWOOD, C. J. 1994. Reduction of annexin-V (placental anticoagulant protein-I) on placental villi of women with antiphospholipid antibodies and recurrent spontaneous abortion. *Am J Obstet Gynecol,* 171**,** 1566-72.

RANDI, A. M. & HOGG, N. 1994. I domain of beta 2 integrin lymphocyte function-associated antigen-1 contains a binding site for ligand intercellular adhesion molecule-1. *J Biol Chem,* 269**,** 12395-8.

RAPETTI, L., CHAVELE, K. M., EVANS, C. M. & EHRENSTEIN, M. R. 2015. B cell resistance to Fas-mediated apoptosis contributes to their ineffective control by regulatory T cells in rheumatoid arthritis. *Ann Rheum Dis,* 74**,** 294-302.

RASCHI, E., TESTONI, C., BOSISIO, D., BORGHI, M. O., KOIKE, T., MANTOVANI, A. & MERONI, P. L. 2003. Role of the MyD88 transduction signaling pathway in endothelial activation by antiphospholipid antibodies. *Blood,* 101**,** 3495-500.

RAVIKUMAR, B., SARKAR, S., DAVIES, J. E., FUTTER, M., GARCIA-ARENCIBIA, M., GREEN-THOMPSON, Z. W., JIMENEZ-SANCHEZ, M., KOROLCHUK, V. I., LICHTENBERG, M., LUO, S., MASSEY, D. C., MENZIES, F. M., MOREAU, K., NARAYANAN, U., RENNA, M., SIDDIQI, F. H., UNDERWOOD, B. R., WINSLOW, A. R. & RUBINSZTEIN, D. C. 2010. Regulation of mammalian autophagy in physiology and pathophysiology. *Physiol Rev,* 90**,** 1383-435.

REDECHA, P., FRANZKE, C. W., RUF, W., MACKMAN, N. & GIRARDI, G. 2008. Neutrophil activation by the tissue factor/Factor VIIa/PAR2 axis mediates fetal death in a mouse model of antiphospholipid syndrome. *J Clin Invest,* 118**,** 3453-61.

REDECHA, P., TILLEY, R., TENCATI, M., SALMON, J. E., KIRCHHOFER, D., MACKMAN, N. & GIRARDI, G. 2007. Tissue factor: a link between C5a and neutrophil activation in antiphospholipid antibody induced fetal injury. *Blood,* 110**,** 2423-31.

REES, F., DOHERTY, M., GRAINGE, M., DAVENPORT, G., LANYON, P. & ZHANG, W. 2016. The incidence and prevalence of systemic lupus erythematosus in the UK, 1999-2012. *Ann Rheum Dis,* 75**,** 136-41.

REMIJSEN, Q., KUIJPERS, T. W., WIRAWAN, E., LIPPENS, S., VANDENABEELE, P. & VANDEN BERGHE, T. 2011a. Dying for a cause: NETosis, mechanisms behind an antimicrobial cell death modality. *Cell Death Differ,* 18**,** 581-8.

REMIJSEN, Q., VANDEN BERGHE, T., WIRAWAN, E., ASSELBERGH, B., PARTHOENS, E., DE RYCKE, R., NOPPEN, S., DELFORGE, M., WILLEMS, J. & VANDENABEELE, P. 2011b. Neutrophil extracellular trap cell death requires both autophagy and superoxide generation. *Cell Res,* 21**,** 290-304.

REPP, R., VALERIUS, T., SENDLER, A., GRAMATZKI, M., IRO, H., KALDEN, J. R. & PLATZER, E. 1991. Neutrophils express the high affinity receptor for IgG (Fc gamma RI, CD64) after in vivo application of recombinant human granulocyte colony-stimulating factor. *Blood,* 78**,** 885-9.

RHODES, B., FURNROHR, B. G., ROBERTS, A. L., TZIRCOTIS, G., SCHETT, G., SPECTOR, T. D. & VYSE, T. J. 2012. The rs1143679 (R77H) lupus associated variant of ITGAM (CD11b) impairs complement receptor 3 mediated functions in human monocytes. *Ann Rheum Dis,* 71**,** 2028-34.

RIBATTI, D., CRIVELLATO, E. & VACCA, A. 2006. The contribution of Bruce Glick to the definition of the role played by the bursa of Fabricius in the development of the B cell lineage. *Clin Exp Immunol,* 145**,** 1-4.

RICHMAN, A. I., SU, E. Y. & HO, G., JR. 1981. Reciprocal relationship of synovial fluid volume and oxygen tension. *Arthritis Rheum,* 24**,** 701-5.

RIISE, T., JACOBSEN, B. K., GRAN, J. T., HAGA, H. J. & ARNESEN, E. 2001. Total mortality is increased in rheumatoid arthritis. A 17-year prospective study. *Clin Rheumatol,* 20**,** 123-7.

RINALDI, N., SCHWARZ-EYWILL, M., WEIS, D., LEPPELMANN-JANSEN, P., LUKOSCHEK, M., KEILHOLZ, U. & BARTH, T. F. 1997a. Increased expression of integrins on fibroblast-like synoviocytes from rheumatoid arthritis in vitro correlates with enhanced binding to extracellular matrix proteins. *Ann Rheum Dis,* 56**,** 45-51.

RINALDI, N., WEIS, D., BRADO, B., SCHWARZ-EYWILL, M., LUKOSCHEK, M., PEZZUTTO, A., KEILHOLZ, U. & BARTH, T. F. 1997b. Differential expression and functional behaviour of the alpha v and beta 3 integrin subunits in cytokine stimulated fibroblast-like cells derived from synovial tissue of rheumatoid arthritis and osteoarthritis in vitro. *Ann Rheum Dis,* 56**,** 729-36.

RITIS, K., DOUMAS, M., MASTELLOS, D., MICHELI, A., GIAGLIS, S., MAGOTTI, P., RAFAIL, S., KARTALIS, G., SIDERAS, P. & LAMBRIS, J. D. 2006. A novel C5a receptor-tissue factor cross-talk in neutrophils links innate immunity to coagulation pathways. *J Immunol,* 177**,** 4794-802.

RIYAPA, D., BUDDHISA, S., KORBSRISATE, S., CUCCUI, J., WREN, B. W., STEVENS, M. P., ATO, M. & LERTMEMONGKOLCHAI, G. 2012. Neutrophil extracellular traps exhibit antibacterial activity against burkholderia pseudomallei and are influenced by bacterial and host factors. *Infect Immun,* 80**,** 3921-9.

ROBBINS, D. L., LEUNG, S., MILLER-BLAIR, D. J. & ZIBOH, V. 1998. Effect of anticardiolipin/beta2-glycoprotein I complexes on production of thromboxane A2 by platelets from patients with the antiphospholipid syndrome. *J Rheumatol,* 25**,** 51-6.

ROBINSON, J., WATSON, F., BUCKNALL, R. C. & EDWARDS, S. W. 1992. Activation of neutrophil reactive-oxidant production by synovial fluid from patients with inflammatory joint disease. Soluble and insoluble immunoglobulin aggregates activate different pathways in primed and unprimed cells. *Biochem J,* 286 (Pt 2)**,** 345-51.

ROBINSON, J. J., WATSON, F., PHELAN, M., BUCKNALL, R. C. & EDWARDS, S. W. 1993. Activation of neutrophils by soluble and insoluble immunoglobulin aggregates from synovial fluid of patients with rheumatoid arthritis. *Ann Rheum Dis,* 52**,** 347-53.

RODESCH, F., SIMON, P., DONNER, C. & JAUNIAUX, E. 1992. Oxygen measurements in endometrial and trophoblastic tissues during early pregnancy. *Obstet Gynecol,* 80**,** 283-5.

ROMAY-PENABAD, Z., AGUILAR-VALENZUELA, R., URBANUS, R. T., DERKSEN, R. H., PENNINGS, M. T., PAPALARDO, E., SHILAGARD, T., VARGAS, G., HWANG, Y., DE GROOT, P. G. & PIERANGELI, S. S. 2011. Apolipoprotein E receptor 2 is involved in the thrombotic complications in a murine model of the antiphospholipid syndrome. *Blood,* 117**,** 1408-14.

ROMAY-PENABAD, Z., CARRERA MARIN, A. L., WILLIS, R., WESTON-DAVIES, W., MACHIN, S., COHEN, H., BRASIER, A. & GONZALEZ, E. B. 2014. Complement C5-inhibitor rEV576 (coversin) ameliorates in-vivo effects of antiphospholipid antibodies. *Lupus,* 23**,** 1324-6.

RONNEFARTH, V. M., ERBACHER, A. I., LAMKEMEYER, T., MADLUNG, J., NORDHEIM, A., RAMMENSEE, H. G. & DECKER, P. 2006. TLR2/TLR4-independent neutrophil activation and recruitment upon endocytosis of nucleosomes reveals a new pathway of innate immunity in systemic lupus erythematosus. *J Immunol,* 177**,** 7740-9.

ROSE, H. M., RAGAN, C. & ET AL. 1948. Differential agglutination of normal and sensitized sheep erythrocytes by sera of patients with rheumatoid arthritis. *Proc Soc Exp Biol Med,* 68**,** 1-6.

ROSETTI, F., CHEN, Y., SEN, M., THAYER, E., AZCUTIA, V., HERTER, J. M., LUSCINSKAS, F. W., CULLERE, X., ZHU, C. & MAYADAS, T. N. 2015. A Lupus-Associated Mac-1 Variant Has Defects in Integrin Allostery and Interaction with Ligands under Force. *Cell Rep*, 10(10), 1655-1664.

ROSSAINT, J., HERTER, J. M., VAN AKEN, H., NAPIREI, M., DORING, Y., WEBER, C., SOEHNLEIN, O. & ZARBOCK, A. 2014. Synchronized integrin engagement and chemokine activation is crucial in neutrophil extracellular trap-mediated sterile inflammation. *Blood,* 123**,** 2573-84.

ROSSI, A. G., SAWATZKY, D. A., WALKER, A., WARD, C., SHELDRAKE, T. A., RILEY, N. A., CALDICOTT, A., MARTINEZ-LOSA, M., WALKER, T. R., DUFFIN, R., GRAY, M., CRESCENZI, E., MARTIN, M. C., BRADY, H. J., SAVILL, J. S., DRANSFIELD, I. & HASLETT, C. 2006. Cyclin-dependent kinase inhibitors enhance the resolution of inflammation by promoting inflammatory cell apoptosis. *Nat Med,* 12**,** 1056-64.

ROTSTEIN, O. D., FIEGEL, V. D., SIMMONS, R. L. & KNIGHTON, D. R. 1988. The deleterious effect of reduced pH and hypoxia on neutrophil migration in vitro. *J Surg Res,* 45**,** 298-303.

ROUBEY, R. A., ROSS, G. D., MERRILL, J. T., WALTON, F., REED, W., WINCHESTER, R. J. & BUYON, J. P. 1991. Staurosporine inhibits neutrophil phagocytosis but not iC3b binding mediated by CR3 (CD11b/CD18). *J Immunol,* 146**,** 3557-62.

ROWLAND, S. L., RIGGS, J. M., GILFILLAN, S., BUGATTI, M., VERMI, W., KOLBECK, R., UNANUE, E. R., SANJUAN, M. A. & COLONNA, M. 2014. Early, transient depletion of plasmacytoid dendritic cells ameliorates autoimmunity in a lupus model. *J Exp Med,* 211**,** 1977-91.

SABROE, I., JONES, E. C., USHER, L. R., WHYTE, M. K. & DOWER, S. K. 2002. Toll-like receptor (TLR)2 and TLR4 in human peripheral blood granulocytes: a critical role for monocytes in leukocyte lipopolysaccharide responses. *J Immunol,* 168**,** 4701-10.

SADIK, C. D., KIM, N. D., IWAKURA, Y. & LUSTER, A. D. 2012. Neutrophils orchestrate their own recruitment in murine arthritis through C5aR and FcgammaR signaling. *Proc Natl Acad Sci U S A,* 109**,** E3177-85.

SALMON, J. E., GIRARDI, G. & HOLERS, V. M. 2002. Complement activation as a mediator of antiphospholipid antibody induced pregnancy loss and thrombosis. *Ann Rheum Dis,* 61 Suppl 2**,** ii46-50.

SALMON, J. E., MILLARD, S., SCHACHTER, L. A., ARNETT, F. C., GINZLER, E. M., GOURLEY, M. F., RAMSEY-GOLDMAN, R., PETERSON, M. G. & KIMBERLY, R. P. 1996. Fc gamma RIIA alleles are heritable risk factors for lupus nephritis in African Americans. *J Clin Invest,* 97**,** 1348-54.

SAMBANDAM, T., BELOUSOVA, M., ACCAVITI-LOPER, M. A., BLANQUICETT, C., GUERCELLO, V., RAIJMAKERS, R. & NICHOLAS, A. P. 2004. Increased peptidylarginine deiminase type II in hypoxic astrocytes. *Biochem Biophys Res Commun,* 325**,** 1324-9.

SANCHEZ-MEJORADA, G. & ROSALES, C. 1998. Signal transduction by immunoglobulin Fc receptors. *J Leukoc Biol,* 63**,** 521-33.

SATTA, N., DUNOYER-GEINDRE, S., REBER, G., FISH, R. J., BOEHLEN, F., KRUITHOF, E. K. & DE MOERLOOSE, P. 2007. The role of TLR2 in the inflammatory activation of mouse fibroblasts by human antiphospholipid antibodies. *Blood,* 109**,** 1507-14.

SATTA, N., KRUITHOF, E. K., FICKENTSCHER, C., DUNOYER-GEINDRE, S., BOEHLEN, F., REBER, G., BURGER, D. & DE MOERLOOSE, P. 2011. Toll-like receptor 2 mediates the activation of human monocytes and endothelial cells by antiphospholipid antibodies. *Blood,* 117**,** 5523-31.

SAVILL, J. 1997. Apoptosis in resolution of inflammation. *J Leukoc Biol,* 61**,** 375-80.

SCHELLEKENS, G. A., DE JONG, B. A., VAN DEN HOOGEN, F. H., VAN DE PUTTE, L. B. & VAN VENROOIJ, W. J. 1998. Citrulline is an essential constituent of antigenic determinants recognized by rheumatoid arthritis-specific autoantibodies. *J Clin Invest,* 101**,** 273-81.

SCHELLEKENS, G. A., VISSER, H., DE JONG, B. A., VAN DEN HOOGEN, F. H., HAZES, J. M., BREEDVELD, F. C. & VAN VENROOIJ, W. J. 2000. The diagnostic properties of rheumatoid arthritis antibodies recognizing a cyclic citrullinated peptide. *Arthritis Rheum,* 43**,** 155-63.

SCHENKEL, A. R., CHEW, T. W. & MULLER, W. A. 2004a. Platelet endothelial cell adhesion molecule deficiency or blockade significantly reduces leukocyte emigration in a majority of mouse strains. *J Immunol,* 173**,** 6403-8.

SCHENKEL, A. R., MAMDOUH, Z., CHEN, X., LIEBMAN, R. M. & MULLER, W. A. 2002. CD99 plays a major role in the migration of monocytes through endothelial junctions. *Nat Immunol,* 3**,** 143-50.

SCHENKEL, A. R., MAMDOUH, Z. & MULLER, W. A. 2004b. Locomotion of monocytes on endothelium is a critical step during extravasation. *Nat Immunol,* 5**,** 393-400.

SCHEPIS, D., GUNNARSSON, I., ELORANTA, M. L., LAMPA, J., JACOBSON, S. H., KARRE, K. & BERG, L. 2009. Increased proportion of CD56bright natural killer cells in active and inactive systemic lupus erythematosus. *Immunology,* 126**,** 140-6.

SCHETT, G. & TEITELBAUM, S. L. 2009. Osteoclasts and arthritis. *J Bone Miner Res,* 24**,** 1142-6.

SCHIEFERDECKER, H. L., ULLRICH, R., WEISS-BRECKWOLDT, A. N., SCHWARTING, R., STEIN, H., RIECKEN, E. O. & ZEITZ, M. 1990. The HML-1 antigen of intestinal lymphocytes is an activation antigen. *J Immunol,* 144**,** 2541-9.

SCHORN, C., JANKO, C., KRENN, V., ZHAO, Y., MUNOZ, L. E., SCHETT, G. & HERRMANN, M. 2012. Bonding the foe - NETting neutrophils immobilize the pro-inflammatory monosodium urate crystals. *Front Immunol,* 3**,** 376.

SELAK, M. A., ARMOUR, S. M., MACKENZIE, E. D., BOULAHBEL, H., WATSON, D. G., MANSFIELD, K. D., PAN, Y., SIMON, M. C., THOMPSON, C. B. & GOTTLIEB, E. 2005. Succinate links TCA cycle dysfunction to oncogenesis by inhibiting HIF-alpha prolyl hydroxylase. *Cancer Cell,* 7**,** 77-85.

SEMENZA, G. L. & WANG, G. L. 1992. A nuclear factor induced by hypoxia via de novo protein synthesis binds to the human erythropoietin gene enhancer at a site required for transcriptional activation. *Mol Cell Biol,* 12**,** 5447-54.

SENGELOV, H., BOULAY, F., KJELDSEN, L. & BORREGAARD, N. 1994a. Subcellular localization and translocation of the receptor for N-formylmethionyl-leucyl-phenylalanine in human neutrophils. *Biochem J,* 299 (Pt 2)**,** 473-9.

SENGELOV, H., KJELDSEN, L., DIAMOND, M. S., SPRINGER, T. A. & BORREGAARD, N. 1993. Subcellular localization and dynamics of Mac-1 (alpha m beta 2) in human neutrophils. *J Clin Invest,* 92**,** 1467-76.

SENGELOV, H., KJELDSEN, L., KROEZE, W., BERGER, M. & BORREGAARD, N. 1994b. Secretory vesicles are the intracellular reservoir of complement receptor 1 in human neutrophils. *J Immunol,* 153**,** 804-10.

SENGELOV, H., NIELSEN, M. H. & BORREGAARD, N. 1992. Separation of human neutrophil plasma membrane from intracellular vesicles containing alkaline phosphatase and NADPH oxidase activity by free flow electrophoresis. *J Biol Chem,* 267**,** 14912-7.

SEQUEIRA, J. F., KESER, G., GREENSTEIN, B., WHEELER, M. J., DUARTE, P. C., KHAMASHTA, M. A. & HUGHES, G. R. 1993. Systemic lupus erythematosus: sex hormones in male patients. *Lupus,* 2**,** 315-7.

SESTAK, A. L., FURNROHR, B. G., HARLEY, J. B., MERRILL, J. T. & NAMJOU, B. 2011. The genetics of systemic lupus erythematosus and implications for targeted therapy. *Ann Rheum Dis,* 70 Suppl 1**,** i37-43.

SHAW, S. K., MA, S., KIM, M. B., RAO, R. M., HARTMAN, C. U., FROIO, R. M., YANG, L., JONES, T., LIU, Y., NUSRAT, A., PARKOS, C. A. & LUSCINSKAS, F. W. 2004. Coordinated redistribution of leukocyte LFA-1 and endothelial cell ICAM-1 accompany neutrophil transmigration. *J Exp Med,* 200**,** 1571-80.

SHEN, B., DELANEY, M. K. & DU, X. 2012. Inside-out, outside-in, and inside-outside-in: G protein signaling in integrin-mediated cell adhesion, spreading, and retraction. *Curr Opin Cell Biol,* 24**,** 600-6.

SHENG, Y., SALI, A., HERZOG, H., LAHNSTEIN, J. & KRILIS, S. A. 1996. Site-directed mutagenesis of recombinant human beta 2-glycoprotein I identifies a cluster of lysine residues that are critical for phospholipid binding and anti-cardiolipin antibody activity. *J Immunol,* 157**,** 3744-51.

SHIMAOKA, M., XIAO, T., LIU, J. H., YANG, Y., DONG, Y., JUN, C. D., MCCORMACK, A., ZHANG, R., JOACHIMIAK, A., TAKAGI, J., WANG, J. H. & SPRINGER, T. A. 2003. Structures of the alpha L I domain and its complex with ICAM-1 reveal a shape-shifting pathway for integrin regulation. *Cell,* 112**,** 99-111.

SHIRAFUJI, N., MATSUDA, S., OGURA, H., TANI, K., KODO, H., OZAWA, K., NAGATA, S., ASANO, S. & TAKAKU, F. 1990. Granulocyte colony-stimulating factor stimulates human mature neutrophilic granulocytes to produce interferon-alpha. *Blood,* 75**,** 17-9.

SHOENFELD, Y., BLANK, M., CERVERA, R., FONT, J., RASCHI, E. & MERONI, P. L. 2006. Infectious origin of the antiphospholipid syndrome. *Ann Rheum Dis,* 65**,** 2-6.

SIMANTOV, R., LASALA, J. M., LO, S. K., GHARAVI, A. E., SAMMARITANO, L. R., SALMON, J. E. & SILVERSTEIN, R. L. 1995. Activation of cultured vascular endothelial cells by antiphospholipid antibodies. *J Clin Invest,* 96**,** 2211-9.

SIMMS, H. H. & D'AMICO, R. 1994. Regulation of whole blood polymorphonuclear leukocyte phagocytosis following hypoxemia and hypoxemia/reoxygenation. *Shock,* 1**,** 10-8.

SIVAKUMAR, B., AKHAVANI, M. A., WINLOVE, C. P., TAYLOR, P. C., PALEOLOG, E. M. & KANG, N. 2008. Synovial hypoxia as a cause of tendon rupture in rheumatoid arthritis. *J Hand Surg Am,* 33**,** 49-58.

SKOGH, T. & PEEN, E. 1993. Lactoferrin, anti-lactoferrin antibodies and inflammatory disease. *Adv Exp Med Biol,* 336**,** 533-8.

SMITH, G. P., SHARP, G. & PETERS, T. J. 1985. Isolation and characterization of alkaline phosphatase-containing granules (phosphasomes) from human polymorphonuclear leucocytes. *J Cell Sci,* 76**,** 167-78.

SMOLEN, J. S., ALETAHA, D., KOELLER, M., WEISMAN, M. H. & EMERY, P. 2007. New therapies for treatment of rheumatoid arthritis. *Lancet,* 370**,** 1861-74.

SMOLEN, J. S. & STEINER, G. 2003. Therapeutic strategies for rheumatoid arthritis. *Nat Rev Drug Discov,* 2**,** 473-88.

SONG, G., YANG, Y., LIU, J. H., CASASNOVAS, J. M., SHIMAOKA, M., SPRINGER, T. A. & WANG, J. H. 2005. An atomic resolution view of ICAM recognition in a complex between the binding domains of ICAM-3 and integrin alphaLbeta2. *Proc Natl Acad Sci U S A,* 102**,** 3366-71.

SORICE, M., LONGO, A., CAPOZZI, A., GAROFALO, T., MISASI, R., ALESSANDRI, C., CONTI, F., BUTTARI, B., RIGANO, R., ORTONA, E. & VALESINI, G. 2007. Anti-beta2-glycoprotein I antibodies induce monocyte release of tumor necrosis factor alpha and tissue factor by signal transduction pathways involving lipid rafts. *Arthritis Rheum,* 56**,** 2687-97.

SPENGLER, J., LUGONJA, B., JIMMY YTTERBERG, A., ZUBAREV, R. A., CREESE, A. J., PEARSON, M. J., GRANT, M. M., MILWARD, M., LUNDBERG, K., BUCKLEY, C. D., FILER, A., RAZA, K., COOPER, P. R., CHAPPLE, I. L. & SCHEEL-TOELLNER, D. 2015. Release of Active Peptidyl Arginine Deiminases by Neutrophils Can Explain Production of Extracellular Citrullinated Autoantigens in Rheumatoid Arthritis Synovial Fluid. *Arthritis Rheumatol,* 67**,** 3135-45.

SPRINGER, T. A. & DUSTIN, M. L. 2012. Integrin inside-out signaling and the immunological synapse. *Curr Opin Cell Biol,* 24**,** 107-15.

SRIRAMARAO, P., VON ANDRIAN, U. H., BUTCHER, E. C., BOURDON, M. A. & BROIDE, D. H. 1994. L-selectin and very late antigen-4 integrin promote eosinophil rolling at physiological shear rates in vivo. *J Immunol,* 153**,** 4238-46.

STAATZ, W. D., RAJPARA, S. M., WAYNER, E. A., CARTER, W. G. & SANTORO, S. A. 1989. The membrane glycoprotein Ia-IIa (VLA-2) complex mediates the Mg++-dependent adhesion of platelets to collagen. *J Cell Biol,* 108**,** 1917-24.

STANLEY, P., BATES, P. A., HARVEY, J., BENNETT, R. I. & HOGG, N. 1994. Integrin LFA-1 alpha subunit contains an ICAM-1 binding site in domains V and VI. *EMBO J,* 13**,** 1790-8.

STEINER, B., PARISE, L. V., LEUNG, B. & PHILLIPS, D. R. 1991. Ca(2+)-dependent structural transitions of the platelet glycoprotein IIb-IIIa complex. Preparation of stable glycoprotein IIb and IIIa monomers. *J Biol Chem,* 266**,** 14986-91.

STEVENSON, K. B., NAUSEEF, W. M. & CLARK, R. A. 1987. The neutrophil glycoprotein Mo1 is an integral membrane protein of plasma membranes and specific granules. *J Immunol,* 139**,** 3759-63.

STHOEGER, Z. M., BEZALEL, S., CHAPNIK, N., ASHER, I. & FROY, O. 2009. High alpha-defensin levels in patients with systemic lupus erythematosus. *Immunology,* 127**,** 116-22.

STRAUB, R. H., BIJLSMA, J. W., MASI, A. & CUTOLO, M. 2013. Role of neuroendocrine and neuroimmune mechanisms in chronic inflammatory rheumatic diseases--the 10-year update. *Semin Arthritis Rheum,* 43**,** 392-404.

STRIETER, R. M., KASAHARA, K., ALLEN, R. M., STANDIFORD, T. J., ROLFE, M. W., BECKER, F. S., CHENSUE, S. W. & KUNKEL, S. L. 1992. Cytokine-induced neutrophil-derived interleukin-8. *Am J Pathol,* 141**,** 397-407.

STROHMEIER, G. R., BRUNKHORST, B. A., SEETOO, K. F., MESHULAM, T., BERNARDO, J. & SIMONS, E. R. 1995. Role of the Fc gamma R subclasses Fc gamma RII and Fc gamma RIII in the activation of human neutrophils by low and high valency immune complexes. *J Leukoc Biol,* 58**,** 415-22.

SUCHARD, S. J., BURTON, M. J. & STOEHR, S. J. 1992. Thrombospondin receptor expression in human neutrophils coincides with the release of a subpopulation of specific granules. *Biochem J,* 284 (Pt 2)**,** 513-20.

SUCHARD, S. J., STETSKO, D. K., DAVIS, P. M., SKALA, S., POTIN, D., LAUNAY, M., DHAR, T. G., BARRISH, J. C., SUSULIC, V., SHUSTER, D. J., MCINTYRE, K. W., MCKINNON, M. & SALTER-CID, L. 2010. An LFA-1 (alphaLbeta2) small-molecule antagonist reduces inflammation and joint destruction in murine models of arthritis. *J Immunol,* 184**,** 3917-26.

SUH, Y. H., SHIN, Y. K., KOOK, M. C., OH, K. I., PARK, W. S., KIM, S. H., LEE, I. S., PARK, H. J., HUH, T. L. & PARK, S. H. 2003. Cloning, genomic organization, alternative transcripts and expression analysis of CD99L2, a novel paralog of human CD99, and identification of evolutionary conserved motifs. *Gene,* 307**,** 63-76.

SULLIVAN, K. E., SURIANO, A., DIETZMANN, K., LIN, J., GOLDMAN, D. & PETRI, M. A. 2007. The TNFalpha locus is altered in monocytes from patients with systemic lupus erythematosus. *Clin Immunol,* 123**,** 74-81.

SUNDD, P., GUTIERREZ, E., KOLTSOVA, E. K., KUWANO, Y., FUKUDA, S., POSPIESZALSKA, M. K., GROISMAN, A. & LEY, K. 2012. 'Slings' enable neutrophil rolling at high shear. *Nature,* 488**,** 399-403.

SUR CHOWDHURY, C., GIAGLIS, S., WALKER, U. A., BUSER, A., HAHN, S. & HASLER, P. 2014. Enhanced neutrophil extracellular trap generation in rheumatoid arthritis: analysis of underlying signal transduction pathways and potential diagnostic utility. *Arthritis Res Ther,* 16**,** R122.

SUZUKI, T., IKARI, K., YANO, K., INOUE, E., TOYAMA, Y., TANIGUCHI, A., YAMANAKA, H. & MOMOHARA, S. 2013. PADI4 and HLA-DRB1 are genetic risks for radiographic progression in RA patients, independent of ACPA status: results from the IORRA cohort study. *PLoS One,* 8**,** e61045.

TAKAGI, J., DEBOTTIS, D. P., ERICKSON, H. P. & SPRINGER, T. A. 2002a. The role of the specificity-determining loop of the integrin beta subunit I-like domain in autonomous expression, association with the alpha subunit, and ligand binding. *Biochemistry,* 41**,** 4339-47.

TAKAGI, J., PETRE, B. M., WALZ, T. & SPRINGER, T. A. 2002b. Global conformational rearrangements in integrin extracellular domains in outside-in and inside-out signaling. *Cell,* 110**,** 599-11.

TAKAGI, J., STROKOVICH, K., SPRINGER, T. A. & WALZ, T. 2003. Structure of integrin alpha5beta1 in complex with fibronectin. *EMBO J,* 22**,** 4607-15.

TAKAHASHI, G. W., ANDREWS, D. F., 3RD, LILLY, M. B., SINGER, J. W. & ALDERSON, M. R. 1993. Effect of granulocyte-macrophage colony-stimulating factor and interleukin-3 on interleukin-8 production by human neutrophils and monocytes. *Blood,* 81**,** 357-64.

TAKAHASHI, K., NAKAMURA, T., ADACHI, H., YAGITA, H. & OKUMURA, K. 1991. Antigen-independent T cell activation mediated by a very late activation antigen-like extracellular matrix receptor. *Eur J Immunol,* 21**,** 1559-62.

TAKEI, H., ARAKI, A., WATANABE, H., ICHINOSE, A. & SENDO, F. 1996. Rapid killing of human neutrophils by the potent activator phorbol 12-myristate 13-acetate (PMA)

accompanied by changes different from typical apoptosis or necrosis. *J Leukoc Biol,* 59**,** 229-40.

TAKEUCHI, O., HOSHINO, K., KAWAI, T., SANJO, H., TAKADA, H., OGAWA, T., TAKEDA, K. & AKIRA, S. 1999. Differential roles of TLR2 and TLR4 in recognition of gram-negative and gram-positive bacterial cell wall components. *Immunity,* 11**,** 443-51.

TAKEUCHI, T., SUZUKI, K., KONDO, T., YOSHIMOTO, K. & TSUZAKA, K. 2012. CD3 zeta defects in systemic lupus erythematosus. *Ann Rheum Dis,* 71 Suppl 2**,** i78-81.

TAMKUN, J. W., DESIMONE, D. W., FONDA, D., PATEL, R. S., BUCK, C., HORWITZ, A. F. & HYNES, R. O. 1986. Structure of integrin, a glycoprotein involved in the transmembrane linkage between fibronectin and actin. *Cell,* 46**,** 271-82.

TAMURA, D. Y., MOORE, E. E., PARTRICK, D. A., JOHNSON, J. L., OFFNER, P. J. & SILLIMAN, C. C. 2002. Acute hypoxemia in humans enhances the neutrophil inflammatory response. *Shock,* 17**,** 269-73.

TANAKA, N., KIM, J. S., NEWELL, J. D., BROWN, K. K., COOL, C. D., MEEHAN, R., EMOTO, T., MATSUMOTO, T. & LYNCH, D. A. 2004. Rheumatoid arthritis-related lung diseases: CT findings. *Radiology,* 232**,** 81-91.

TANAKA, S., MAEDA, S., HASHIMOTO, M., FUJIMORI, C., ITO, Y., TERADAIRA, S., HIROTA, K., YOSHITOMI, H., KATAKAI, T., SHIMIZU, A., NOMURA, T., SAKAGUCHI, N. & SAKAGUCHI, S. 2010. Graded attenuation of TCR signaling elicits distinct autoimmune diseases by altering thymic T cell selection and regulatory T cell function. *J Immunol,* 185**,** 2295-305.

TAPPER, H. & GRINSTEIN, S. 1997. Fc receptor-triggered insertion of secretory granules into the plasma membrane of human neutrophils: selective retrieval during phagocytosis. *J Immunol,* 159**,** 409-18.

TEICHMANN, L. L., OLS, M. L., KASHGARIAN, M., REIZIS, B., KAPLAN, D. H. & SHLOMCHIK, M. J. 2010. Dendritic cells in lupus are not required for activation of T and B cells but promote their expansion, resulting in tissue damage. *Immunity,* 33**,** 967-78.

TERVAERT, J. W., MULDER, L., STEGEMAN, C., ELEMA, J., HUITEMA, M., THE, H. & KALLENBERG, C. 1993. Occurrence of autoantibodies to human leucocyte elastase in Wegener's granulomatosis and other inflammatory disorders. *Ann Rheum Dis,* 52**,** 115-20.

TIAN, H., MCKNIGHT, S. L. & RUSSELL, D. W. 1997. Endothelial PAS domain protein 1 (EPAS1), a transcription factor selectively expressed in endothelial cells. *Genes Dev,* 11**,** 72-82.

TIAN, L., KILGANNON, P., YOSHIHARA, Y., MORI, K., GALLATIN, W. M., CARPEN, O. & GAHMBERG, C. G. 2000. Binding of T lymphocytes to hippocampal neurons through ICAM-5 (telencephalin) and characterization of its interaction with the leukocyte integrin CD11a/CD18. *Eur J Immunol,* 30**,** 810-8.

TKALCEVIC, J., NOVELLI, M., PHYLACTIDES, M., IREDALE, J. P., SEGAL, A. W. & ROES, J. 2000. Impaired immunity and enhanced resistance to endotoxin in the absence of neutrophil elastase and cathepsin G. *Immunity,* 12**,** 201-10.

TODD, R. F., 3RD, ARNAOUT, M. A., ROSIN, R. E., CROWLEY, C. A., PETERS, W. A. & BABIOR, B. M. 1984. Subcellular localization of the large subunit of Mo1 (Mo1 alpha; formerly gp 110), a surface glycoprotein associated with neutrophil adhesion. *J Clin Invest,* 74**,** 1280-90.

TOMASONI, S., NORIS, M., ZAPPELLA, S., GOTTI, E., CASIRAGHI, F., BONAZZOLA, S., BENIGNI, A. & REMUZZI, G. 1998. Upregulation of renal and systemic cyclooxygenase-2 in patients with active lupus nephritis. *J Am Soc Nephrol,* 9**,** 1202-12.

TOTOSON, P., MAGUIN-GATE, K., PRATI, C., WENDLING, D. & DEMOUGEOT, C. 2014. Mechanisms of endothelial dysfunction in rheumatoid arthritis: lessons from animal studies. *Arthritis Res Ther,* 16**,** 202.

TRAVERS, J. & ROTHENBERG, M. E. 2015. Eosinophils in mucosal immune responses. *Mucosal Immunol,* 8**,** 464-75.

TSAN, M. F. 1980. Phorbol myristate acetate induced neutrophil autotoxicity. *J Cell Physiol,* 105**,** 327-34.

TSUJIMURA, Y., OBATA, K., MUKAI, K., SHINDOU, H., YOSHIDA, M., NISHIKADO, H., KAWANO, Y., MINEGISHI, Y., SHIMIZU, T. & KARASUYAMA, H. 2008. Basophils play a pivotal role in immunoglobulin-G-mediated but not immunoglobulin-E-mediated systemic anaphylaxis. *Immunity,* 28**,** 581-9.

TSURUTA, D., HOPKINSON, S. B., LANE, K. D., WERNER, M. E., CRYNS, V. L. & JONES, J. C. 2003. Crucial role of the specificity-determining loop of the integrin beta4 subunit in the binding of cells to laminin-5 and outside-in signal transduction. *J Biol Chem,* 278**,** 38707-14.

TUCCI, M., QUATRARO, C., LOMBARDI, L., PELLEGRINO, C., DAMMACCO, F. & SILVESTRIS, F. 2008. Glomerular accumulation of plasmacytoid dendritic cells in active lupus nephritis: role of interleukin-18. *Arthritis Rheum,* 58**,** 251-62.

UEDA, T., RIEU, P., BRAYER, J. & ARNAOUT, M. A. 1994. Identification of the complement iC3b binding site in the beta 2 integrin CR3 (CD11b/CD18). *Proc Natl Acad Sci U S A,* 91**,** 10680-4.

ULRICH, V., GELBER, S. E., VUKELIC, M., SACHARIDOU, A., HERZ, J., URBANUS, R. T., DE GROOT, P. G., NATALE, D. R., HARIHARA, A., REDECHA, P., ABRAHAMS, V. M., SHAUL, P. W., SALMON, J. E. & MINEO, C. 2016. ApoE Receptor 2 Mediation of Trophoblast Dysfunction and Pregnancy Complications Induced by Antiphospholipid Antibodies in Mice. *Arthritis Rheumatol,* 68**,** 730-9.

URBAN, C. F., REICHARD, U., BRINKMANN, V. & ZYCHLINSKY, A. 2006. Neutrophil extracellular traps capture and kill Candida albicans yeast and hyphal forms. *Cell Microbiol,* 8**,** 668-76.

VALENCIA, X., YARBORO, C., ILLEI, G. & LIPSKY, P. E. 2007. Deficient CD4+CD25high T regulatory cell function in patients with active systemic lupus erythematosus. *J Immunol,* 178**,** 2579-88.

VAN DER WOUDE, D., RANTAPAA-DAHLQVIST, S., IOAN-FACSINAY, A., ONNEKINK, C., SCHWARTE, C. M., VERPOORT, K. N., DRIJFHOUT, J. W., HUIZINGA, T. W., TOES, R. E. & PRUIJN, G. J. 2010. Epitope spreading of the anti-citrullinated protein antibody response occurs before disease onset and is associated with the disease course of early arthritis. *Ann Rheum Dis,* 69**,** 1554-61.

VAN KOOYK, Y., WEDER, P., HEIJE, K., DE WAAL MALEFIJT, R. & FIGDOR, C. G. 1993. Role of intracellular Ca2+ levels in the regulation of CD11a/CD18 mediated cell adhesion. *Cell Adhes Commun,* 1**,** 21-32.

VARGAS-ROJAS, M. I., CRISPIN, J. C., RICHAUD-PATIN, Y. & ALCOCER-VARELA, J. 2008. Quantitative and qualitative normal regulatory T cells are not capable of inducing suppression in SLE patients due to T-cell resistance. *Lupus,* 17**,** 289-94.

VAUPEL, P., BRAUNBECK, W. & THEWS, G. 1973. Respiratory gas exchange and pO2-distribution in splenic tissue. *Adv Exp Med Biol,* 37A**,** 401-6.

VEGA-OSTERTAG, M., CASPER, K., SWERLICK, R., FERRARA, D., HARRIS, E. N. & PIERANGELI, S. S. 2005. Involvement of p38 MAPK in the up-regulation of tissue factor on endothelial cells by antiphospholipid antibodies. *Arthritis Rheum,* 52**,** 1545-54.

VEGA, F. M., FRUHWIRTH, G., NG, T. & RIDLEY, A. J. 2011. RhoA and RhoC have distinct roles in migration and invasion by acting through different targets. *J Cell Biol,* 193**,** 655-65.

VERPOORT, K. N., VAN GAALEN, F. A., VAN DER HELM-VAN MIL, A. H., SCHREUDER, G. M., BREEDVELD, F. C., HUIZINGA, T. W., DE VRIES, R. R. & TOES, R. E. 2005. Association of HLA-DR3 with anti-cyclic citrullinated peptide antibody-negative rheumatoid arthritis. *Arthritis Rheum,* 52**,** 3058-62.

VILLANUEVA, E., YALAVARTHI, S., BERTHIER, C. C., HODGIN, J. B., KHANDPUR, R., LIN, A. M., RUBIN, C. J., ZHAO, W., OLSEN, S. H., KLINKER, M., SHEALY, D., DENNY, M. F., PLUMAS, J., CHAPEROT, L., KRETZLER, M., BRUCE, A. T. & KAPLAN, M. J. 2011.

Netting neutrophils induce endothelial damage, infiltrate tissues, and expose immunostimulatory molecules in systemic lupus erythematosus. *J Immunol,* 187**,** 538-52.

VINCENT, C., NOGUEIRA, L., SEBBAG, M., CHAPUY-REGAUD, S., ARNAUD, M., LETOURNEUR, O., ROLLAND, D., FOURNIE, B., CANTAGREL, A., JOLIVET, M. & SERRE, G. 2002. Detection of antibodies to deiminated recombinant rat filaggrin by enzyme-linked immunosorbent assay: a highly effective test for the diagnosis of rheumatoid arthritis. *Arthritis Rheum,* 46**,** 2051-8.

VINOGRADOVA, O., VELYVIS, A., VELYVIENE, A., HU, B., HAAS, T., PLOW, E. & QIN, J. 2002. A structural mechanism of integrin alpha(IIb)beta(3) "inside-out" activation as regulated by its cytoplasmic face. *Cell,* 110**,** 587-97.

VOLKHOLZ, H. J., HOPER, J., BRUNNER, M., FRANK, K. H., HARRISON, D. K., ELLERMANN, R. & KESSLER, M. 1984. Measurement of local PO2 and intracapillary hemoglobin oxygenation in lung tissue of rabbits. *Adv Exp Med Biol,* 169**,** 633-41.

VORDENBAUMEN, S., FISCHER-BETZ, R., TIMM, D., SANDER, O., CHEHAB, G., RICHTER, J., BLECK, E. & SCHNEIDER, M. 2010. Elevated levels of human beta-defensin 2 and human neutrophil peptides in systemic lupus erythematosus. *Lupus,* 19**,** 1648-53.

VOSSENAAR, E. R., NIJENHUIS, S., HELSEN, M. M., VAN DER HEIJDEN, A., SENSHU, T., VAN DEN BERG, W. B., VAN VENROOIJ, W. J. & JOOSTEN, L. A. 2003. Citrullination of synovial proteins in murine models of rheumatoid arthritis. *Arthritis Rheum,* 48**,** 2489-500.

WALKER, B. A., HAGENLOCKER, B. E., STUBBS, E. B., JR., SANDBORG, R. R., AGRANOFF, B. W. & WARD, P. A. 1991. Signal transduction events and Fc gamma R engagement in human neutrophils stimulated with immune complexes. *J Immunol,* 146**,** 735-41.

WALLBERG-JONSSON, S., JOHANSSON, H., OHMAN, M. L. & RANTAPAA-DAHLQVIST, S. 1999. Extent of inflammation predicts cardiovascular disease and overall mortality in seropositive rheumatoid arthritis. A retrospective cohort study from disease onset. *J Rheumatol,* 26**,** 2562-71.

WALMSLEY, S. R., COWBURN, A. S., CLATWORTHY, M. R., MORRELL, N. W., ROPER, E. C., SINGLETON, V., MAXWELL, P., WHYTE, M. K. & CHILVERS, E. R. 2006. Neutrophils from patients with heterozygous germline mutations in the von Hippel Lindau protein (pVHL) display delayed apoptosis and enhanced bacterial phagocytosis. *Blood,* 108**,** 3176-8.

WALMSLEY, S. R., PRINT, C., FARAHI, N., PEYSSONNAUX, C., JOHNSON, R. S., CRAMER, T., SOBOLEWSKI, A., CONDLIFFE, A. M., COWBURN, A. S., JOHNSON, N. & CHILVERS, E. R. 2005. Hypoxia-induced neutrophil survival is mediated by HIF-1alpha-dependent NF-kappaB activity. *J Exp Med,* 201**,** 105-15.

WANG, F., WANG, C. L., TAN, C. T. & MANIVASAGAR, M. 1997. Systemic lupus erythematosus in Malaysia: a study of 539 patients and comparison of prevalence and disease expression in different racial and gender groups. *Lupus,* 6**,** 248-53.

WANG, G. L., JIANG, B. H., RUE, E. A. & SEMENZA, G. L. 1995. Hypoxia-inducible factor 1 is a basic-helix-loop-helix-PAS heterodimer regulated by cellular O2 tension. *Proc Natl Acad Sci U S A,* 92**,** 5510-4.

WANG, J. S. & LIU, H. C. 2009. Systemic hypoxia enhances bactericidal activities of human polymorphonuclear leuocytes. *Clin Sci (Lond),* 116**,** 805-17.

WANG, Y., WYSOCKA, J., SAYEGH, J., LEE, Y. H., PERLIN, J. R., LEONELLI, L., SONBUCHNER, L. S., MCDONALD, C. H., COOK, R. G., DOU, Y., ROEDER, R. G., CLARKE, S., STALLCUP, M. R., ALLIS, C. D. & COONROD, S. A. 2004. Human PAD4 regulates histone arginine methylation levels via demethylimination. *Science,* 306**,** 279-83.

WARCHOL, T., LIANERI, M., LACKI, J. K., OLESINSKA, M. & JAGODZINSKI, P. P. 2011. ITGAM Arg77His is associated with disease susceptibility, arthritis, and renal symptoms in systemic lupus erythematosus patients from a sample of the Polish population. *DNA Cell Biol,* 30**,** 33-8.

WATTS, G. M., BEURSKENS, F. J., MARTIN-PADURA, I., BALLANTYNE, C. M., KLICKSTEIN, L. B., BRENNER, M. B. & LEE, D. M. 2005. Manifestations of inflammatory arthritis are critically dependent on LFA-1. *J Immunol,* 174**,** 3668-75.

WEISMAN, M. H. 2002. Newly diagnosed rheumatoid arthritis. *Ann Rheum Dis,* 61**,** 287-9.

WELLS, G., BECKER, J. C., TENG, J., DOUGADOS, M., SCHIFF, M., SMOLEN, J., ALETAHA, D. & VAN RIEL, P. L. 2009. Validation of the 28-joint Disease Activity Score (DAS28) and European League Against Rheumatism response criteria based on C-reactive protein against disease progression in patients with rheumatoid arthritis, and comparison with the DAS28 based on erythrocyte sedimentation rate. *Ann Rheum Dis,* 68**,** 954-60.

WEST, B. C., ROSENTHAL, A. S., GELB, N. A. & KIMBALL, H. R. 1974. Separation and characterization of human neutrophil granules. *Am J Pathol,* 77**,** 41-66.

WIESENER, M. S., JURGENSEN, J. S., ROSENBERGER, C., SCHOLZE, C. K., HORSTRUP, J. H., WARNECKE, C., MANDRIOTA, S., BECHMANN, I., FREI, U. A., PUGH, C. W., RATCLIFFE, P. J., BACHMANN, S., MAXWELL, P. H. & ECKARDT, K. U. 2003. Widespread hypoxia-inducible expression of HIF-2alpha in distinct cell populations of different organs. *FASEB J,* 17**,** 271-3.

WIESENER, M. S., TURLEY, H., ALLEN, W. E., WILLAM, C., ECKARDT, K. U., TALKS, K. L., WOOD, S. M., GATTER, K. C., HARRIS, A. L., PUGH, C. W., RATCLIFFE, P. J. & MAXWELL, P. H. 1998. Induction of endothelial PAS domain protein-1 by hypoxia: characterization and comparison with hypoxia-inducible factor-1alpha. *Blood,* 92**,** 2260-8.

WILLIS, R., SEIF, A. M., MCGWIN, G., JR., MARTINEZ-MARTINEZ, L. A., GONZALEZ, E. B., DANG, N., PAPALARDO, E., LIU, J., VILA, L. M., REVEILLE, J. D., ALARCON, G. S. & PIERANGELI, S. S. 2012. Effect of hydroxychloroquine treatment on pro-inflammatory cytokines and disease activity in SLE patients: data from LUMINA (LXXV), a multiethnic US cohort. *Lupus,* 21**,** 830-5.

WILLIS, V. C., GIZINSKI, A. M., BANDA, N. K., CAUSEY, C. P., KNUCKLEY, B., CORDOVA, K. N., LUO, Y., LEVITT, B., GLOGOWSKA, M., CHANDRA, P., KULIK, L., ROBINSON, W. H., AREND, W. P., THOMPSON, P. R. & HOLERS, V. M. 2011. N-alpha-benzoyl-N5-(2-chloro-1-iminoethyl)-L-ornithine amide, a protein arginine deiminase inhibitor, reduces the severity of murine collagen-induced arthritis. *J Immunol,* 186**,** 4396-404.

WILSON, W. A., GHARAVI, A. E., KOIKE, T., LOCKSHIN, M. D., BRANCH, D. W., PIETTE, J. C., BREY, R., DERKSEN, R., HARRIS, E. N., HUGHES, G. R., TRIPLETT, D. A. & KHAMASHTA, M. A. 1999. International consensus statement on preliminary classification criteria for definite antiphospholipid syndrome: report of an international workshop. *Arthritis Rheum,* 42**,** 1309-11.

WIPKE, B. T. & ALLEN, P. M. 2001. Essential role of neutrophils in the initiation and progression of a murine model of rheumatoid arthritis. *J Immunol,* 167**,** 1601-8.

WONG, C. K., HO, C. Y., LI, E. K. & LAM, C. W. 2000. Elevation of proinflammatory cytokine (IL-18, IL-17, IL-12) and Th2 cytokine (IL-4) concentrations in patients with systemic lupus erythematosus. *Lupus,* 9**,** 589-93.

WOODFIN, A., VOISIN, M. B., IMHOF, B. A., DEJANA, E., ENGELHARDT, B. & NOURSHARGH, S. 2009. Endothelial cell activation leads to neutrophil transmigration as supported by the sequential roles of ICAM-2, JAM-A, and PECAM-1. *Blood,* 113**,** 6246-57.

WORTHEN, G. S., AVDI, N., BUHL, A. M., SUZUKI, N. & JOHNSON, G. L. 1994. FMLP activates Ras and Raf in human neutrophils. Potential role in activation of MAP kinase. *J Clin Invest,* 94**,** 815-23.

WU, C. H., HSIEH, S. C., LI, K. J., LU, M. C. & YU, C. L. 2007. Premature telomere shortening in polymorphonuclear neutrophils from patients with systemic lupus erythematosus is related to the lupus disease activity. *Lupus,* 16**,** 265-72.

WU, J., EDBERG, J. C., REDECHA, P. B., BANSAL, V., GUYRE, P. M., COLEMAN, K., SALMON, J. E. & KIMBERLY, R. P. 1997. A novel polymorphism of FcgammaRIIIa (CD16) alters receptor function and predisposes to autoimmune disease. *J Clin Invest,* 100**,** 1059-70.

WURM, H. 1984. beta 2-Glycoprotein-I (apolipoprotein H) interactions with phospholipid vesicles. *Int J Biochem,* 16**,** 511-5.

XIAO, T., TAKAGI, J., COLLER, B. S., WANG, J. H. & SPRINGER, T. A. 2004. Structural basis for allostery in integrins and binding to fibrinogen-mimetic therapeutics. *Nature,* 432**,** 59-67.

XIE, J., LI, R., KOTOVUORI, P., VERMOT-DESROCHES, C., WIJDENES, J., ARNAOUT, M. A., NORTAMO, P. & GAHMBERG, C. G. 1995. Intercellular adhesion molecule-2 (CD102) binds to the leukocyte integrin CD11b/CD18 through the A domain. *J Immunol,* 155**,** 3619-28.

XIONG, J. P., STEHLE, T., DIEFENBACH, B., ZHANG, R., DUNKER, R., SCOTT, D. L., JOACHIMIAK, A., GOODMAN, S. L. & ARNAOUT, M. A. 2001. Crystal structure of the extracellular segment of integrin alpha Vbeta3. *Science,* 294**,** 339-45.

XIONG, J. P., STEHLE, T., ZHANG, R., JOACHIMIAK, A., FRECH, M., GOODMAN, S. L. & ARNAOUT, M. A. 2002. Crystal structure of the extracellular segment of integrin alpha Vbeta3 in complex with an Arg-Gly-Asp ligand. *Science,* 296**,** 151-5.

XU, C., MAO, D., HOLERS, V. M., PALANCA, B., CHENG, A. M. & MOLINA, H. 2000. A critical role for murine complement regulator crry in fetomaternal tolerance. *Science,* 287**,** 498-501.

YAKUBENKO, V. P., YADAV, S. P. & UGAROVA, T. P. 2006. Integrin alphaDbeta2, an adhesion receptor up-regulated on macrophage foam cells, exhibits multiligand-binding properties. *Blood,* 107**,** 1643-50.

YALAVARTHI, S., GOULD, T. J., RAO, A. N., MAZZA, L. F., MORRIS, A. E., NUNEZ-ALVAREZ, C., HERNANDEZ-RAMIREZ, D., BOCKENSTEDT, P. L., LIAW, P. C., CABRAL, A. R. & KNIGHT, J. S. 2015. Release of neutrophil extracellular traps by neutrophils stimulated with antiphospholipid antibodies: a newly identified mechanism of thrombosis in the antiphospholipid syndrome. *Arthritis Rheumatol,* 67**,** 2990-3003.

YAMASHITA, T., OHNEDA, O., NAGANO, M., IEMITSU, M., MAKINO, Y., TANAKA, H., MIYAUCHI, T., GOTO, K., OHNEDA, K., FUJII-KURIYAMA, Y., POELLINGER, L. & YAMAMOTO, M. 2008. Abnormal heart development and lung remodeling in mice lacking the hypoxia-inducible factor-related basic helix-loop-helix PAS protein NEPAS. *Mol Cell Biol,* 28**,** 1285-97.

YANG, N., ISBEL, N. M., NIKOLIC-PATERSON, D. J., LI, Y., YE, R., ATKINS, R. C. & LAN, H. Y. 1998a. Local macrophage proliferation in human glomerulonephritis. *Kidney Int,* 54**,** 143-51.

YANG, R. B., MARK, M. R., GRAY, A., HUANG, A., XIE, M. H., ZHANG, M., GODDARD, A., WOOD, W. I., GURNEY, A. L. & GODOWSKI, P. J. 1998b. Toll-like receptor-2 mediates lipopolysaccharide-induced cellular signalling. *Nature,* 395**,** 284-8.

YANG, W., ZHAO, M., HIRANKARN, N., LAU, C. S., MOK, C. C., CHAN, T. M., WONG, R. W., LEE, K. W., MOK, M. Y., WONG, S. N., AVIHINGSANON, Y., LIN, I. O., LEE, T. L., HO, M. H., LEE, P. P., WONG, W. H., SHAM, P. C. & LAU, Y. L. 2009. ITGAM is associated with disease susceptibility and renal nephritis of systemic lupus erythematosus in Hong Kong Chinese and Thai. *Hum Mol Genet,* 18**,** 2063-70.

YE, Z., MA, N., ZHAO, L., JIANG, Z. Y. & JIANG, Y. F. 2014. Differential expression of natural killer activating and inhibitory receptors in patients with newly diagnosed systemic lupus erythematosus. *Int J Rheum Dis*.

YI, Y., MCNERNEY, M. & DATTA, S. K. 2000. Regulatory defects in Cbl and mitogen-activated protein kinase (extracellular signal-related kinase) pathways cause persistent hyperexpression of CD40 ligand in human lupus T cells. *J Immunol,* 165**,** 6627-34.

YIPP, B. G., PETRI, B., SALINA, D., JENNE, C. N., SCOTT, B. N., ZBYTNUIK, L. D., PITTMAN, K., ASADUZZAMAN, M., WU, K., MEIJNDERT, H. C., MALAWISTA, S. E., DE BOISFLEURY CHEVANCE, A., ZHANG, K., CONLY, J. & KUBES, P. 2012. Infection-induced NETosis is a dynamic process involving neutrophil multitasking in vivo. *Nat Med,* 18**,** 1386-93.

YOUNG, A. & KODURI, G. 2007. Extra-articular manifestations and complications of rheumatoid arthritis. *Best Pract Res Clin Rheumatol,* 21**,** 907-27.

YOUNG, A., KODURI, G., BATLEY, M., KULINSKAYA, E., GOUGH, A., NORTON, S. & DIXEY, J. 2007. Mortality in rheumatoid arthritis. Increased in the early course of disease, in ischaemic heart disease and in pulmonary fibrosis. *Rheumatology (Oxford),* 46**,** 350-7.

YOUSEFI, S., MIHALACHE, C., KOZLOWSKI, E., SCHMID, I. & SIMON, H. U. 2009. Viable neutrophils release mitochondrial DNA to form neutrophil extracellular traps. *Cell Death Differ,* 16**,** 1438-44.

YU, F., WHITE, S. B., ZHAO, Q. & LEE, F. S. 2001. HIF-1alpha binding to VHL is regulated by stimulus-sensitive proline hydroxylation. *Proc Natl Acad Sci U S A,* 98**,** 9630-5.

ZANDMAN-GODDARD, G., SOLOMON, M., ROSMAN, Z., PEEVA, E. & SHOENFELD, Y. 2012. Environment and lupus-related diseases. *Lupus,* 21**,** 241-50.

ZENG, B. X., FUJIWARA, H., SATO, Y., NISHIOKA, Y., YAMADA, S., YOSHIOKA, S., UEDA, M., HIGUCHI, T. & FUJII, S. 2007. Integrin alpha5 is involved in fibronectin-induced human extravillous trophoblast invasion. *J Reprod Immunol,* 73**,** 1-10.

ZHANG, H., CASASNOVAS, J. M., JIN, M., LIU, J. H., GAHMBERG, C. G., SPRINGER, T. A. & WANG, J. H. 2008. An unusual allosteric mobility of the C-terminal helix of a high-affinity alphaL integrin I domain variant bound to ICAM-5. *Mol Cell,* 31**,** 432-7.

ZHANG, K. & CHEN, J. 2012. The regulation of integrin function by divalent cations. *Cell Adh Migr,* 6**,** 20-9.

ZHANG, P., LU, L., YAO, Q., LI, Y., ZHOU, J., LIU, Y. & DUAN, C. 2012. Molecular, functional, and gene expression analysis of zebrafish hypoxia-inducible factor-3alpha. *Am J Physiol Regul Integr Comp Physiol,* 303**,** R1165-74.

ZHANG, P., YAO, Q., LU, L., LI, Y., CHEN, P. J. & DUAN, C. 2014. Hypoxia-inducible factor 3 is an oxygen-dependent transcription activator and regulates a distinct transcriptional response to hypoxia. *Cell Rep,* 6**,** 1110-21.

ZHAO, H., JIANG, Y., CAO, Q., HOU, Y. & WANG, C. 2012. Role of integrin switch and transforming growth factor Beta 3 in hypoxia-induced invasion inhibition of human extravillous trophoblast cells. *Biol Reprod,* 87**,** 47.

ZHAO, M. H., JONES, S. J. & LOCKWOOD, C. M. 1995. Bactericidal/permeability-increasing protein (BPI) is an important antigen for anti-neutrophil cytoplasmic autoantibodies (ANCA) in vasculitis. *Clin Exp Immunol,* 99**,** 49-56.

ZHAO, M. H. & LOCKWOOD, C. M. 1996. Azurocidin is a novel antigen for anti-neutrophil cytoplasmic autoantibodies (ANCA) in systemic vasculitis. *Clin Exp Immunol,* 103**,** 397-402.

ZHOU, L., LEE, D. H., PLESCIA, J., LAU, C. Y. & ALTIERI, D. C. 1994. Differential ligand binding specificities of recombinant CD11b/CD18 integrin I-domain. *J Biol Chem,* 269**,** 17075-9.

ZHOU, M., TODD, R. F., 3RD, VAN DE WINKEL, J. G. & PETTY, H. R. 1993. Cocapping of the leukoadhesin molecules complement receptor type 3 and lymphocyte function-associated antigen-1 with Fc gamma receptor III on human neutrophils. Possible role of lectin-like interactions. *J Immunol,* 150**,** 3030-41.

ZHOU, M. J., LUBLIN, D. M., LINK, D. C. & BROWN, E. J. 1995. Distinct tyrosine kinase activation and Triton X-100 insolubility upon Fc gamma RII or Fc gamma RIIIB ligation in human polymorphonuclear leukocytes. Implications for immune complex activation of the respiratory burst. *J Biol Chem,* 270**,** 13553-60.

ZHOU, Y., FISHER, S. J., JANATPOUR, M., GENBACEV, O., DEJANA, E., WHEELOCK, M. & DAMSKY, C. H. 1997. Human cytotrophoblasts adopt a vascular phenotype as they

differentiate. A strategy for successful endovascular invasion? *J Clin Invest,* 99**,** 2139-51.

ZHOU, Y., WU, J., KUCIK, D. F., WHITE, N. B., REDDEN, D. T., SZALAI, A. J., BULLARD, D. C. & EDBERG, J. C. 2013. Multiple lupus-associated ITGAM variants alter Mac-1 functions on neutrophils. *Arthritis Rheum,* 65**,** 2907-16.

ZHU, J. & SPRINGER, T. A. 2013. Complete integrin headpiece opening in eight steps. *J Cell Biol,* 201**,** 1053-68.

ZHU, J., YAMANE, H. & PAUL, W. E. 2010. Differentiation of effector CD4 T cell populations (*). *Annu Rev Immunol,* 28**,** 445-89.

ZIMMERMANN, M., ARRUDA-SILVA, F., BIANCHETTO-AGUILERA, F., FINOTTI, G., CALZETTI, F., SCAPINI, P., LUNARDI, C., CASSATELLA, M. A. & TAMASSIA, N. 2016. IFNalpha enhances the production of IL-6 by human neutrophils activated via TLR8. *Sci Rep,* 6**,** 19674.

ZINKERNAGEL, A. S., PEYSSONNAUX, C., JOHNSON, R. S. & NIZET, V. 2008. Pharmacologic augmentation of hypoxia-inducible factor-1alpha with mimosine boosts the bactericidal capacity of phagocytes. *J Infect Dis,* 197**,** 214-7.

ZUND, G., NELSON, D. P., NEUFELD, E. J., DZUS, A. L., BISCHOFF, J., MAYER, J. E. & COLGAN, S. P. 1996. Hypoxia enhances stimulus-dependent induction of E-selectin on aortic endothelial cells. *Proc Natl Acad Sci U S A,* 93**,** 7075-80.

ZWERINA, J., HAYER, S., REDLICH, K., BOBACZ, K., KOLLIAS, G., SMOLEN, J. S. & SCHETT, G. 2006. Activation of p38 MAPK is a key step in tumor necrosis factor-mediated inflammatory bone destruction. *Arthritis Rheum,* 54**,** 463-72.

Appendix I: General Materials and Equipment

15ml conical centrifuge tube	Falcon centrifuge tube (Corning, UK)
50ml conical centrifuge tube	Falcon centrifuge tube (Corning, UK)
1.5ml microcentrifuge tubes	Epindorf tubes (VWR, UK)
Centrifuges	Sorvall Legend XT (Thermo Scientific, UK)
	Megafuge 40R (Thermo Scientific, UK)
Plate reader	Tecan GENios Spectra FLUOR (Tecan UK Ltd., UK)
NanoDrop	ND-1000 Spectrophotometer (LabTech International, UK)

Appendix II: General Buffers

For all buffers prepared, solutions were made using distilled and deionised water (ddH_2O) and adjusted to the correct pH with 0.1M HCl or 0.1M NaOH. Buffers were then either autoclaved or filter sterilised with a 0.22μm syringe driven filter unit (Millipore, Ireland).

Phosphate-buffered saline (PBS) pH 7.4.

One PBS tablet (Life Technologies, UK) was added per 500ml ddH_2O and allowed to completely dissolve before being autoclaved prior to use. For assays requiring PBS washes, a 10xPBS stock solution was prepared using the PBS tablets and diluted using ddH_2O. To make PBS/0.1% Tween-20, 1ml of Tween-20 was added to 1L PBS.

Tris-buffered saline (TBS) pH 7.4.

A 10xTBS stock solution was prepared by dissolving 160g of NaCl (1.37M), 4g of KCl (0.27M) and 60g (0.25M) of Tris base in 1.8L of ddH_2O. The solution was subsequently adjusted to pH 7.4 and made up to 2L. The 10xTBS stock solution was autoclaved and diluted to 1xTBS using ddH_2O. To prepare TBS/0.1% Tween-20, 1ml of Tween-20 was added to 1L TBS.

Sodium HEPES buffer

To prepare the HEPES buffer, 4.77g HEPES (20mM) (Sigma, UK), 8,18g NaCl (140mM) (Sigma, UK), 2g glucose (2mg/ml) (Sigma, UK) and 3g BSA (0.3% w/v) (Sigma, UK) was dissolved in 800ml ddH_2O. The pH was adjusted to pH 7.4 using 10M NaOH before making the solution up to 1L with ddH_2O. The HEPES buffer was subsequently filter sterilised through a 0.22μm vacuum driven filter unit (Millipore, Ireland) and stored at 4^oC

IgG Binding buffer

The binding buffer used for IgG purification is a 0.1M phosphate buffer comprised of both monobasic and dibasic buffers. First 13.9g of monobasic sodium phosphate (0.2M) (Sigma, UK) was dissolved in 500ml of ddH_2O and 28.4g dibasic sodium phosphate (0.2M) (Sigma,

UK) was dissolved in 1L of ddH_2O to make the two stock solutions, which were subsequently autoclaved. To make the working binding buffer, 117ml of the 0.2M monobasic phosphate buffer was added to 183ml of the dibasic phosphate buffer that was adjusted to pH 7.2 and made up to 600ml with ddH_2O to achieve a 0.1M phosphate buffer. The binding buffer was filter sterilised through with a 0.22µm syringe driven filter unit (Millipore, Ireland) when required.

IgG Elution buffer

To elute bound IgG, a 0.1M glycine buffer (elution buffer) was used. To prepare this a 1M stock solution was prepared in 800ml of ddH_2O and the pH adjusted to pH 3.0 and made up to 1L. The stock solution was autoclaved and working solutions were prepared by diluting further using ddH_2O. The elution buffer was filter sterilised through with a 0.22µm syringe driven filter unit (Millipore, Ireland) before use.

IgG Neutralisation buffer

A neutralisation buffer was used to neutralise the acidic elution buffer to prevent denaturation of purified IgG. This was a 1M solution of Tris (Sigma, UK) that hade been adjusted to pH 9.0 and autoclaved. As with the other solutions, the neutralisation buffer was filtered sterilised through with a 0.22µm syringe driven filter unit (Millipore, Ireland) prior to use.

Appendix III: Publications Arising From This Thesis

Conference Abstracts

Khawaja, A.A., Pericleous, C. Ripoll, V.M., Porter, J.C. and Giles, I. Different autoimmune rheumatic disease IgG have differential effects upon neutrophil binding, activation and neutrophil extracellular trap formation. *Arthritis Rheumatol*. 2015; 67(s10)

Khawaja, A.A., Pericleous, C., Thomas, L.W., Ashcroft, M., Giles, I. and Porter, J.C. Hypoxia increases neutrophil extracellular trap formation and adhesion to endothelial cells. *Immunology*. 2014; **143(s2):** 1-200

Khawaja, A.A., Pericleous, C., Thomas, L.W., Ashcroft, M., Giles, I. and Porter, J.C. Hypoxia modulates peptidyl arginine deiminase 4 activity and neutrophil extracellular trap formation. *Arthritis & Rheumatism*. 2014; **66:** S1-S1402

Journal Articles

Cambridge, G., Moura, R.A., Santos, T., **Khawaja, A.A.**, Polido-Pereira, J., Canhão, H., Leandro, M.J. and Fonseca, J.E. Expression of the Inherently Autoreactive Idiotope 9G4 on Autoantibodies to Citrullinated Peptides and on Rheumatoid Factors in Patients with Early and Established Rheumatoid Arthritis. *PLoS One*. 2014; **9(9):** e107513