The interaction of type 1 and 2 interferons with neutrophils in Juvenile-onset Systemic Lupus Erythematosus (JSLE)

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Declaration

I declare that this thesis is the result of my own work. The material contained in this thesis has not been presented, nor currently being presented, either wholly or in part for any other degree or qualification. The contribution of others is made explicit where this has taken place.

This research was carried out in the Institute in the Park, Alder Hey children's NHS Foundation Trust, and the Department of Women's and Children's Health, Institute of Translational Medicine, University of Liverpool.

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Dedication

This is dedicated to everyone who has ever felt they couldn't do it. Whatever 'it' is. For all those who have suffered imposter syndrome, anxiety, not being good enough. You're stronger than you think and amazing just the way you are.

Abstract

The interaction of type 1 and 2 IFNs with neutrophils in JSLE

Sophie Lindsay Irwin

Introduction: Juvenile-onset Systemic Lupus Erythematosus (JSLE) is a multisystem autoimmune disease characterised by an increase in nuclear autoantigens and subsequent increased production in autoantibodies. Neutrophil function and apoptosis is dysregulated in JSLE, and thus neutrophils are thought to be an important factor in JSLE pathogenesis. Type 1 and 2 interferons (IFNs) have been shown to be increased in SLE and JSLE serum, and an IFN and granulocyte genetic signature within JSLE patients has indicated a potential interaction of IFNs and neutrophils within JSLE. However, specific mechanisms of IFNs on neutrophil apoptosis and function within JSLE is yet to be elucidated.

Aim: To investigate the interaction of IFNs with neutrophil function, apoptosis and signalling pathways, with specific focus on how these interactions may change in an inflammatory disease such as JSLE.

Methods: Neutrophils were isolated from whole blood from healthy adult volunteers and children with and without JSLE. Neutrophils were left naïve or primed with TNFα or IFNs, and subsequently stimulated with IFNs and other stimulants. Phagocytosis, apoptosis, activation states and receptor expression were analysed using flow cytometry. Intracellular signalling proteins were analysed using Western blotting. Chemotaxis was analysed using a transwell assay in the presence or absence of chemokines. NETosis was analysed using confocal microscopy and DNA quantification.

Results: An *in vitro* model was developed to investigate the role of IFNs on a range of key neutrophil functions. IFNs were shown to have little effect on specific aspects of chemotaxis, phagocytosis and NETosis of healthy volunteer neutrophils. IFNs were shown to be anti-apoptotic towards naïve neutrophils from healthy adult volunteers but could either lose this ability or induce apoptosis when neutrophils were primed with TNFα. The anti-apoptotic effect observed was via reduced cleavage of caspase 3 but not through the stability of MCL1. Patient serum (including paediatric control) activated neutrophils from healthy adult volunteers, but this had no significant effect on neutrophil apoptosis downstream. Priming with TNFα reduced IFNAR1 expression on neutrophils from healthy adult volunteers but did not change the expression of other IFN receptor chains. There was no difference in IFN receptor chain expression on neutrophils from JSLE patients compared to paediatric controls. Following priming with TNFα, there was a reduction of STAT3 phosphorylation and an increase in STAT1 phosphorylation in neutrophils from healthy adult volunteers.

Conclusion: IFNs have an important influence on neutrophil apoptosis. In primed neutrophils, lower concentrations of IFNs lose their anti-apoptotic ability and can induce apoptosis at high concentrations. This may reflect what is happening at sites of inflammation in active patients. Drug treatment may help to reduce this potentially IFN-related increase in neutrophil apoptosis in JSLE patients. Patients with inactive JSLE disease, likely due to their medication, had similar rates of apoptosis to matched controls. Activation of healthy adult neutrophils reduced the expression of IFNAR1 alone, which may contribute to the differential IFN induced phosphorylation of anti-apoptotic STAT3 and pro-apoptotic STAT1. This manipulation of the IFN signalling pathway through TNFα priming (and therefore through activation of neutrophils in inflammatory diseases) may contribute to the dual IFN effect on apoptosis, with STAT1 possibly involved in increased neutrophil apoptosis in JSLE. Inhibition of STAT1 may therefore be therapeutically beneficial in JSLE.

Contents

Appendices

Figures

Tables

Abbreviations

A	Adenosine
AB	Antibody
ACR	American College of Rheumatology
AE	Adverse effects
AIDS	Acquired Immune Deficiency Syndrome
ANA	Anti-nuclear antibody
Anti-Sm	Anti-Smith antibody
Apaf1	Apoptotic protease activating factor 1
APC	Allophycocyanin
ATP	Adenosine triphosphate
BAFF	B-cell activating factor
BALF	Bronchoalveolar lavage fluid
BCL2	B cell lymphoma 2
Bcl-XL	B-cell lymphoma-extra large
BILAG	British Isles Lupus Assessment Group
BSA	Bovine serum albumin
C	Cytosine
C3/C4	Complement component 3/4
CCL	Chemokine (C-C motif) ligand
CCR	CC chemokine receptors
CD	Cluster of differentiation
CGP	Good Clinical Practice
cIAP	Cellular inhibitor of apoptosis
CNS	Central nervous system

COPD	Chronic obstructive pulmonary diseases
CR	Complement receptor
CXCL	Chemokine (C-X-C motif) ligand
CXCR	CXC chemokine receptor
D	Aspartic acid
DAPI	4', 6-diamidino-2-phenylindole
DBD	DNA binding domain
DED	Death effector domain
DISC	Death inducing signalling complex
DMARD	Disease-modifying anti-rheumatic drug
DMEM	Dulbecco's modified eagle's medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dsDNA	Double-stranded deoxyribonucleic acid
DTT	Dithiothreitol
dUTP	2´-deoxyuridine, 5´-triphosphate
EAE	Experimental autoimmune encephalomyelitis
E.coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
EMSA	Electrophoretic mobility shift assay
ELISA	Enzyme-linked immunosorbent assay
ESI1	Epithelial stromal interaction 1
ESR	Erythrocyte sedimentation rate
FADD	Fas-associated protein with death domain

FasL	Fas ligand
FBS	Foetal bovine serum
FcγR	Fragment crystallisable γ receptor
FERM	Four point one, ezrin, radixin, moesin
FGF	Fibroblast growth factor
FITC	Fluorescein isothiocyanate
FL	Fluorescence channel
FLAG	aspartic acid-tyrosine-lysine-aspartic acid-aspartic acid-lysine [DYKDDDDK] epitope
FLIL10R1/ λR1	FLAG-IL-10 receptor 1/λ receptor 1
fMLP	N-formyl-methionyl-leucyl-phenylalanine
FS	Forward scatter
g	Gram
G	Guanine
GAF	Gamma interferon activation factor
GAS	Gamma interferon activation site
GCSF	Granulocyte colony stimulating factor
GMCSF	Granulocyte macrophage stimulating factor
GROα	Growth regulated oncogene α
GVHD	Graft Versus Host Disease
HBSS	Hanks' balanced salt solution
HDN	High density neutrophils
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HI-PHS	Heat inactivated pooled human serum
HLA	Human leukocyte antigen

HLA-DR	human leukocyte antigen - antigen D Related
HMGB1	High mobility group box 1
Hr	Hour
HRP	Horseradish peroxidase
IAP	Inhibitor of apoptosis
IBF	Ibuprofen
IFIT3	Interferon-induced protein with tetratricopeptide repeats 3
IFITM	Interferon-induced transmembrane proteins
IFN	Interferon
IFNAR	Interferon α receptor
IFNGR	Interferon γ receptor
Ig	Immunoglobulin
IL	Interleukin
IP10	IFNγ-induced protein 10
IRF	Interferon regulatory factor
ISG	Interferon stimulated gene
ISGF	Interferon stimulated gene factor
ISM	Interferon signature matrix
IV	Intravenous
IVIG	Intravenous immunoglobulin
JAK	Janus kinase
JH	Janus kinase homology
JSLE	Juvenile-onset Systemic Lupus Erythematosus
K	Lysine
kDA	Kilodaltons

Ki	kinase
KIR	Kinase inhibitory region
L	Leucine
LDG	Low density granulocytes
LDN	Low density neutrophils
Lk	Linker
LPS	Lipopolysaccharide
M	Molar
mAB	Monoclonal antibody
Mac-1	Macrophage-1 antigen
MAPK	Mitogen-activated protein kinase
MASP	Mannose binding lectin-associated proteins
MBL	Mannose binding lectin
MCL1	Myeloid cell leukaemia 1
MCP	Monocyte chemoattractant protein
MCSF	Macrophage colony stimulating factor
mDC	Myeloid dendritic cell
MDM2	Mouse double minute 2 homolog
MEF	Mouse embryonic fibroblast
mg	Milligram
MIG	Monokine induced by γ IFN
MIP1α	Macrophage inflammatory protein 1 α
Min	Minute
ml	Millilitre
MMP9	Matrix metallopeptidase 9

MPO	Myeloperoxidase
mtDNA	Mitochondrial deoxynucleic acid
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
mRNA	Messenger ribonucleic acid
mUnits	Milliunits
mM	MilliMolar
MyD88	Myeloid differentiation primary response 88
N	Number
NADPH oxidase	Nicotinamide adenine dinucleotide phosphate oxidase
NE	Neutrophil elastase
NET	Neutrophil extracellular trap
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
ng	Nanogram
NHS	National Health Service
NIHR	National Institute for Health Research
NK cell	Natural killer cell
NKT cell	Natural killer T cell
NLR	Nod-like receptor
nM	NanoMolar
nm	Nanometre
NOD mice	Non-obese diabetic mice
nRNP	Nuclear ribonucleoprotein
OAS1A	2'-5' Oligoadenylate synthetase 1A
OCP	Oral contraceptive pill
pAB	Polyclonal antibody

PAMP	Pathogen associated molecular pattern
pBILAG	Paediatric adaption of the British Isles Lupus Assessment Group
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PD1/PDCD1	Programmed cell death 1
pDC	Plasmacytoid dendritic cell
PE	Phycoerythrin
PEG	Polyethylene glycol
PET	Polyethylene terephthalate
PI	Propidium iodide
PI-PLC	Phosphatidylinositol-specific phospholipase C
PI3 kinase	Phosphoinositide 3 kinase
PINP	Polyethylene glycol (PEG)ylated immune-conjugated polylactide coglycolide nanoparticle
PK	Pharmacokinetic
PKB	Protein kinase B
PKC	Protein kinase C
PLC	Phospholipase C
PLGA	Polylactide coglycolide
PMA	Phorbol myristate acetate
PPR	Pattern recognition receptors
PolyHEMA	Poly(2-hydroxyethyl methacrylate)
pSTAT	Phosphorylated signal transducer and activator of transcription
PTPN22	Protein tyrosine phosphatase, non-receptor type 22
R	Receptor

RA	Rheumatoid arthritis
RANTES	Regulated on activation, normal T cell expressed and secreted
RBC	Red blood cell
REC	Research ethics committee
RLR	Rig-1-like receptor
RNP	Ribonucleoprotein
RPM	Revolutions per minute
RPMI 1640 media	Roswell Park Memorial Institute 1640 media
ROS	Reactive oxygen species
S.aureus	*Staphylococcus aureus*
S. pyogenes	*Streptococcus pyogenes*
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
Sec	Second
SELENA-SLEDAI	Safety of Estrogens in Lupus Erythematosus National Assessment - Systemic Lupus Erythematosus Disease Activity Index
SH2	Src homology 2
siRNA	Small interfering ribonucleic acid
SLE	Systemic Lupus Erythematosus
SLEDAI	Systemic Lupus Erythematosus Disease Activity Index
SLEDAI-2K	Systemic Lupus Erythematosus Disease Activity Index 2000
SLICC	Systemic Lupus International Collaborating Clinics
snRNP	Small nuclear ribonucleo proteins
Src	SRC proto-oncogene, non-receptor tyrosine kinase
SOCS	Suppressor of cytokine signalling

SRI	SLE response index
SS	Side scatter
SSA	Sjögren's syndrome type A
SSB	Sjögren's syndrome type B
ssDNA	Single stranded deoxyribonucleic acid
STAT	Signal transducer and activator of transcription
T	Thymine
TAD	Transactivation domain
TBS	Tris base saline
TBST	Tris base saline (0.1%) tween 20
TE buffer	Tris EDTA buffer
TGFβ	Transforming growth factor β
Th cells	T helper cells
TIR	Toll/interleukin-1 receptor
TIRAP/Mal	Toll–interleukin 1 receptor domain–containing adapter protein/MyD88 adapter-like
TLR	Toll-like receptor
TMPD	2, 6, 10, 14 Tetramethylpentadecane
TNF	Tumour necrosis factor
TRAIL	Tumour necrosis factor-related apoptosis-inducing ligand
T_{reg}	Regulatory T cell
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labelling
Tyk2	Tyrosine kinase
U/ml	Unit per millilitre
UK	United Kingdom

USA	United States of America
USP18	Ubiquitin specific peptidase 18
w/	With
xg	Gravity
xIAP	X-linked inhibitor of apoptosis protein
Y	Tyrosine
α	Alpha
β	Beta
γ	Gamma
δ	Delta
κ	Kappa
λ	Lambda
ψ	Psi
ψK	Pseudokinase
µg	Microgram
µl	Microlitre
µM	MicroMol

Chapter 1: Literature Review

1.1. What is lupus?

Systemic Lupus Erythematosus, or SLE, is a multisystem autoimmune disease with a wide range of clinical and immunological manifestations (1). On a cellular level, SLE is characterized by hyperactive B cells, and subsequent autoantibody production directed against nuclear autoantigens (2). It has been demonstrated that T lymphocytes play a role in autoantibody formation (3). It is unclear what causes SLE or how the disease progresses. However, it is thought that there are hormonal, genetic and environmental factors contributing to the pathogenesis of SLE (4-6).

Juvenile-onset SLE (JSLE), in which onset of lupus occurs before 18 years of age, is rarer than adult-onset SLE and is generally more severe than its adult counterpart, both at diagnosis and over the disease course (7). The spectrum of disease differs between JSLE and adult-onset SLE, with children having more active renal, neuropsychiatric and haematological disease and increased associated disease activity and organ damage (7). Up to 20% of SLE patients develop the disease in childhood and thus JSLE produces an important research area to be investigated (8).

1.1.1. The UK JSLE Cohort Study

The United Kingdom (UK) JSLE Study Group is a collaborative group of paediatric rheumatologists, nephrologists, and other specialities including basic scientists from multiple centres, which was started in 2006, and is led by the University of Liverpool, based at Alder Hey Children's Foundation NHS Trust. The UK JSLE Cohort Study aims is to bring together a comprehensive team of experts to investigate the 'clinical characteristics and immunopathology of JSLE', which includes a database of clinical and demographic data, and a biobank of blood products and urine (https://www.liverpool.ac.uk/translational-medicine/research/ukjsle/about/).

The majority of adult SLE and JSLE patients are female, with approximately 85% overall of the UK JSLE Cohort Study being girls (9). The median age at diagnosis within the Cohort is 12.6 years, and the median time from onset of symptoms to diagnosis is 3.2 months (9). Male JSLE patients are generally younger at disease presentation than females (9). Although the majority of JSLE patients in the UK JSLE Cohort Study are Caucasian (approximately 50%), there is a significant incidence of JSLE in non-Caucasian populations, including of black African and Asian ethnic origin (9). Non-Caucasian patients tend to be younger than Caucasian

patients, and there is a tendency of increased nephritis at presentation in Chinese, Black African and mixed ethnicity patients (9). Some 40% of patients have a family history of autoimmune disease, with 15% of families having lupus (9). In a multicentre analysis, which included adult-onset SLE and the UK JSLE Cohort Study patients, although females represented the majority of cases, this gender ratio in favour of females was less evident in the younger patient populations (10). There is a higher proportion of males with JSLE than with adult-onset SLE, with younger children (and those over the age of 60) having the lowest male-to -female ratio (10). Older patients tended to be Caucasian, and a higher proportion of Asian patients were found in JSLE patient population (10).

1.1.2. Epidemiology of SLE

Childhood-onset SLE is a rare condition with an incidence of 6-30 per 100,000 children per year (11), with some 15-20% of patients with SLE having disease onset in childhood. The incidence of SLE has been shown to depend on many demographic factors such as geography, gender, age and ethnicity (12). In general, the incidence of SLE is more prevalent in females which ranged from 2:1 to 15:1, depending on population studied (12). SLE in females peaks around the third to seventh decades of life, whereas SLE in males peak around the fifth to seventh decades of life (12).

1.1.3. Clinical symptoms of JSLE

The American College of Rheumatology (ACR) has devised a set of 11 criteria for SLE classification, whereby at least four are required to diagnose SLE (13, 14). Some of the most common features in JSLE using the ACR criteria are renal, neuropsychiatric, immunogenic and hematologic features, as well as non-erosive arthritis and malar rash (13, 14). Hematologic features include lymphopenia, leukopenia, haemolytic anaemia and thrombocytopenia (13, 14). The immunological characteristics include: anti-double stranded deoxynucleic acid (dsDNA), anti-Smith (Sm) and anti-phospholipid antibodies (12, 13). The paediatric British Isles Lupus Assessment Group (pBILAG)-2004 disease activity score is made up of specific domains which describe the organ involvement which may be affected by JSLE, and which can be assessed by a series of questions and clinical assessments assigned to each of these organs (15). These scores assess whether JSLE disease was active (assigned an A or B), improving (assigned a C), inactive but with previous involvement (assigned a D) or inactive with no previous disease involvement (assigned an E) at time of assessment (15). These letter classifications can have numerical assignments, and these numerical assignments are totalled to create an overall pBILAG score (15). Overall, active disease may be defined as a patient

having an A or B in any organ domain, whereas inactive disease may be defined as a patient having a D or E in all organ domains (16).

Other organ system involvement and damage can involve: ophthalmic (ocular cataract and retinal change), neurological, pulmonary (including hypertension, fibrosis, and pulmonary infarction), cardiac (including cardiomyopathy), vascular (including venous thrombosis) gastrointestinal (including gastrointestinal infarction), muscoskeletal (including muscular atrophy), cutaneous (including alopecia) and diabetes (13, 14, 17).

In comparing UK JSLE Cohort Study patients to a population of adult SLE patients looked after in a large, tertiary referral centre, there were no significant clinical differences in the prevalence of rash or photosensitivity (10). JSLE patients tended to have more alopecia and oral rashes, whereas adults were more likely to have arthritis and serositis, although there was a reduction in serositis in the mature age group (10). JSLE patients had significantly more lupus nephritis than any other group, and a trend that JSLE patients had more neurological disease (10). Lymphopenia became more common with age, whereas thrombocytopenia and haemolytic anaemia were both more prevalent in JSLE (10). Cardiovascular events were more common and had longer duration in adult-onset SLE than in JSLE and additionally, mortality was higher in adult-onset SLE than in JSLE (10). A diagram summarising an overview of the systems affected is shown in Figure 1.1.

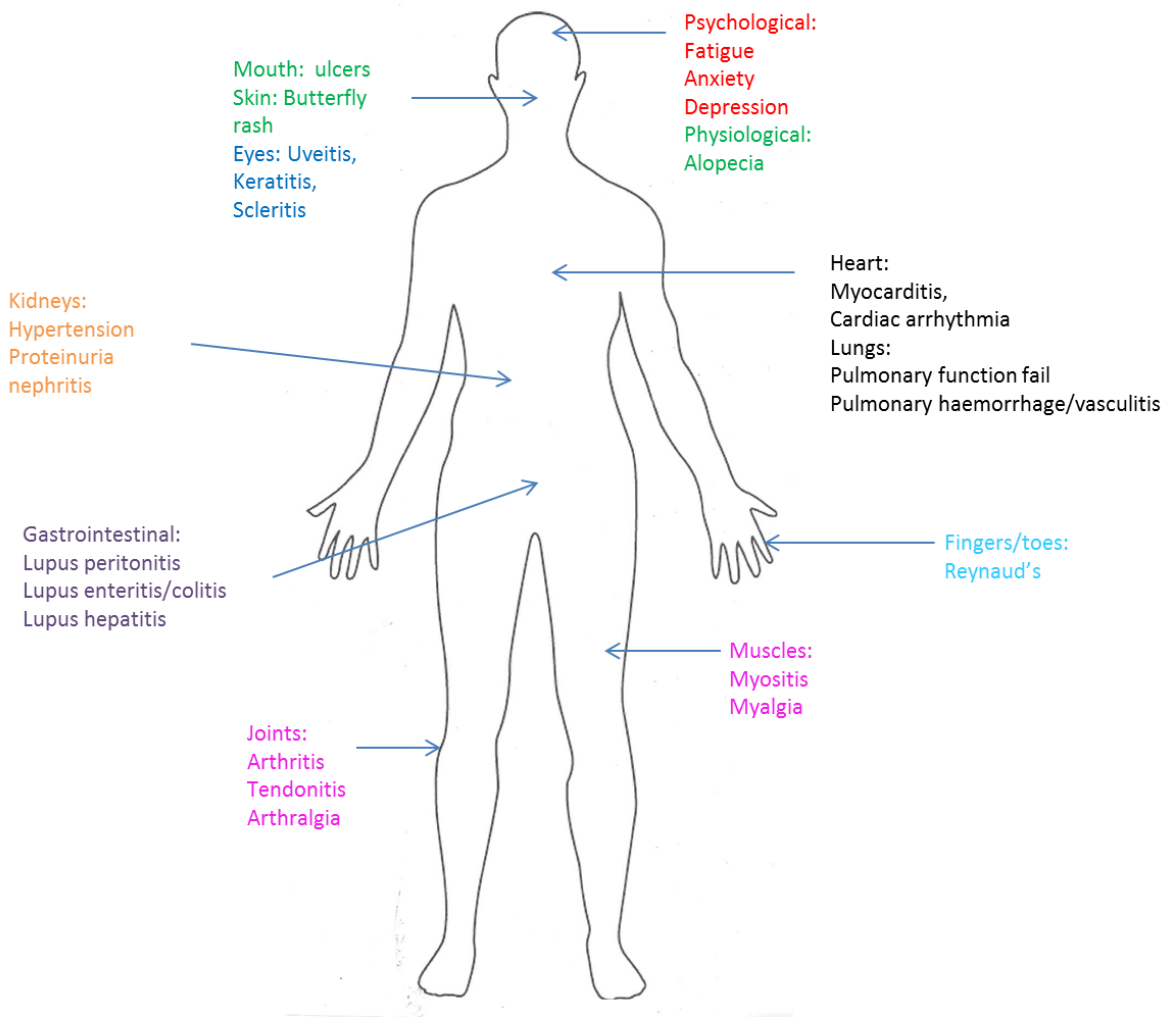


Figure 1.1. Organ systems affected by JSLE and their manifestations. Although not a comprehensive list for systems or symptoms, this illustrative figure denotes each symptom classified by organ system (neurological = red, mucocutaneous = green, ophthalmic = dark blue, renal = orange, gastrointestinal = purple, musculoskeletal = pink, vasculitis = light blue, and cardio and respiratory = black). Whether these symptoms are improving, or worsening will contribute to pBILAG classification. (Human body outline = http://humananatomychart.us/anatomy-human-body-diagram/anatomy-human-body-diagram-printable-human-body-outline-174/).

1.1.4. Aetiology of JSLE

SLE is characterised by many clinical characteristics but also cellular components. Clinically, as noted these can involve many organ systems, which include articular, skin, pleuro-pulmonary, gastrointestinal and renal findings (18). On a cellular level, increased gamma (γ)-globulins, anaemia, anti-nuclear antibodies (ANA), anti-dsDNA and anti-histone antibodies can be detected, which can help in the diagnosis of JSLE (18). Although SLE can affect individuals of any age, as a group, the UK JSLE Study Group focus specifically on the pathogenesis of JSLE, and as such, the majority of this thesis will have this patient group as the focus of investigation.

Within the UK JSLE Cohort Study, 98% of JSLE patients were positive for ANA (9). Compared to UK adult patients, JSLE patients had more anti-dsDNA, anti-ribonucleoprotein (RNP) and anti-Sm autoantibodies, and low complement component (C)3 was more common (10). Anti-nuclear autoantibodies can help diagnostically and they also can indicate or predict SLE activity (19). Microarray technology has been used to analyse such autoantibodies which were found to be higher in JSLE than healthy controls, and include commonly used antibodies such as those directed against dsDNA, histones and RNP (19). Recently, it was also shown that that autoantibodies against other factors are increased in JSLE such as B-cell activating factor (BAFF) (19). BAFF is a member of the tumour necrosis factor (TNF) family and in particular stimulates the growth of B cells (20). BAFF autoantibodies, in particular, have been shown to be associated with active disease, emphasising the importance of autoantibodies and autoantigens in the development of JSLE (19). Autoantibodies such as those directed against dsDNA and histone H1 have been shown to be higher in patients with lupus nephritis, indicating a role of autoantibodies in the development of particular organ damage (19). Therefore, the role of autoantibodies, and investigation into the processes that contribute to autoantibody development, such as dysregulated apoptosis, is important for understanding the development of JSLE.

1.1.5. Genetics of JSLE

Both adult-onset SLE and JSLE have important genetic as well as environmentally-induced factors underpinning their disease pathogenesis. Studies have shown that certain genetic variants and polymorphisms may contribute to JSLE pathogenesis or predispose patients to the development of the disease (21-27), as discussed in detail below. A genetic variant of the *oestrogen receptor 1* was found to significantly more frequent in JSLE patients than healthy controls, which may be associated with the high percentage of female patients (9, 21). Interestingly, within an adult cohort of SLE patients, there is a higher frequency of an

oestrogen receptor 2 allele, suggesting that age of onset may rely on different genetic causes (21).

Dysregulation of the immune system is important in the development of JSLE. Of interest is the role of the inflammasome (28, 29). It has been shown that genes that may be important in inflammasome assembly are differently distributed between JSLE patients and controls, and specific genes, such as *interleukin (IL)-1B* gene, are significantly associated with JSLE (22, 23). It was found that there were various differences in polymorphisms in genotype, haplotype and alleles in the *IL-4* gene and a haplotype polymorphism association within the *IL-6* gene in JSLE patients compared to controls (23, 24). Interleukins (ILs) are a large family of cytokines that can act in a paracrine or autocrine way, and can modulate cellular growth, differentiation and activation during immune responses (30). Other gene polymorphisms such as the -28C/G polymorphism in the chemokine *regulated on activation, normal T cell expressed and secreted (RANTES)* promoter is more frequent in JSLE patients, and patients with the C/G or G/G genotypes at RANTES –28 had a higher presence of JSLE markers such as ANA, and lower levels of C3 (26, 31). These data indicate that the dysregulated immune response found in JSLE, is due, in part at least to genetic variants. This theory is supported by other studies, including investigation of the A allele of a *PD1* gene polymorphism which has been found to be more frequent in JSLE than healthy controls (25). It was also shown that a G/G genotype and a G allele in the PD1 gene were significantly less frequent in JSLE than healthy controls, suggesting that there can be a variety of genetic differences in just one gene that can contribute to JSLE, highlighting the complexity of this disease (25). Programmed cell death 1 (PDCD1 or PD1) which the *PD1* gene can encode, is involved in central and peripheral tolerance and acts as an inhibitory immune receptor which can modulate self-reactive T cells (25, 32). Therefore, the decrease intolerance, and possible over activation of the immune system in JSLE may be partly genetic.

Other genes have been found to have variants that are more associated with JSLE in certain ethnic populations. In a Mexican population of JSLE patients, the *protein tyrosine phosphatase, non-receptor type 22 (PTPN22) 1858* C/T genotype and the *PTPN221858* T allele was more frequent in JSLE patients than controls (27). This suggests that, within a Mexican population at least, there are specific components within signalling pathways that are genetically altered within JSLE, and this is contributing, with the genetic variants suggested above, to an altered immune response within JSLE, which may be true of other JSLE populations.

1.2. Immune system

1.2.1. Overview

The immune system is made up of multiple cell-types and tissues. The cells are grouped into those of the innate and adaptive immune systems (33). The different types of immune cells can be individually distinguished by their morphology including shape and size of the nuclei, pattern of granules and cluster of differentiation (CD) markers.

The cells of the innate immune system comprise of neutrophils, macrophages, natural killer (NK) cells, mast cells and dendritic cells (33). The main purpose of the innate immunity is to act as the body's 'first line of defence' against infection and injury. The adaptive immune system is more specific; T and B cells 'memorise' specific antigens of pathogens or foreign factors, and are thus able to target these in a very swift, direct and precise manner. However, previous infection of these 'recognised' pathogens is required to produce this adaptive immune system memory. B-cells produce antibodies that recognise invading microbes, and can rapidly remove the microbe if it has previously invaded. The adaptive immune system can be activated by the innate immune system via cytokines or antigens of invading pathogens and is particularly useful if the innate immune system is unable to effectively deal with and/or remove any pathogens (33).

Antigens are molecules that can induce an immune response (33). An invading organism express antigens on their surface that the immune system can recognise as foreign through interaction with antibodies or receptors. Host cells also express antigens and generally, these are not immunogenic. However, within autoimmunity, host antigens can elicit an immune response without the presence of an invading pathogen; these are termed autoantigens (33).

Cytokines are small molecules that are secreted by all immune cells in response to a certain stimulus (33). They can have an effect on the cell that produces them (known as an autocrine effect) and are important in cell-to-cell signalling. Cytokines are involved in cell growth, differentiation, chemotaxis, activation and cytotoxicity. Different cytokines may have similar biological roles, whereas other cytokines may have opposing effects on cells, depending on the environment or stimulus (33). The action of cytokines may vary considerably depending upon their concentration and that of other cytokines present in the general or specific cytokine milieu, the cells producing them, and the cells they are influencing.

1.2.2. Innate immune system

The innate immune system is usually the first line of defence when pathogens infect the body (34). Although quick to respond, the innate immune system has quite a generic role, and cannot adapt to attack specific pathogens, or contribute to long term immunity (34).

1.2.2.1. Neutrophils

Neutrophils are the most abundant circulating leukocyte and are involved in early inflammatory reactions (34, 35). The cytoplasm contains granules that are filled with enzymes and microbicidal substances, and thus are termed 'granulocytes' (34). Immature neutrophils are produced in the bone marrow and mature in the presence of granulocyte colony stimulating factor (GCSF), a process termed granulopoiesis (34, 35). Standardly, adults will have $1x10^{11}$ circulating neutrophils, which, although can live between a few hours and a few days, it has been suggested that neutrophils have a typical half-life of 6-8hrs (34, 36). At sites of infection, neutrophils will migrate into the tissue, and function through phagocytosis or neutrophil extracellular traps (NET)osis to remove invading pathogens (34, 37, 38). The function of neutrophils is described in greater detail in Section 1.3.

1.2.2.2. Low density granulocytes

Low density granulocytes were first described in 1986, which were initially termed 'low buoyant density granulocytes' (39, 40). These granulocytes were found typically in the PBMC layer of fractioned adult SLE blood, and correlated with disease activity (39, 40). Within a study by Bennett *et al*., microarray analysis detected a high expression of neutrophil genes within the PBMC layer of fractioned JSLE patient blood, and was thought that these genes were from immature neutrophils present in this PBMC layer (40, 41). A negative selection technique by Denny *et al*., was developed to purify the low density neutrophils, which the group termed Low density granulocytes (LDGs), and it was shown that LDGs were present in the PBMC fraction of all SLE patients (40, 42).

LDGs have been observed to have less segmented nuclei than normal density neutrophils, with hetero- and euchromatin delineated, and various granulocytes observed in the cytoplasm (40). Surface marker wise, LDGs express high CD15, and low CD14, which can distinguish the LDGs from CD14 high and CD15 low monocytes (40). LDGs also express the mature granulocyte markers CD10 and CD16, but only express the immature granulocyte marker CD33 weakly, suggesting LDGs aren't simply immature granulocytes and are a sub type of mature granulocytes (40). In comparison to healthy control neutrophils, both LDGs

and autologous lupus neutrophils have an activated phenotype; this was shown through the upregulation of CD66b and CD11b (40).

LDGs have increased ability to form NETs compared to control/normal density neutrophils (40). Additionally, LDGs have a decreased phagocytosis function, which may be contributing to the increased NETosis seen (40).

1.2.3. Cytokines and their role in the innate and adaptive immune system

Cytokines are small proteins that are used to signal between cells and influence different functions such as growth, chemotaxis, activation and can regulate the immune system as a whole (33). Immune cytokines can be grouped depending on which cell type produces it or its function. However, cytokines are not exclusively produced by immune cells, and can have effects on cells of different lineages (43-45). ILs are produced by leukocytes, monokines are produced by myeloid cells, lymphokines are produced by lymphocytes, chemokines are involved in cell migration and IFNs protected against invading viruses, and are able to activate cells and modulate immunity (for details on IFNs, see 1.5) (33).

Lymphokines are lymphocyte growth factors, which include IL-2, IL-3 and IL4 (33). IL-2 is made by T cells and functions as a T cell growth factor. IL-3 has an important role in haematopoiesis, IL-4 is produced by Th2 and mast cells, and has a role in growth and differentiation for Th2 and B cells (33). IL-5, which like IL-4 is produced by Th2 and mast cells, is involved in B cell activation and IgA production (33). IL-10, another cytokine produced by Th2 cells, induces the response of Th2 cells (33).

Monokines have been indicated to be critical to immune defence and inflammation functions (33). IL-1, TNFα and IL-6 can activate lymphocytes, phagocytes and the vascular endothelium (33). IL-8 is also a chemokine, as it is chemotactic for neutrophils (33). IL-12 can activate NK cells to produce IFNγ (33).

Chemokines are small specialist cytokines that are produced by many cells in response to either invading pathogen or physical damage to the tissue (33). They can activate and cause migration of cells that express the appropriate receptors to the site of infection or damage, and are involved in the migration of leukocytes into the tissue (33). Chemokines are divided into two groups; CC chemokines (proteins with two adjacent cysteines near the amino terminus) attract monocytes, and CXC (proteins with two amino terminal cysteines separated by another amino acid) can attract polymononuclear cells such as neutrophils (31, 33).

Other cytokines, which don't come under the classifications above, include colony stimulating factors, that influence the development, differentiation and expansion of myeloid cells (33). Granulocyte macrophage stimulating factor (GMCSF) induces the differentiation of myeloid cells into both monocyte and granulocyte cells, whereas GCSF and macrophage colony stimulating factor (MCSF) differentiate myeloid cells into either granulocyte or monocyte cells respectively (33). TGFβ is involved in the inhibition of B and T cell growth, TNFβ is deemed cytotoxic (33).

1.2.4. The complement system

The complement system was first defined in the 1890s, where it was shown that factors aided bacteria killing by heat stable antibodies found in the serum (46). The complement system encompasses more than 30 different proteins that are either present within the blood or are associated with membranes (46). Once the complement system is activated, a cascade of enzyme reactions leads cleavage of some glycoproteins (or complement components, classified by 'C' and a number) into active fragments (for example, C3 is cleaved into C3a and C3b), which then can activate components downstream (33). This leads to the lysis of invading pathogens, or at least some protection against such pathogens (33). It has been shown that the complement system has a role in both the innate and adaptive immune systems (46).

The complement system involves three pathways; the alternative, the classical and the lectin, which all involve C3 (46). The alternative pathway is activated by carbohydrates, lipids and proteins found on foreign surfaces which certain complement proteins are associated with specific pathogens (33, 46). The classical pathway is activated by the formation of immune complexes to invading pathogens or other foreign antigens through which antibodies are bound to either an antigen or the invading pathogen (33, 46). The lectin pathway is initiated by the binding of mannose binding lectin (MBL) or ficolin to carbohydrates on pathogen surfaces (46). MBL and ficolin create complexes in the serum with MBL-associated proteins (MASP); MASP1 has been shown to cleave C2m and thus is thought to help enhance complement activation (46).

Complement proteins have been shown to have roles in many cell signalling and processes (46). The major functions of the complement system include: an initiation of the acute inflammation through the activation of mast cells (which involves C3a and C5a); attraction of neutrophils through chemotaxis to the site of infection (which involves C5a); enhanced

attachment of the pathogen to the phagocyte (which involves C3a); and finally killing of the pathogen activating lysis (which involves C9) (33).

1.3. Neutrophil functions

The specific focus of this thesis is the investigation of neutrophils and their function in relation to JSLE, and the following sections describe the functions of neutrophils that may be important in JSLE pathogenesis. Key characteristics of neutrophils include their role in: chemotaxis, phagocytosis, NETosis, and apoptosis, which will be considered further below.

1.3.1. Neutrophil Chemotaxis

Neutrophils migrate by a process called chemotaxis (depicted in Figure 1.2) by which they move towards host or bacterial chemokines (chemotactic cytokines; see Table 1.1) or chemoattractants. These chemoattractants/chemokines have a hierarchy on neutrophil migration, which is dependent on the strength of the chemotactic signal and this has been particularly seen with IL-8 (which can be released from macrophages and epithelial cells) and N-formyl-methionyl-leucyl-phenylalanine (fMLP) (a bacterial product) (47-50). This hierarchy helps neutrophils to migrate through the vasculature endothelium to the general site of inflammation created by other leukocytes such as macrophages via chemokines such as IL-8, and then migrate specifically to the site of invading infections via chemoattractants such as fMLP (47). This suggests an importance of a range of chemokines in promoting migration of neutrophil to the appropriate site of infection, and both bacterial- and host-originated chemokines are needed to attract neutrophils.

Table 1.1. Most common neutrophil chemoattractants.

Chemoattractant	Intracellular pathway	Reference
fMLP	p38/MAPK	(47)
IL-8	PI3K/PKB	(47, 51)
C5a	PI3K/PKB	(51, 52)
Growth-regulated oncogene (GRO)α/β/γ	PI3K/PKB*	(53-55)
Leukotriene B4	PI3K/PKB*	(56-58)
IFNγ	PI3K/PKB*, #	(59, 60)

*most likely signalling pathway.

#IFNγ chemotactic ability on neutrophils found in mice.

There are two pathways that are activated in chemotaxis, the PI3K/protein kinase B (PKB) and p38/MAPK pathways, and which of these is specifically activated is dependent on the chemokine present (47). Chemoattractants such as fMLP function through the p38/MAPK pathway, and the intermediary chemokines function through the PI3K/PKB pathway (47). Additionally, fMLP has been indicated to signal through the PI3K/PKB pathway although no published data suggests IL-8 signals through p38/MAPK (55). Neutrophils preferentially migrate to end target chemoattractants (chemoattractants that attract neutrophils to the precise area of infection) such as fMLP compared to intermediary ones (chemoattractants that attract neutrophils to the general area of infection) such as IL-8 (47); this may be due to the observed inhibitory effect of fMLP on the PI3K/PKB pathway, which was independent of the IL-8 receptor (47). This suggests that IL-8 from other cells will attract neutrophils, but once in close enough proximity, the neutrophils will switch pathways, and migrate specifically to fMLP – producing bacteria and not accumulate unnecessarily with host cells within the general site of infection.

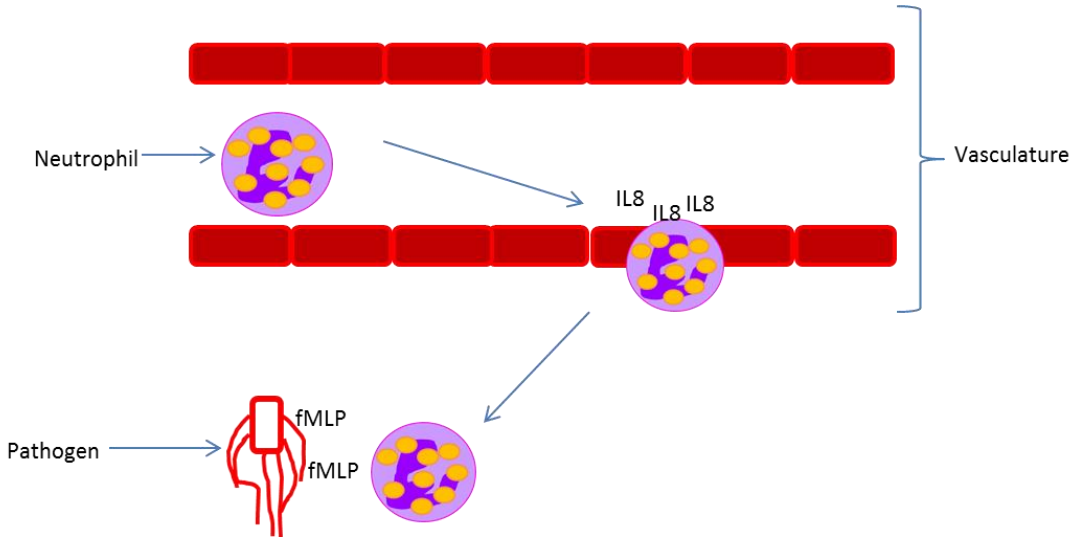


Figure 1.2. Mechanism of neutrophil chemotaxis. Neutrophils migrate from the vasculature to the general site of infection via chemotaxis towards IL-8. Neutrophils then migrate sepcifically to the invading pathogen (red outline) within the tissue via chemotaxis along a gradient of concentration of fMLP. The vascular eptithelial cells are in red, and in regards to the neutrophils, the cytoplasm is in light purple, the plasma membrane is in pink, the nucleus is in purple, and granules are in yellow.

1.3.2. Phagocytosis function

Neutrophils eliminate pathogens and cell debris by phagocytosis (Section 1.2.2.1) (Figure 1.3). Neutrophils recognise foreign particles through receptors (such as Fcγ receptors and complement receptors (61)); once the receptors are activated, particles are internalised and degradation pathways within the neutrophil are triggered to degrade the particles (38). Neutrophils are able to phagocytise opsonised (a process by which particular are coated with immune complexes that bind to immune cell receptors) and non-opsonised particles. Fc receptors and β2 integrins are the main opsonisation receptors on neutrophils, and bind to Ig and complement-coated particles (38). Of note, the process of opsonisation occurs when a pathogen or foreign molecule is coated by particular molecules, such as C3b and antibodies such as IgG, to allow the pathogen to be phagocytosed more easily (33).

Fcγ receptors are the most characterised in regards to phagocytic internalisation, and require phosphorylation of tyrosine residues (38). These phosphorylated tyrosines allow for docking of proteins with src homology 2 (SH2) domains such as Syk, which has been deemed essential for IgG-opsonised particle phagocytosis (38). These activate phosphoinositide kinases and PLC, which again are required for phagocytosis (38). This pathway induces the polymerisation of actin and thus the membrane of the phagocytosing cell is reconfigured, which then enables the host cell to engulf particles (38). Endomembranes are delivered to the particle to form a phagosomal cup (38).

The phagosome, a vesicle that is formed around the engulfed phagocyte, is matured from the phagosomal cup, by which it obtains necessary complexes to kill and dispose of the particles (38). These include microbicidal enzymes, vacuolar ATPases and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (38). The phagosome is made up through the fusion of secondary vesicles and granules, which contain microbial peptides and proteolytic enzymes (38). Granules include primary, which contains myeloperoxidase (MPO), secondary which contain lactoferrin, tertiary which contain gelatinase, and secondary vesicles which contain albumin, and express alkaline phosphatase on the membrane (38). Changes in cytosolic calcium are required for granule secretion and fusion in neutrophils in the process of phagocytosis (38).

Neutrophils generate reactive oxygen species upon activation, which are generated by NADPH oxidase (38). Assembly occurs during ingestion of particles and a superoxide anion is created, which can be converted to hydrogen peroxide and hypochlorous acid, which can act as effective antimicrobial agents (38).

Within neutrophils, by using compounds such as phosphatidylinositol-specific phospholipase C (PI-PLC), specific roles of each phagocytosis receptor can be investigated. PI-PLC can specifically affect the phagocytic receptor FcγIIIb, but not other phagocytic receptors, such as FcγII (62). Priming is the process by which cytokines can pre-determine or enhance certain processes immune cells conduct or change the state of activation of these cells. When primed (where neutrophils are pre-treated to become activated or have an altered function to that of naïve neutrophils) neutrophils from healthy volunteers were shown to have an increased respiratory burst when incubated with immune complexes *in vitro* (62). However, depletion of the FcγIIIb receptor on these healthy volunteer neutrophils reduced the primed neutrophils' respiratory burst but did not significantly reduce neutrophil related bacterial killing of heat-inactivated serum-opsonised *Staphylococcus aureus* (*S. aureus)*, nor neutrophil phagocytosis of serum-opsonised, propidium iodide-stained *S. aureus in vitro* (62). Additionally, depletion of the FcγIIIb receptor did not have any effect on the phagocytosis of non-opsonised or serum-opsonised latex particles, but did reduce the phagocytosis of IgG-coated beads, showing that FcγIIIb has a major role in phagocytosis of IgG coated particles (62). Depletion of the FcγIIIb receptor did not have any effect on the activation of the respiratory burst during phagocytosis of non-opsonised or serum-opsonised latex particles, but did reduce the respiratory burst during phagocytosis of IgG-coated beads (62). Blocking of the FcγII and the FcγIIIb receptor had a slight and equal delay on activation of the respiratory burst when neutrophils were phagocytising un opsonised and serum opsonised latex beads, whereas blocking the FcγII and the FcγIIIb receptor decreased respiratory burst, with blocking the FcγIIIb receptor more so than the FcγII receptor (62).

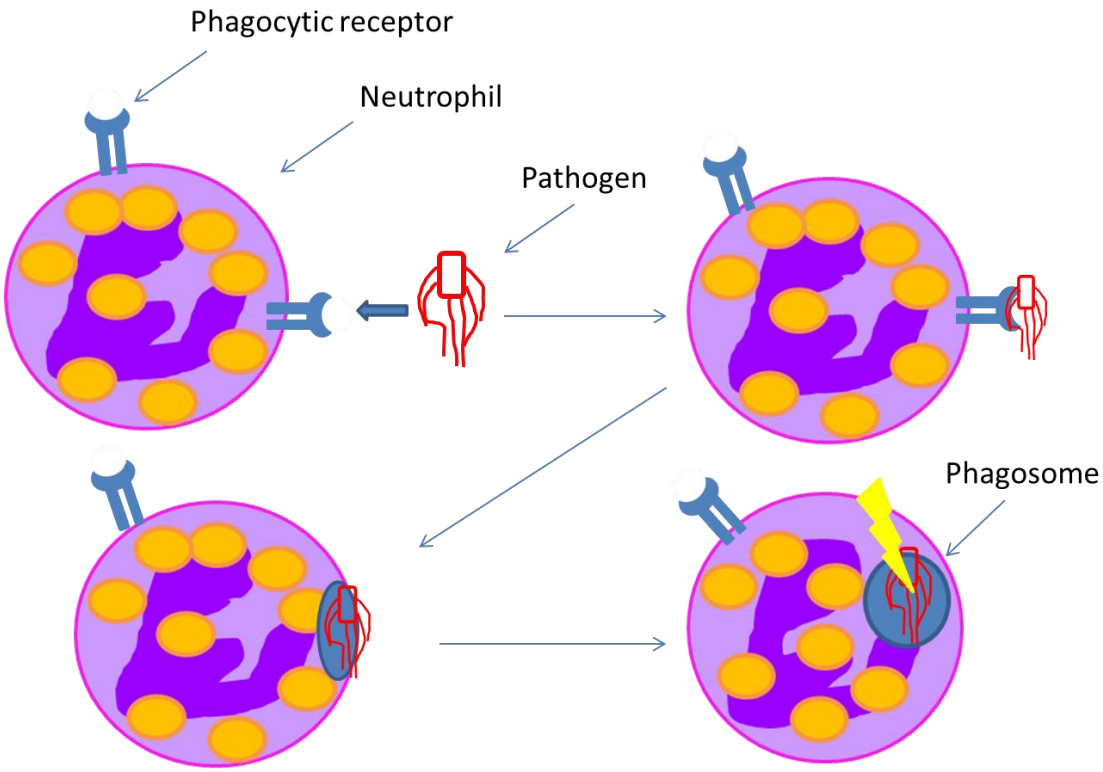


Figure 1.3. General mechanism of neutrophil phagocytosis. Neutrophils detect pathogens (red outline) and foreign particles through various receptors (such as Fcγ receptors and β2 integrins, depicted as 'phagocytic receptor'), engulf them and kill them using various enzymes within a phagosome (shown in blue). The cytoplasm is in light purple, the plasma membrane is in pink, the nucleus is in purple, and granules are in yellow.

1.3.3. NETosis function

NETosis has been described as a form of cell death that lead to the formation of neutrophil extracellular traps (NETs) by which chromatin and associated proteins are released into the extracellular space to trap and kill bacteria (Figure 1.4) (37, 63). NETs are released from activated neutrophils, and when stimulated with cytokines or products such as IL-8, probal myristate acetate (PMA) or LPS, the neutrophils became flat and their membranes protruded (37). NETs, which when measured can form large threads up to 50nm long, contained proteins from primary granules, such as neutrophil elastase (NE), cathepsin G and MPO, and proteins from secondary and tertiary granules such as lactoferrin and gelatinase (37). Additionally, DNA is the main structural component of NETs, which can be dismantled using DNase (37). Using confocal microscopy, antibody staining of NETing neutrophils (upon stimulation with PMA, IL-8 or LPS) demonstrates that histones H1, H2A, H2B, H3 and H4 are also part of the NET structure (37). NETs can associate with both gram-negative and gram-positive bacteria, which are thought to be 'trapped' in the NETs (37). Proteases and neutrophil elastase within the NETs target bacterial virulence factors, and therefore NETs can attack bacteria once trapped (37). NETs material, which included granule proteins and histones are required for bactericidal activity (37).

The mechanism of action of NETs has been studied extensively in the mouse model. This has allowed exploration of underlying mechanisms through *in vivo* studies and mouse knockout models. These can provide some insight into the possible processes of NETosis taking place in humans in an *in vivo* environment. NETs induced by *S. aureus* in exteriorised mouse skin can be visualised as sheets of extracellular DNA (64). The NETs formed are extravascular and induced by systemic and not localised infection. This migration into tissue may enhance DNA release, and prevent NETs spreading through the blood and causing damage (64). NET material, specifically histones and neutrophil elastase, is released upon injection of *S. aureus* and *Streptococcus pyogenes (S. pyogenes)*, whilst histone stained areas expanded akin to the spread of extracellular DNA also observed, indicating that the observed reaction was NET production (64). Injection of dead bacteria into the model also induced NET formation, indicating that neutrophil recognition of bacteria material is sufficient to induce NETosis (64). Using mouse knockouts, toll-like receptor (TLR)2 and C3 have been shown to be important in regulating NET formation, although neither alone was sufficient to induce NETs in isolation (64). NETing neutrophils have been shown to be viable and have an intact chemotactic function, and still be able to phagocytose (64).

Of note, NADPH oxidase is required for NETosis to occur in pulmonary aspergillosis and once committed to NETosis, neutrophils are not then able to undergo any apoptosis (65). This was shown by the de-condensation of chromatin (a hallmark of NETosis) in contrast to chromatin condensation (a hallmark of apoptosis; Section 1.3.4) and the absence of cleaved caspase 3 (the apoptosis effector protein; Section 1.3.4) (65). HMGB1 can induce NETosis through a TLR4 dependent manner (66). This is likely to be important in the context of JSLE, as it has been shown that HMGB1 is up-regulated in JSLE plasma (67, 68). This increase in HMGB1 may increase NETosis in JSLE, a function that may contribute to JSLE pathogenesis.

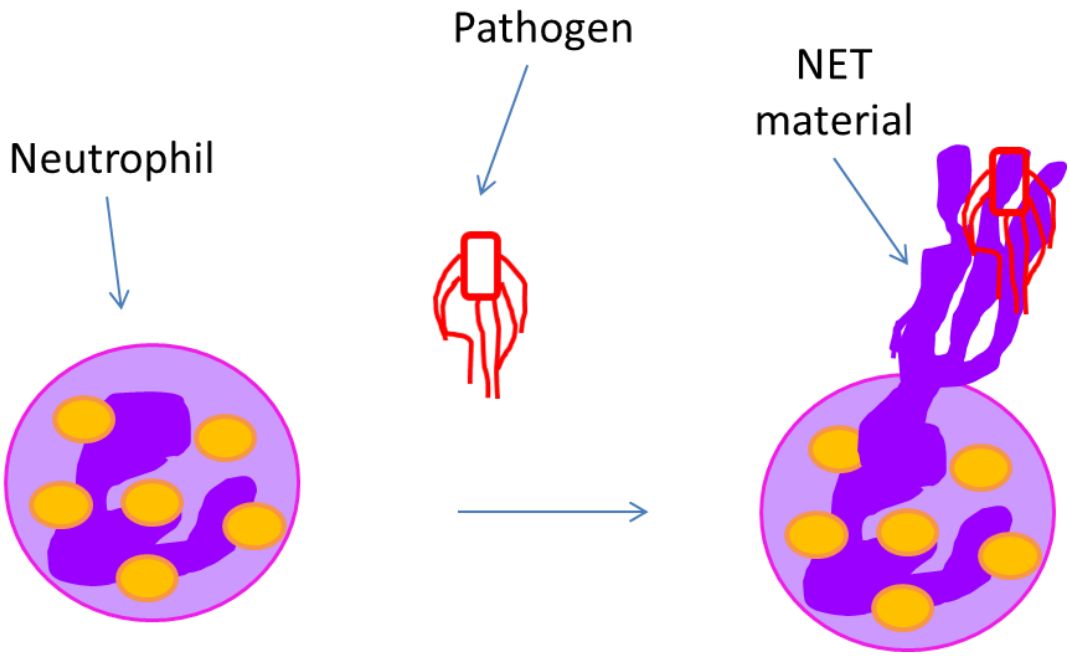


Figure 1.4. Diagram depicting the process of NETosis. Neutrophils release chromatin, which contains DNA, histones and granule proteins, which can trap and kill invading pathogens (red outline). The cytoplasm is in light purple, the plasma membrane is in pink, the nucleus and nuclear material is in purple, and granules are in yellow.

1.3.4. Neutrophil Apoptosis

Apoptosis was first characterised in 1972 by J.F. Kerr (69). Apoptosis is the process of programmed cell death (depicted in Figure 1.5), and a natural phenomenon and integral to the cell cycle. Many animal models, cell lines and *in vitro* experimentation of isolated human or animal cells have been used to investigate apoptosis.

Apoptosis is integral to many processes within the body. During embryogenesis, apoptosis has been sure to have a role in organ shape development, and helps structure fingers and toes (70). Additionally, it has been shown that both the nervous system and the immune system arise through overproduction of cells; cells which don't fully develop into differentiated nerve or immune cells undergo apoptosis to maintain homeostasis (70). Within adults, cell production and differentiation from stem cells for the majority of cell types is constant; apoptosis of cells is required to maintain the balance of cell populations (70). Additionally, apoptosis is required to remove old or damaged cells (70).

Dysregulated apoptosis is associated with many diseases. For example, increased apoptosis has been associated with diseases such as Acquired Immune Deficiency Syndrome (AIDs), neurodegenerative diseases and some autoimmune diseases such as Graft Versus Host Disease (GVHD) (70). Decreased or inhibited apoptosis has been shown in other diseases, such as cancer and importantly for this thesis, SLE and JSLE (70-72).

Using electron microscopy of both human and animal cells, apoptosis has been characterised by the formation of apoptotic bodies, via the condensation of chromatin and membrane blebbing, which forms apoptotic bodies (69). The apoptotic bodies are then taken up phagocytic cells, and then are broken down in phagosomes (69). There are two main pathways within apoptosis, the extrinsic pathway and the intrinsic pathway, and the signalling pathways controlling the apoptotic process take place via the cleavage of certain caspases from inactive procaspases (73).

The intrinsic pathway within apoptosis depends on the release of cytochrome C from the mitochondria (74). This release of cytochrome c from the mitochondria into the cytoplasm the result of the interaction of apoptotic protein Bax with the mitochondria, resulting in a pore formation in the outer membrane (75). Once released, the cytochrome C interacts with apoptotic protease activating factor 1 (Apaf1) and ATP to form an apoptosome (74). This apoptosome recruits and subsequently cleaves procaspase 9, resulting in the release of the active caspase 9 (74). Caspase 9 subsequently cleaves procaspase 3, resulting in the release of active apoptotic effector caspase, caspase 3, which induces apoptosis (74).

The extrinsic pathway within apoptosis is dependent on the interaction of a death ligand, such as Fas, to an associated death receptor (73). This binding leads to the recruitment of Fas-associated protein with a death domain (FADD) to the receptor; FADD interacts with procaspase 8 and procaspase 10 through the interaction death effector domains (DED) on both components, creating death inducing signal complex (DISC) (73). The DISC formation cleaves procaspase 8 and active caspase 8 subsequently cleaves procaspase 3, resulting in the release of active apoptotic effector caspase, caspase 3, which induces apoptosis (76).

The fine balance in pro- and apoptotic proteins that regulate apoptosis. In addition to the pro-apoptosis proteins, caspases and p53 described above, there are many anti-apoptotic proteins involved in the apoptosis signalling, an important one being B cell lymphoma (BCL)2. BCL2 can inhibit the release of cytochrome c from mitochondria, inhibiting subsequent apoptosis (73). Neutrophils typically do not have BCL2, but rather the delay in apoptosis in neutrophils is via the stability of myeloid cell leukaemia (MCL)1, another member of the BCL2 family (77). Other anti-apoptotic such as members of the inhibitors of apoptosis proteins (IAP) family including cellular (c)IAP1, cIAP2 and x-linked (x)IAP are found in neutrophils (78). These can inhibit apoptosis by inhibiting cleaved caspases, such as caspase 3, 7 and 9 (79).

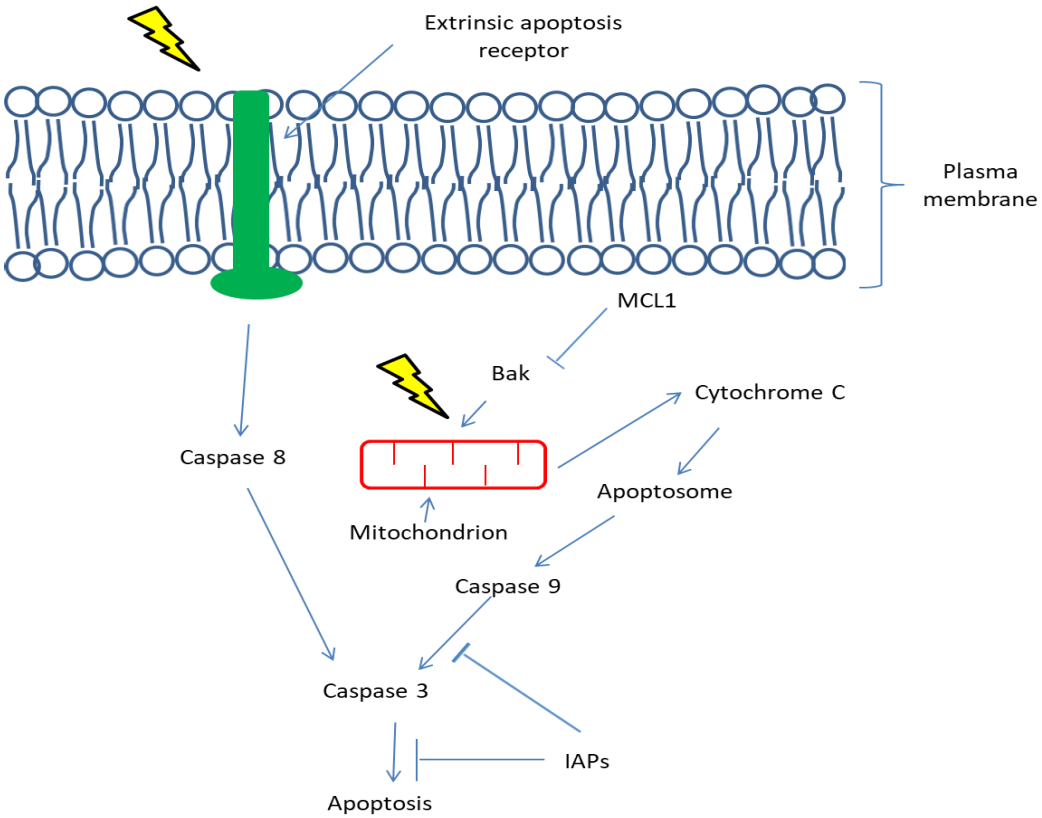


Figure 1.5. Mechanisms controlling apoptosis. The extrinsic pathway, through receptors on the plasma membrane, and the intrinsic pathway, via the release of cytochrome c from the mitochondria, and the FADD independent pathway, via genotoxic stress and p53, induce apoptosis through the cleavage of caspases. MCL1 and IAPs can inhibit apoptosis at various stages of apoptosis by interacting and inhibiting various apoptotic proteins and factors.

1.3.5. Neutrophil priming

Priming of neutrophils encompasses an exposure to a priming agent, which modulates an enhanced activation and/or function by subsequent stimuli (80). It has been thought that priming of neutrophils is important in host defence, but also associated with certain diseases, whereby priming is linked to disease severity or activity (80).

It is known that certain cytokines, such as TNFα and GMCSF, are known to prime neutrophil function such as respiratory burst (81), and this priming can lead to activated neutrophils, a phenotype commonly found in inflammatory diseases such as SLE (82, 83). Through priming, TNFα and GMCSF can influence the expression of proteins in neutrophils (84), and this may influence how other cytokines influence neutrophil function.

TNFα and GMCSF can prime healthy volunteer neutrophils to undergo a respiratory burst *in vitro* via the upregulation of p47 phosphorylation and TNFα priming can elevate the effect of other signalling pathways such as fMLP on the respiratory burst (85-88).

GMCSF has also been shown to enhance TLR2 expression and subsequent TLR2-mediated IL-8 responses in healthy volunteer neutrophils *in vitro* (89). Along with TNFα and GMCSF, IFNγ can also prime neutrophils; priming neutrophils *in vitro* from healthy volunteer and malignant melanoma patients with GMCSF, TNFα and IFNγ increases the neutrophil cytokine production of IL-1β, IL-6 and TNFα upon subsequent opsonized zymosan stimulation (90). These same processes resulting in the priming of neutrophils may be important in the pathogenesis of SLE and JSLE. *Ex vivo*, it has been shown that TLR2 is increased in $CD4^+$ T cells in patients with SLE compared to healthy controls, which enhance immune reactivity, and TLR2 is required for autoantibody production and development of renal disease in a lupus mouse model (91, 92). Thus, activated neutrophils in JSLE may have increased TLR2 expression, which may contribute to dysregulated neutrophil function. Increased numbers of activated neutrophils can be seen in SLE. This activated neutrophil phenotype may contribute to the increased cytokine production observed in SLE, and thus may have a contributory role to the development of a pro-inflammatory environment in these patients (93, 94).

Published data has demonstrated that *ex vivo* neutrophils from individuals with SLE are more activated than neutrophils from healthy individuals (83). Neutrophils from individuals with JSLE may also demonstrate increased neutrophil activation in comparison to neutrophils from healthy individuals. To investigate the role of priming of neutrophils in the aetiopathogenesis of JSLE, an experimental model would need to use both naïve and activated neutrophils. To measure neutrophil priming, which models neutrophil activation,

particular protein markers of neutrophil activation/priming can be analysed using flow cytometry. Upon *in vitro* priming of neutrophils, activation markers undergo changes in expression. For example, expression of CD11b is typically increased, and CD62L is typically decreased. Priming agents such as TNFα can initiate these changes, which can additionally influence the downstream expression and activation of other proteins in the relevant signalling pathways (85, 86, 95, 96). Of note, CD11b is an integrin that forms part of the Mac-1, an integrin receptor, also known as CC chemokine receptor (CCR)3 and is involved in functions such as chemotaxis and phagocytosis (97-100). CD62L (L-Selectin) is an adhesion molecule that can mediate leukocyte rolling (i.e. migration within the blood) (101). It is apparent that *in vitro*, and probably *in vivo* priming and subsequent activation of neutrophils may influence downstream neutrophil function, and other signalling pathways via changes in expression or activation of certain membrane or intracellular proteins. This altered function, which may include dysregulated apoptosis and pro-inflammatory cytokine production, may contribute to the pathogenesis of JSLE.

1.4. Immune system in autoimmune diseases

Autoimmunity occurs when an individual's immune system becomes highly activated towards self-antigens (autoantigens) and as a consequence the surrounding tissue becomes injured (34). One organ can become affected (organ-specific, such as type 1 diabetes), or multiple organs can become affected (systemic, such as SLE). Autoimmune diseases are characterised by immune complexes, auto reactive T cells and circulating autoantibodies. Autoantigens are ever present in autoimmune disease and amplify and sustain immune responses which cause the disease to be chronic. However, clinical and pathological features can differ and diseases are characterised by the dominant autoimmune features (34).

1.4.1. Immune system in autoimmune diseases other than JSLE

There is a fine signalling balance between the innate and adaptive immune systems, by which the innate system immediately attacks any pathogens, and also signals to the adaptive immune system, which can have a delayed response (102). DCs are important in this link between innate and adaptive immune system as they are critical in promoting primary T and B cell responses to invading pathogens (102). T cells that are reactive to autoantigens, and such are named self-reactive T cells are usually removed in the thymus, although some are found in the periphery (102). These cells are usually dealt with through mechanisms, such downregulation of self and non-self-antigens by $CD4^+$ cells containing the IL-2 receptor α

chains, so that they are tolerated and therefore do not react with host cells and so avoid triggering an immune response (102, 103). However, intercurrent infections and up-regulated stimulation of antigen presenting cells can break this tolerance and can prime the activation of self-reactive T cells which may lead to autoimmunity (102).

Macrophages can also contribute to the development of autoimmunity, through production of such cytokines such as TNFα, IL-1 and IL-6, and chemokines such as CCL5, CXCL9 and CXCL10, which can promote recruitment of Th1 cells and NK cells to the site (104). Neutrophils can produce NETs (see 1.3.3) which contain histones, DNA and HMGB1, that may contribute to autoimmunity underlying diseases such as JSLE and rheumatoid arthritis (RA) (104) (Section 1.5.3.2 and Section 3.1).

In the example of type 1 diabetes, T cells attack insulin producing pancreatic beta cells, and it has been suggested that TLR-mediated innate immune responses can contribute to diabetes induction in mice and that $CD4^+$ and $CD8^+$ T cells also promote further inflammation in this tissue (102, 104). Innate immune cells such as γδ T cells, macrophages and neutrophils may play an essential role in the pathogenesis of type 1 diabetes, through the production of cytokines that promote β-cell apoptosis, (which leads to the loss of β-cell and reduction in insulin production) and increase the recruitment of the T cells that destroy remaining β-cells (104). Diabetic ketoacidosis may trigger an inflammatory response, as it may increase the levels of CXCL8, IL-1β and TNFα (105). Additionally, M1 macrophages can initiate pancreatic β cell death, via the production of ROS, and can also activate T cells within diabetes (104). It was suggested that number of neutrophils are reduced in the blood of patients with type 1 diabetes (106). Increased recruitment of neutrophils noted in the pancreatic tissue in neonatal non-obese diabetic (NOD) mice may explain reduced circulating neutrophils found in diabetic patients (106, 107). Increased neutrophil infiltration into the pancreas may contribute to β-cell destruction, and subsequent diabetic development.

Within the example of RA, environmental factors may be a cause of molecular changes to proteins, and a breakdown of immune self-tolerance (104). These factors can also lead to autoantibody development towards citrullinated protein antigens (108). Many cells are involved in the pathogenesis of RA, including T and B cells, neutrophils and fibroblasts (104). As they are usually the first at the site of inflammation, neutrophils may play a role in tissue damage and initiate inflammation in RA, as neutrophil depletion in animal models could block joint inflammation and swelling in RA (104). Neutrophils are abundant in synovial fluid in active RA and can release high concentrations of oxidants such as ROS, cytokines such as

TNFα and granules containing proteases and myeloperoxidases into the synovial fluid or directly on cartilage surfaces (104). NETs are increased in peripheral blood and synovial fluid in RA patients compared to healthy individuals (peripheral blood) and patients with osteoarthritis (synovial fluid) respectively, and are a source of the citrullinated proteins that act as antigens, such as vimentin, and it has been shown that RA serum auto-antibodies can cause RA and control neutrophils to undergo NETosis (108).

Within a model of experimental autoimmune encephalomyelitis (EAE), an animal modal of multiple sclerosis, $CD4^+$ T cells mediate the progression of the disease (102). Activation of innate immune cells via microbial TLR ligands was deem critical for priming of $CD4^+$ T cells and progression of EAE (102). Induction of EAE requires signalling through myeloid differentiation primary response 88 (MyD88) (102). Myeloid DCs (mDCs) were also shown to induce the expansion of IL-17 producing $CD4^+$ T cells within EAE (102). pDCs however were found in central nervous system (CNS) and prevent mDC-induced pro-inflammatory $CD4^+$ T cells responses via IL-17 and IFNγ (102). Additionally CD8α- mDCs can suppress myelin-specific T cell function, and therefore the severity of EAE by producing anti-inflammatory IL-10 (102).

1.4.2. Immune system in adult onset SLE and JSLE

The role of the immune system in SLE is highly diverse and involves many aspects. It is typically characterised by the development of autoantigens directed against nuclear autoantigens, and the loss of tolerance by other cells to these autoantigens (109). Exposure to antigens activates T and B cells and their subsequent proliferation (109). B cells produce autoantibodies that can contribute to tissue injury and can take up autoantigens which then allow exposure of more autoantigens to auto-reactive T cells (109). It is of particular note that the main mechanism of interest for B cells in particular is through the interaction of autoantigens and TLRs (109). Therefore, unpicking the immune system in regards to SLE and JSLE pathogenesis is fundamental to understanding the disease, and finding potential therapeutic targets.

1.4.3. Role of neutrophils in adult onset SLE and JSLE

Within healthy individuals, neutrophils mature in the bone marrow, and once mature are released into the blood stream (104). Neutrophils then migrate to the tissues to perform immune functions; once complete and with no further stimulus, they undergo apoptosis and are cleared by macrophages to maintain homeostasis (104). This death and clearance is

efferocytosis, and its decreased function, and decreased clearance of neutrophil apoptotic debris, may contribute to autoimmunity such as SLE (104).

Dysregulated neutrophil apoptosis clearance has been shown to be particularly heightened in both SLE and JSLE and have been indicated as sources of autoantigens such as dsDNA, again through the exposure of chromatin (71, 72, 110). As there is indication that neutrophils are a source of autoantigens due to being particularly both NETotic and apoptotic in JSLE, neutrophils are potentially fundamental to JSLE (111).

Phagocytosis, and in particular neutrophil phagocytosis, has been shown to be reduced in SLE and JSLE (112-115). The decreased phagocytosis function of neutrophils and other immune cells such as macrophages may contribute to the reduced clearance of the increased neutrophil apoptotic debris (112, 113, 115). This indicates that dysregulated phagocytosis may also be an important contributor to the build-up of autoantigens that may be coming from apoptotic material.

Neutrophil migration, or chemotaxis, is an important function within immune responses. However, it has been indicated that neutrophil chemotaxis is dysregulated in SLE and JSLE (112, 116, 117). Many demographical and clinical aspects have been shown to contribute to dysregulated chemotaxis in SLE and JSLE; however, consensus of published data showed that neutrophil chemotaxis is generally reduced in SLE and JSLE, although one study suggests an increase in chemokines in SLE patient plasma (112, 116-118). This may result in accumulation of neutrophils at sites of inflammation and cause tissue damage that contributes to JSLE pathogenesis.

1.5. Interferons and SLE

IFNs are cytokines, categorised into 3 groups; type 1 encompasses the biggest group and includes IFNα and IFNβ, type 2 includes IFNγ only, and type 3 includes IFNλ only (120). The initial role for type 1 IFNs in particular involves antiviral effects, and type 2 expands on this, and can have roles in development of host protection against pathogens, and anti-tumour immune response and can also amplify antiviral activity by IFNα or IFNβ (121). Type 3 IFNs can modulate Th1 and Th2 responses in the form of IL-13 and IFNγ secretion (122).

IFNα is one of the main type 1 IFNs and is produced by activated pDCs upon interaction with other cells, such as neutrophils (123). Specifically, autoantigens such as dsDNA and mitochondrial (mt)DNA expressed by neutrophils can activate pDCs, and these then can

produce an increased amount of IFNα (123). IFNα has been correlated with SLE severity and increases T cell activation and B cell activation and survival (124-126).

IFNβ is an early response cytokine after viral infections, as shown through the increase of its messenger ribonucleic acid (mRNA), and has been shown to be important for positive feedback loop for the expression of IFNα in mouse fibroblasts (127). Both pDCs and myeloid DCs produce IFNβ upon activation through TLRs (128).

IFNγ is the only type 2 interferon, and is structurally different from the type 1 interferons and signals through a different receptor (129). Many cells produce IFNγ, including T cells, which are involved in adaptive immune response, and NK cells, which are involved in early responses (129). IFNγ production is controlled by cytokines, of note IL-12 and IL-18 and negative regulators of IFNγ include IL-4 and TGFβ (129).

1.5.1. JAK/STAT pathway

The Janus kinase (JAK)/ signal transducers and activation of transcription proteins (STAT) pathway regulates a host of processes (including cell proliferation, migration and apoptosis) and is the principal pathway for a wide variety of cytokines (130-133). The JAK family comprises of four proteins, JAK1, JAK2, JAK3 and tyrosine kinase (Tyk)2 (130). The JAKs range from 120kDa to 140kDa in size, and comprise seven conserved JAK homology (JH) domains (134). Two carboxyl-terminal JH regions contain a kinase and pseudo kinase domain, whereby activation is through phosphorylation of tyrosines within an inactivation loop (134). Four amino-terminal JH regions contains four point one, ezrin, radixin, moesin (FERM) domain that associates with receptors via the proline-rich, membrane-proximal box1/box2 domain on the receptors. An SH2 related domain lies between the psuedokinase and FERM domains, although the function remains unknown (134). The structure is depicted in Figure 1.6.

The STAT family comprises of seven proteins; STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b and STAT6 (135). STATs range from 750 to 900 amino acids in size, STATs have a similar structure consisting of a amino-terminal domain, coiled-coil domain, a DNA binding domain, a linker domain, an SH2 domain, a phosphotyrosyl tail segment (whereby a tyrosine is phosphorylated upon STAT activation), and with the exception of two STAT isoforms, STAT1β and STAT4β, have a transactivation domain (134, 136). The amino terminal, approximately 125 residues, is implicated to be involved in homodimers of inactive STAT dimers, and is important in cooperative binding to DNA (see below) (134). The coiled-coil domain,

consisting of approximately residues 135-315, creates a large hydrophilic surface that binds regulators (134). The DNA binding domain, consisting of residues 320-480, contains a β-barrel immunoglobulin fold that can bind to the gamma interferon activation site (GAS) family of enhancers (which STAT1 dimers bind) (134). The corresponding domain in STAT1/STAT2 heterodimers is yet to be determined (134). The adjacent residues, 480-580, is the linker domain and maintains the right conformation between the DNA-binding and dimerization domains (134). The SH2 domain (consisting of residues 575-680) is the most conserved domain, and the tyrosine activation domain (at approximately residue 700) is situated directly adjacent to the SH2 domain (134). Lastly, the carboxyl terminal, which differs between STATs, contains the transactivation domain (134). Within resting cells, STATs form inactive homodimers, and remain in the cytoplasm (134). Activation of the STATs is via an initial activation and phosphorylation of the JAK proteins, and as such phosphorylation of the STATs and their subsequent dimerization (136). Activated dimers translocate to the nucleus and bind to specific gene sequences (134); STAT1 homodimers via IFNγ stimulations bind to GAS family of enhancers, whereas type 1 IFN stimulation results in STAT1/STAT2 heterodimers which form interferon stimulated gene factor (ISGF)-3 with interferon regulatory factor (IRF)-9 and bind to the ISRE family of enhancers (134).

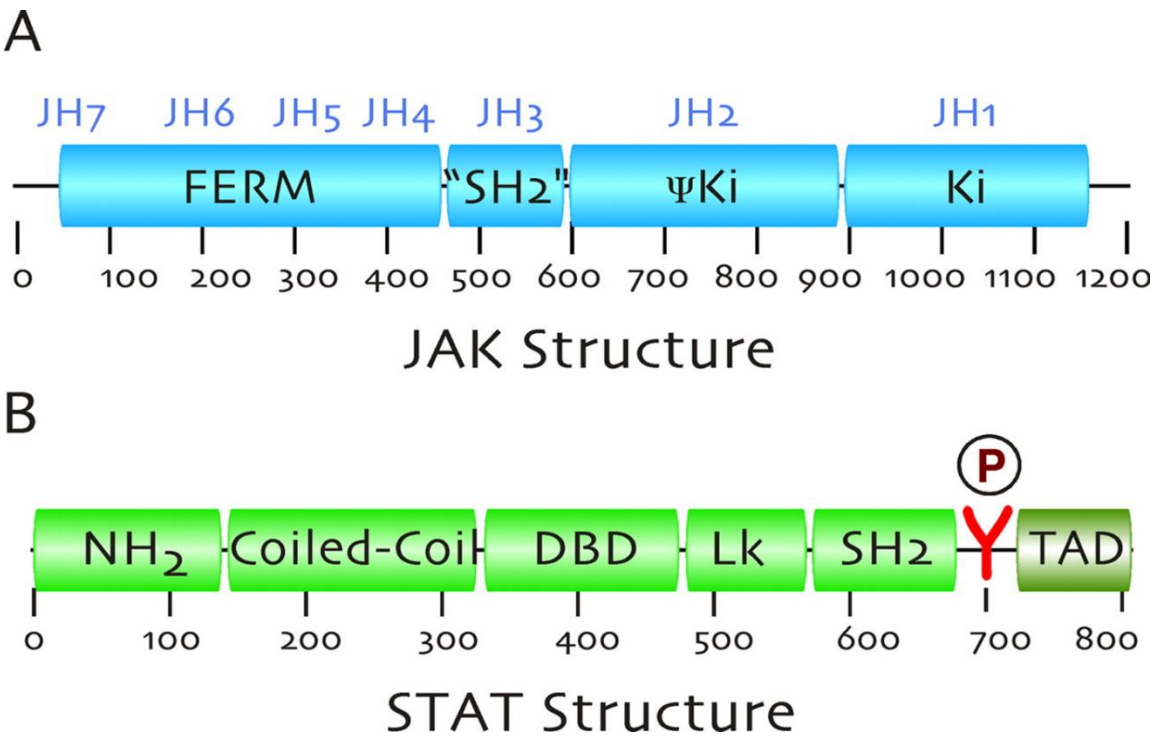


Figure 1.6. Depiction of the structures of JAK (A) and STAT (B) families (by Schindler *et al.* (134)). The JAK structure constitutes JH domains, which create a FERM domain, a SH2-related domain ("SH2"), a pseudo kinase domain (ψKi) and a kinase domain (Ki). The STAT structure contains an amino-terminal domain (NH_2), a coiled-coil domain, a DNA-binding domain (DBD), a linker domain (Lk), an SH2 domain, a phosphotyrosyl tail segment/tyrosine activation domain (Y) and a transcriptional activation domain (TAD).

The dimerization of STAT1 in particular is via the phosphorylated tyrosine within the SH2 domain and interaction of alanine in position 77 (137). Additionally, substitution of alanine to phenylalanine at position 77 leads to dimerization of unphosphorylated STAT1 in a conformation entitled 'anti-parallel' (137). The parallel conformation arises through the stabilisation of SH2-phophtryosine interactions, and tyrosine-phosphorylated STAT1 dimers are able to switch between anti-parallel and parallel conformations (137). pSTATs then translocate to the nucleus to activate transcription of target genes, the target being dependent on cytokine stimulus and the specific STAT (136). Cooperative DNA binding is the principle of switching between occupied and non-occupied promotor states, and STAT1 cooperative binding is important in recruiting gamma interferon activation factor (GAF) to gene promotors, and subsequent IFN gene expression (137). Mutations within the N domain (i.e. substitution of alanine to phenylalanine at position 77 within a mouse model) disrupts IFNγ-related STAT1-cooperative DNA-binding, and can lead to increase in bacterial infections such as Listeria monocytogenes within mouse macrophages (137).

In vitro, STAT1 has been shown to be pro-apoptotic in a variety of cells, including A431, Hela and HT29 and published data have shown that this can be via the up regulation of Fas and Fas ligand (FasL) expression or through expression of caspase 1 (138, 139). STAT2 was shown to be particularly important in antitumor effects of type 1 IFNs (140). STAT3 is considered anti-apoptotic, and has been shown to induce type 1 and 2 IFN mediated anti-apoptosis in neutrophils (141). STAT4 is important in the differentiation and proliferation of Th1 cells (142). STAT5 was shown to be involved in suppression of the tumour protein, p53, and enhanced Mouse double minute 2 homolog (MDM2), and thus may be important in regulating apoptosis and contribute to cell survival (143) STAT6 is involved in the induction of regulatory T (T_{reg}) cells, and Th2 immunity promotion (144, 145).

Ligands such as IFNs activate the signalling pathway by initially dimerising the receptor subunits, such as IFNα receptor (IFNAR) and IFNγ receptor (IFNGR), these receptors are then able to phosphorylate JAK tyrosine kinases, which associate with the receptor (see Figure 1.6) (146-149). Different JAK kinases associate with different receptors, as JAK1 and JAK2 are shown to be phosphorylated by IFNγ, whereas JAK1 and Tyk2 are phosphorylated by IFNα, contributing to the dynamic signalling of the JAK/STAT pathway (148, 150). STATs can associate with the receptor and can be phosphorylated on the SH2 domain by the JAK kinases (149, 151). Again, different STATs associate with different JAKs and receptors, such as STAT2 with IFNα receptor allowing for multiple cytokines to exert different effects (149). Phosphorylated STATs can dimerise through their SH2 domains and the phosphorylated sites

and are able to enter the nucleus via importin alpha 5 and 7 pathway, and bind to specific regulatory sequences to activate or repress the transcription of target genes (151-153). The JAK/STAT pathway is involved in neutrophil apoptosis and up-regulates apoptosis genes in JSLE.

1.5.1.1. STAT3

Within healthy volunteer neutrophils, in an *in vitro* setting, it was shown that 1000U/ml GMCSF can induce the JAK/STAT pathway, via JAK2 and STAT3, as shown by an electrophoretic mobility shift assay (EMSA) (154). This *in vitro* STAT3 phosphorylation by GMCSF was shown to reduce the level of neutrophil apoptosis, an observation also seen in JSLE-serum activated neutrophils (110, 154). *In vitro* STAT3 depletion by an antisense oligonucleotide increased the level of apoptosis after GMCSF stimulation, indicating an important STAT3 role in GMCSF delayed apoptosis (154).

MCL1 protein and mRNA was also increased upon GMCSF treatment, and it was confirmed *in vitro* that GMCSF delayed healthy volunteer neutrophil apoptosis via increased MCL1 stability (77, 154). These studies highlight a potential mechanism by which the GMCSF delays apoptosis via the phosphorylation of STAT3 and an increased stability of MCL1. Additionally, it has been shown *in vitro* that IFNs can be anti-apoptotic in healthy volunteer neutrophils, which is through the phosphorylation of STAT3 and up-regulation of cIAP2 (141). IFNs may delay apoptosis, particularly in the neutrophils of healthy individuals, and this may be via the phosphorylation of STAT3 and leading to increased stability of MCL1. In JSLE neutrophils, however, STAT3 may be down regulated, and its potential effect on MCL1 and delayed apoptosis may be reduced.

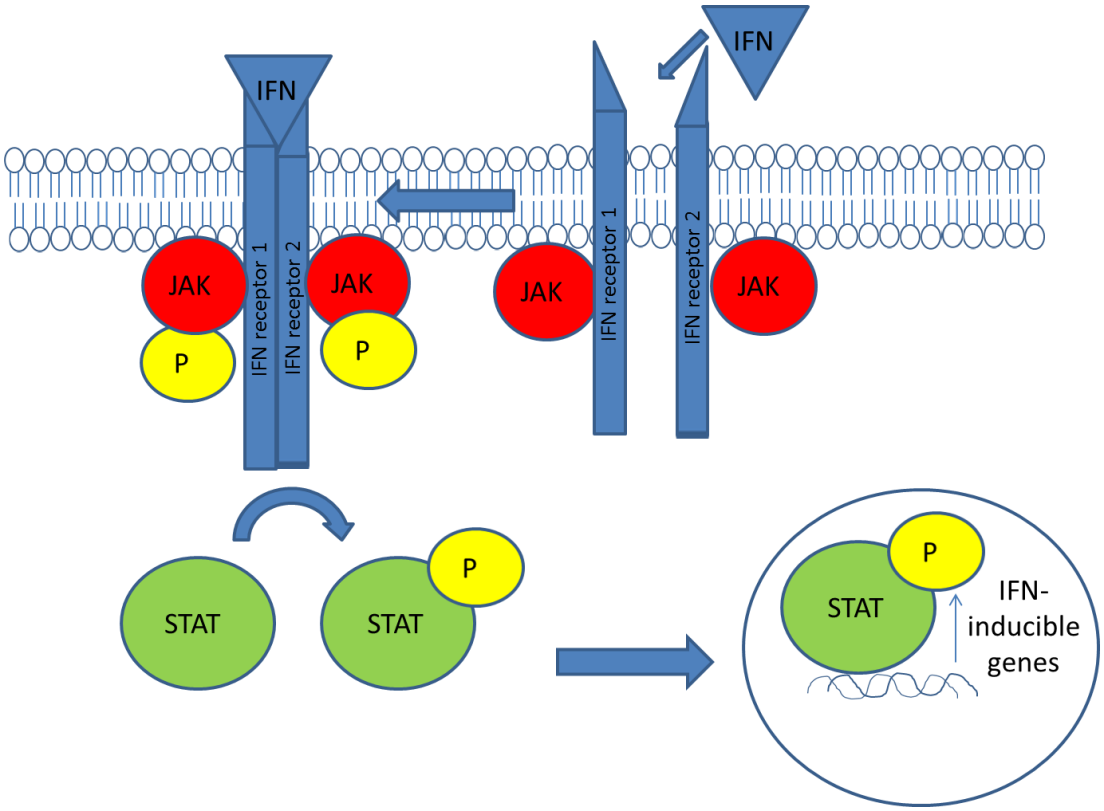


Figure 1.7. The JAK/STAT pathway. IFNs bind to the IFN receptor, which allows for dimerization. JAKs associate with the receptor and phosphorylate each other. This then leads to the phosphorylation of STAT1, which transports to the nucleus and up-regulates IFN-inducible genes.

1.5.1.2. Interplay of STATs within IFN signalling

The effect of IFN signalling depends on the phosphorylation and interaction of STATs with each other and downstream components, which results in a variety of different processes. It has been shown that IFN signalling can lead to augmented TLR signalling within macrophages, through the activation of STAT1 (155). Interestingly, within STAT1 deficient macrophages, IFNγ and IFNβ can suppress TLR signalling, through the reduction of activated NFκB and subsequent production of cytokines such at TNFα and IL-12. This correlated with sustained phosphorylation of STAT3 (155), suggesting a switch in IFN/STAT signalling. Additionally, it has been shown that in the absence of STAT1, IFNγ can activate STAT3 via SRC-family kinases within mouse embryo fibroblasts (MEFs) (156). STAT1 and STAT3 have similar structures and activation domains, and this leads to STAT1 and 3 activating overlapping but distinct genes; this explains how, in STAT1-null MEFs, STAT3 drives the expression of STAT1 genes (156). Contextually, this may be important in regards to apoptosis; STAT1 has been shown to be pro-apoptotic, and STAT3 has been shown to be anti-apoptotic (138, 141). STAT2, which is usually associated with type 1 IFN signalling, has been identified to be part of the IFNγ pathway, and can inhibit STAT1, but not STAT3, in multiple pathways (157). The study by *Ho et al.* suggest that this STAT2 related STAT1 inhibition may allow for a shift towards STAT3 related signalling, and therefore may be important in IFN-related apoptosis signalling (157). Additionally, STAT1 mediated cytokine function depends on 'free' STAT1 and that although STAT1 is present in excess compared to STAT2, IFN stimulation increases STAT1:STAT2 dimers (157), and therefore likely to be regulating the amount of activated STAT1 signalling. These dynamics may be dysregulated within SLE and JSLE, in particular in regards to JSLE apoptosis.

1.5.1.3. SOCSs

Suppressors of cytokine signalling (SOCS) proteins were shown to be STAT target genes that can antagonise STAT activation, resulting in a negative feedback loop (134). SOCS1, SOCS2 and SOCS3 can inhibit the IFNγ/STAT1, IL-12/STAT4, IL-4/STAT6. GH/STAT5 and IL-6/STAT3 pathways (134).

Understanding of the mechanisms of SOCS proteins have been derived from SOCS1 and 3, which have overlapping functions but some unique differences (158). Both SOCS1 and 3 can block JAK/STAT signalling through inhibition of JAK enzyme; SOCS1 and SOCS3 interact with the phosphotyrosine found in in the JAK catalytic loop and has also been shown to interact phosphotyrosine residues on the IFNα receptor 1 chain (IFNAR1) and IFNγ receptor 1 chain (IFNGR1) (158).

Kinase inhibitory region (KIR) of SOCS 1 and 3 (a 12 amino acid domain located on the amino terminal, adjacent to the SH2) is required for inhibition of JAK kinase activity (158). It was shown to work as a pseudo-substrate, and blocking the catalytic cleft of the JAK to prevent further enzymic activity (158). The SOCS family is typically defined by a 40 amino acid SOCS box motif, which is generally located at the carboxyl terminus (158). The SOCS box consists of three α helices which are attached to an E3 ubiquitin ligase complex (158). Within this region, the SOCS box also contains an E1 ubiquitin-activating enzyme, and an E2 ubiquitin-conjugating enzyme, which contribute to the polyubiquitination and proteasomal degradation of the proteins which SOCS bind to (158). The SOCS box of SOCS1 has been shown to ubiquitinate targets such as JAK2, signalling for degradation (158). The induction of SOCS protein expression by cytokine stimulation has been implicated to be STAT dependent (158). Cytokine stimulation rapidly induces SOCS1 and SOCS3 expression and both are degraded upon termination of said signalling, suggesting that the half-life SOCS protein is tightly regulated (158).

1.5.2. Interferon receptors

IFNs signal through receptors on membrane surfaces to activate the JAK/STAT pathway. The type 1 IFNs signal through the IFNα receptor (IFNAR). However, it has been shown that the potencies and effects of this binding and signal vary between IFNs (159, 160). Type 2 IFNs signal similarly through the IFNγ receptor (IFNGR) (161, 162). Both receptors consist of two distinct chains, and the expression and dimerization of these chains are important in how the signalling pathway is activated (146, 147). In particular, it has been shown that the IFNGR chains have differential expression on different immune cell lines, and this differentiation can contribute to whether IFNγ induces a signalling cascade to induce cell proliferation or apoptosis in isolated healthy volunteer T cells and the T cell line (161, 162).

1.5.3. Roles of IFNs on neutrophil function in JSLE

Although this is covered in in subsequent chapters in more detail (see Chapter 3 and 4), it is of note that the IFNs have each been shown to have significant effects on neutrophil apoptosis, NETosis, chemotaxis and phagocytosis, which all of have been indicated to be dysregulated in SLE, and therefore could be possible contributors to the pathogenesis of both SLE and JSLE (71, 72, 112, 116, 117, 163).

1.5.3.1. IFNs and Apoptosis in JSLE

IFNs have been shown to be both pro- and anti-apoptotic *in vitro*, and this is dependent on the signalling pathway, whereby STAT1 is pro-apoptotic, and STAT3 is anti-apoptotic (138,

141, 164-166). This may also depend on the activation state of neutrophils. Neutrophils have been shown to be activated in SLE, and probably also JSLE patient neutrophils (83). Cytokines such as GMCSF, TNFα and IFNγ can prime/activate neutrophils *in vitro* and this can alter the signalling pathways, both extracellular and intracellular protein expressions, and neutrophil function (81, 85, 86, 90, 95, 167-169). Thus, it is likely that the probable activation of JSLE patient neutrophils may account for the up regulation of the pro-apoptotic proteins shown within JSLE patients neutrophils *in vitro* (170). It is also possible that IFNs are involved in the up-regulation of neutrophil apoptosis within JSLE due to their role in pro-apoptosis, and the high IFN genetic signature and high IFN serum levels found within SLE and JSLE patients (41, 68, 72, 171-174) (Section 1.4.2).

1.5.3.2. IFNs and NETosis in JSLE

It has been suggested that IFNs prime neutrophils to undergo NETosis *in vitro* (175). However, it is important to note that it is likely that NETosis is more important for the increase in IFNα production from pDCs found in JSLE patients and *in vitro* in particular. This may contribute in turn to the increased IFNα concentration measured within JSLE serum, would could lead to more neutrophils being primed for NETosis, creating a positive feedback loop (68, 172, 175, 176). It would therefore be of importance to investigate the role of IFNs on NETosis, either as priming agents or whether they stimulate NETosis in naïve or TNFα-primed neutrophils.

1.5.3.3. IFNs and chemotaxis in JSLE

IFNs have been shown to have an influence on chemotaxis, although this can be differential depending on IFN and cell type. *In vitro*, IFNβ was shown to reduce healthy volunteer PMBC migration towards chemokines RANTES, macrophage inflammatory protein (MIP)1α and monocyte chemoattractant protein (MCP)1, and in particular can reduce healthy volunteer T cell chemotaxis (177, 178). Alternatively, *in vitro*, IFNγ can up-regulate healthy volunteer neutrophil chemotaxis through the upregulation of CCR1 and CCR3, which recognise chemokines such as MCP3 and RANTES, however, IFNγ had no effect on healthy adult T cell chemotaxis *in vitro* (178-182). It is suggested that IFNγ in particular has an influence on neutrophil chemotaxis, and it would be of interest to investigate the role of IFNs on neutrophil chemotaxis, and how this may relate to dysregulated neutrophil chemotaxis seen in SLE, and possibly in JSLE (116, 117).

1.5.3.4. IFNs and phagocytosis in JSLE

IFNs can affect phagocytosis. However, this effect can be differential depending on the type of IFN, the targeted immune cell, the phagocytic pathway being followed, and the particle

being phagocytosed. For example, within mouse peritoneal macrophages, IFNs have a differential effect on phagocytosis *in vitro*; IFNα/β can increase the phagocytosis of *Escherichia coli (E.coli)*, whereas IFNγ reduced this phagocytosis (183). Both IFNα and IFNγ have been shown to up-regulate neutrophil phagocytosis of *S. aureus in vitro* (184). Of note, many receptors are involved in phagocytosis, and *in vivo* administration of IFNγ in healthy volunteers has been shown to influence receptors such as the FcγRI on healthy volunteer neutrophils *ex vivo*, which in turn may have an effect on phagocytosis *in vitro* (185). Thus, IFNs may be involved in the dysregulated neutrophil phagocytosis seen in JSLE (112).

1.5.4. Implications of IFN/JAK/STAT pathway in JSLE

1.5.4.1. IFN expression and SLE/JSLE

*I*t has previously been shown that there was both an increase in type 1 and 2 IFNs in the serum of SLE patients, and a recent study has shown that IFNγ was increased in SLE serum (171, 186). In recent years, it has been shown that IFNα is increased in JSLE serum (68, 172) and it may be that IFNγ is also raised in JSLE.

Both adult-onset SLE and JSLE are characterised by an increased granulocyte and IFN gene signature (a set of genes deemed associated with granulocytes or stimulated by IFNs), with distant type 1 IFN genes and distinct type 2 IFN genes elevated in SLE patients (41, 174) suggesting an interaction between raised IFNs within the serum and neutrophils contribute to the pathogenesis of JSLE. Increased granulocyte genes within JSLE PBMCs includes *defensin alpha 3* (*defa3)*, which has antibacterial functions, and *eosinophil derived neurotoxin* (41). IFN associated genes that were found upregulated in JSLE PBMCs included genes that code for Tumour necrosis factor-related apoptosis-inducing ligand (TRAIL), IRF-7b and inferferon stimulated gene (ISG)-15 (41). Additionally, IFNAR may have an important role in the increased type 1 IFN signature expression seen in JSLE (41, 187). Of note, healthy neutrophils stimulated with JSLE serum had dysregulated genes found in both the TLR and IFN pathways, which include an increase in *irf-7,* and *stat1*, and a decrease in *apoptotic peptidase activating factor (apaf1)*; over half of these genes correlated with that of JSLE neutrophils (163). It also been shown that there were variations of IFN scores within adult SLE patients, ranging from absent to strong, and strength of expression IFN genes could correlate with disease activity, and biomarkers such as dsDNA (174). Importantly, although there were genes that were more associated with either type 1 or type 2 IFN stimulation, there were genes that were up regulated by both type 1 or type 2 IFNs within SLE (174). This

suggests that not only do the IFNs have a genetic role in SLE pathogenesis; it is likely that SLE and JSLE pathogenesis develop through a combination of IFNs and not just one type alone.

It should be of note that there is an IFN 'high' signature (increased expression of IFN-related genes) and an IFN 'low' signature (no increase in expression of IFN-related genes) within SLE, whereby in the IFN low signature patients have similar gene expression to that of control patients (188). Within this study, it was showed that the IFN high signature associated more with African-American ancestry and a high level of dsDNA and low complement levels (188). The IFN high signature patients also had more active disease, and therefore IFN genes are likely to be important in SLE pathogenesis (188). Certain analytes within the serum are differentially expressed between SLE and control patients; for example, chemokine CCL20 and fibroblast growth factor (FGF)2 are down regulated in SLE regardless of IFN signature, whereas cytokines IL-5, IL-6, and IL-15 are up-regulated in SLE regardless of IFN signature (188). Additionally, certain analytes correlated more with IFN high than IFN low patients; for example, CCL2, CXCL9, CXCL11 and IL-18 were significantly up-regulated in high IFN patients, but not low IFN patient, compared to healthy controls, they were significantly up regulated compared to IFN low patients also (188). Many IFN-regulated analytes correlated with disease activity and anti-dsDNA antibodies (188). Many of these analytes were chemokines, and suggests not only that the patients are undergoing a chemokine 'storm' induced by IFNs, but also that there was a chemokine gene score that correlated with disease activity (188). Certain chemokines, such as CXCL11 and CCL3 were significantly increased in patients with renal disease compared to those without (188). Interestingly, CXCL8/IL-8 (a neutrophil chemokine) was negatively correlated with active haematological involvement (188). This may correlate with the neutropenia commonly found in SLE patients (189). This study highlights a role of IFN signature within SLE; inducing certain chemokines that: a) can act as biomarkers and b) may lead to recruitment of leukocytes to tissues and organs and become involved in disease activity. However, the study also highlights that, although IFNs seem to be important in SLE, a subset of patients have IFN signatures similar to healthy controls, and therefore IFNs are not the sole cause of SLE pathogenesis, and other cytokines and factors must be investigated.

Within a longitudinal study, there was indication of increased IFNα activity in serum of SLE patients precedes their official diagnosis, and elevation of this activity correlates with autoantibodies, and has been shown to follow accumulation of autoantibodies (190). IFNγ, and its associated mediators was also found to be up-regulated in patient serum before SLE was fully evident , and correlates with IFNα activity (190). Additionally, IFNγ, and type 2 IFN

mediator, IFNγ induced protein (IP)10, were up regulated prior to accumulation of autoantibodies and IFNα activity (190). It was also suggested that type 2 IFN mediators and accumulation of autoantibodies can predict SLE classification, and also contribute to the increased IFNα activity seen (190). Thus, this indicates an importance of both types of IFNs in the initial development of SLE and highlights not only a potential biomarker of SLE pathogenies, it highlights that IFNs and any signalling pathway and mediators are important therapeutic targets.

1.5.4.2. STAT1 expression and SLE/JSLE

Studies have strongly indicated an important role of STAT1 in increased apoptosis. Overexpression of STAT1 in lung cancer cells increased the rate of apoptosis upon IFNγ induction *in vitro* (191). IFNγ can induce STAT1 activation subsequent to apoptosis in HeLa cells *in vitro* (138). Cycloheximide, an apoptosis inducer (192), can increase IFN-induced STAT1 mRNA expression in healthy volunteer peripheral blood mononuclear cells (PBMCs) *in vitro* (193) indicating a link between STAT1 and any potential increase in apoptosis within PBMCs. An increase in STAT1 activation maybe a pivotal factor in any possible IFN induced neutrophil apoptosis within JSLE is part of the IFN/JAK/STAT pathway, it may be the link between IFNs and neutrophil apoptosis (72).

Data has shown that STAT1 mRNA and protein expression was increased in PBMCs by IFNα and IFNγ *in vitro* (193). These studies show that IFNs in particular are able to induce increased STAT1 mRNA and protein expression in PMBCs, so this indicates that it is likely that the factors found in the serum may be causing an increase in STAT1 expression within JSLE neutrophils, rather than a genetic factor (193). So, it would be important to know if IFNs or other pro-inflammatory factors, such as TNFα, are shown to up-regulate STAT1 in any inflammatory conditions, especially adult-onset SLE and JSLE.

Coincidently, increased STAT1 expression occurs in both RA and adult-onset SLE PBMCs (194, 195). In RA, IFNs and TNFα up regulate STAT1 expression in healthy volunteer and RA PBMCs *in vitro* (195). IFNγ increase the proportion of phosphorylated (p)STAT1 in RA monocytes compared to healthy controls *in vitro*, and lead to an increase in IFN inducible genes (195). In this model, an increase in both STAT1 and pSTAT1 in PBMCs in adult-onset SLE compared to healthy volunteer neutrophils *ex vivo*, and IFNγ was able to effectively phosphorylate more STAT1 in SLE PBMCs than in healthy controls *in vitro* (194). This up-regulation of STAT1 in SLE PBMCs positively correlated with dsDNA levels, indicating a link between STAT1 and disease activity (194). It is possible that in inflammatory diseases, such as RA and adult-onset SLE,

cytokines such as TNFα in RA, and IFNs in adult-onset SLE, and possible JSLE, cause an increase in STAT1, as indicated by these studies. Thus, in JSLE, the increase in IFNs in the serum is not only increasing the expression of STAT1, but also increasing the phosphorylation of STAT1. As such, in JSLE, STAT1 is the prominent downstream protein from IFN stimulation in neutrophils, and this may result in the observed increase in neutrophil apoptosis (72).

STAT1 has also been implicated in the mouse model of lupus nephritis, in which the expression of both STAT1 and pSTAT1 were higher in cells lupus nephritis mice compared to healthy control mice *ex vivo* (196). A high level of STAT1 is present in the mesangial cells of lupus nephritis in a mice model, and both IFNγ and IFNα were able to phosphorylate STAT1 in these cells *in vitro*, albeit IFNα less so than IFNγ (196). This phosphorylation was inhibited by AG490 *in vitro*, a JAK2 and JAK3 inhibitor (details found here: https://www.tocris.com/products/ag-490_0414), indicating a IFN/JAK role in STAT1 phosphorylation (196). Interestingly, an increase in the suppressor of cytokine signalling (SOCS)1 and 3 mRNA and SOCS3 protein occurs in the kidney tissue of lupus nephritis mice compared to healthy controls (196). This mouse model indicates an important potential link between IFNs, STAT1 and lupus nephritis pathogenesis. These studies indicate that STAT1 pathway seems to be paramount in SLE and lupus nephritis pathogenesis. Additionally, the STAT1 function pro-apoptosis highlights its potential to become a therapeutic target in SLE and JSLE patients. Inhibition of the STAT1 pathway may dampen disease progression through a particular reduction in the otherwise increased neutrophil apoptosis, that has been hypothesised to contribute to the autoantigens that are characteristic of SLE and JSLE.

1.5.4.3. Inhibition of the JAK/STAT pathway

JAK inhibitors have been used to inhibit the JAK/STAT pathway and have been used in lupus mouse models to treat SLE and specifically lupus nephritis (197, 198). CP-690,550 (tofacitinib) is a JAK inhibitor that can reduce proteinuria and prevent glomerular and tubular lesions in mice with lupus nephritis and SLE prone mice (197, 198). Tofacitinib can decrease anti-dsDNA levels, complement and IgG glomerular deposits, and macrophage and T cell infiltration into the glomeruli (197, 198). Tofacitinib could also reduce the number of effector/memory T cells and activated T cells in spleens of lupus prone mice (197). Importantly, tofacitinib inhibited the expression of a range of STAT genes (STAT1, STAT2, STAT3, STAT4 and STAT5a) in mice with lupus nephritis (198). Additionally, tofacitinib significantly reduced gene expressions of IL-6, IL-2 and IFNα in kidneys of lupus prone mice, and reduced IFNγ gene expression within whole blood, although this was not significant (197). IFN-induced genes encoding for interferon-induced transmembrane proteins (*ifitm2* and *ifitm3)*, interferon-induced protein

with tetratricopeptide repeats 3 (*ifit3)* , 2'-5' oligoadenylate synthetase 1A (*oas1a*) and interferon stimulated gene 15 (*isig-15*) were supressed in splenic T cells in tofacitinib treated mice (197). Tofacitinib also reduced systemic circulating cytokines, with a significant reduction in IFNα, TNFα and IL-17 levels. Thus, within SLE, and particularly in regard to lupus nephritis, inhibiting the JAK/STAT pathway reduces many inflammatory markers and cytokines that contribute to SLE development. This shows that the JAK/STAT pathway is important in SLE and targeting different components may be of therapeutic benefit.

JAK inhibitors have differential effects on neutrophil functions (199). Tofacitinib, and another JAK inhibitor, baricitinib (a JAK1/JAK2 inhibitor) (both inhibitors are used to treat RA: see https://www.nice.org.uk/guidance/ta480 and https://www.nice.org.uk/guidance/ta466 for details) were shown to reduce both the IFNγ and GMCSF induced a delay in apoptosis in neutrophils from healthy volunteers at 20hrs *in vitro* (199). Both JAK inhibitors reduced GMCSF-induced STAT3 phosphorylation and IFNγ-induced STAT1 phosphorylation in neutrophils from healthy volunteers *in vitro* (199). *In vitro*, both inhibitors reduced random RA neutrophil chemotaxis and baricitinib decreased RA neutrophil chemotaxis towards IL-8, although chemotaxis towards fMLP was not affected (199). Additionally, neither IL-8 nor fMLP induced STAT1 or STAT3 phosphorylation *in vitro* (199). Neither inhibitor reduced the production of ROS by RA neutrophils in response to fMLP *in vitro*, however ROS was increased in RA neutrophils incubated with tofacitinib and stimulated with PMA and both inhibitors increased ROS in GMCSF primed RA neutrophils *in vitro* (199). This study shows the JAK/STAT pathway is important in a variety of neutrophil functions, particularly in regards to the inflammatory disease RA, and in particular, its inhibition leads to an altered apoptosis effect in regards to GMCSF and IFNγ. Thus, within this thesis, it would be of upmost importance to investigate if IFNs influence apoptosis in particular through the JAK/STAT pathway, and whether the inhibition of the pathway may alter the increased apoptosis seen in JSLE patients and therefore offers potential as a therapeutic target.

However, although JAK inhibitors are well established for inhibiting the JAK/STAT pathway, it was shown that many STATs are inhibited at the gene level in the kidney of a mouse model of lupus nephritis by using JAK inhibitors and both STAT1 and STAT3 phosphorylation can be inhibited by using JAK inhibitors in stimulated healthy volunteer neutrophils *in vitro* (198, 199). As STAT1 and STAT3 are important in IFN-related apoptosis, and importantly have opposite effects on this process, in order to differentiate IFN related effects on apoptosis, more specific inhibitors than JAK inhibitors are required (138, 141). Briefly, two inhibitors

haven been indicated to inhibit STAT1, fludarabine phosphate and S1495, and will be used as STAT1 inhibited in this study (200-202). Their roles are discussed later within this thesis.

1.6. Summary

Neutrophils are important in both health and disease. Within SLE and JSLE, the dysregulation of their life cycle and functions may be linked to auto-antigen exposure. Reduced neutrophil chemotaxis in these diseases may lead to their accumulation in tissues which may lead to organ damage such as that associated with lupus nephritis. Both the neutrophil and IFN-related gene signatures are upregulated in JSLE, indicating a potential interaction between IFNs and neutrophils that may contribute to SLE pathogenesis. Importantly, increased IFN protein concentrations have been detected in the serum of individuals with SLE and JSLE, supporting the hypothesis that IFNs are important in the disease processes underpinning SLE and JSLE. IFNs have been shown to prime neutrophils to undergo NETosis, which in turns stimulates pDCs to increase IFNα production. This may in turn contribute to the increased IFNα protein level found in JSLE patients and prime more neutrophils to undergo NETosis. Neutrophil chemotaxis and phagocytosis have also been shown to be dysregulated in JSLE, and IFNs have been implicated to alter these functions, particularly through influencing neutrophil receptor expression. IFNs signal via the JAK/STAT pathway, and different members of the STAT family have differential effects on apoptosis. Apoptosis is increased and dysregulated in JSLE patients and may lead to increased autoantigen exposure. STAT1 in particular has been shown to be increased in SLE and may induce a pro-apoptotic signal. The effect of inhibition of STAT1 activity on downstream signalling effects on apoptosis may highlight it as a potential therapeutic target within JSLE patients.

1.7. Hypothesis

The interaction of IFNs and neutrophils in JSLE contributes to disease pathogenesis. This may be via the influence of IFNs on neutrophil function and apoptosis, due to changes in the IFN signalling pathway.

1.8. Aims and objectives

Overall Aim: To investigate the interaction of IFN sub-types with neutrophils, and how IFNs can influence neutrophil function and apoptosis in the context of JSLE.

Aim 1: To investigate the effect of IFNs on chemotaxis, phagocytosis and NETosis in combination with other factors.

- Objective 1: Using transwells and manual counting using a haemocytometer and light microscopy, measure the rate of a) naïve healthy adult neutrophil migration towards IFNs and known neutrophil chemoattractants, IL-8 and fMLP; and b) IFN-primed healthy adult neutrophils towards known neutrophil chemoattractants, IL-8 and fMLP.

- Objective 2: Using flow cytometry, measure the rate of healthy adult neutrophil phagocytosis of *E.coli* bioparticles after IFN priming.

- Objective 3: Using confocal microscopy and DNA quantification (via Quant-iT™ PicoGreen™ dsDNA assay kit), measure NETosis after a) IFN-priming and subsequent LPS stimulation of healthy adult neutrophils and b) stimulation of IFNs on naïve and TNFα-primed healthy adult neutrophils.

Aim 2: To investigate the role of IFNs on neutrophil apoptosis.

- Objective 1: Using flow cytometry, quantify the rate of apoptosis on naïve and TNFα-primed healthy adult neutrophils following a 6hr incubation with a high concentration of IFNs.

- Objective 2: Using flow cytometry, quantify the rate of apoptosis on naïve and TNFα-primed healthy adult neutrophils following a 6hr incubation with IFNs, and measure the downstream expression of anti-apoptotic MCL1 (at 6hrs) and caspase 3 (at 20hrs) using Western blotting.

- Objective 3: Using flow cytometry, quantify the rate of apoptosis on naïve and patient sera-primed healthy adult neutrophils following a 6hr incubation with a lower concentration IFNs.

- Objective 4: Using flow cytometry, quantify the rate of apoptosis on paediatric control patient and JSLE patient neutrophils following a 6hr incubation with a lower concentration IFNs.

Aim 3: To investigate the IFN signalling pathway in naïve and primed neutrophils and its relation to apoptosis.

- Objective 1: Using flow cytometry, measure the IFN receptor expression on naïve and TNFα-primed healthy adult neutrophils.
- Objective 2: Using flow cytometry, measure the IFN receptor expression on paediatric control and JSLE patient neutrophils.
- Objective 3: Using Western blotting, measure the relative expression of pSTAT1 and pSTAT3 in naïve and TNFα-primed healthy adult neutrophils.
- Objective 4: Using both flow cytometry and Western blotting, measure the ability of fludarabine phosphate and S1459 to reduce pSTAT1/β actin expression ratio (and therefore possibly inhibiting pSTAT1) within healthy adult neutrophils and their effect on the rate of neutrophil apoptosis.

Chapter 2: Technical methods

2. Part A. Ethics, recruitment and patient data

2.1. Patient Cohort

Clinical data and bio-samples used in studies included in this thesis were collected within the appropriate regulatory approvals of 'UK JSLE Cohort Study and Repository', which started in 2006. The aim of this study is to investigate the 'clinical characteristics and immunopathology of JSLE'. The study is co-ordinated by the University of Liverpool and collects data and samples from 21 centres throughout the UK. Demographic and clinical data are obtained during routine clinical appointments using standardised data collection sheets that are then uploaded onto a secure, central database managed by the University of Liverpool. In addition to this, whole blood DNA, serum, plasma and urine are collected for analysis and/or biobank storage from multiple sites. Specifically, whole blood samples for the isolation of immune cells are collected from patients attending the Department of Rheumatology at Alder Hey Children's NHS Foundation Trust, Liverpool, UK. These samples are collected generally on the National Institute for Health Research (NIHR) Alder Hey Clinical Research Facility for Experimental Medicine and processed in the University of Liverpool laboratories within the 'Institute in the Park', Alder Hey Children's NHS Foundation Trust. Additionally, paediatric and adult healthy control samples are collected for comparison within the overarching regulatory permissions for the study. As of August 2017, when the laboratory work on this thesis was completed, 659 JSLE patients and 610 paediatric control patients have been recruited to the study.

2.2. Ethics

This study formed part of the UK JSLE Cohort Study & Repository that received full ethical approval by the North West - Liverpool East Research Ethics Committee (REC: 6/Q1502/77) (Appendix A1). The study is sponsored by Alder Hey Children's NHS Foundation Trust. Written informed patient/parental assent/consent was obtained in accordance with the Declaration of Helsinki (203). The study protocol stipulates that informed consent is required from patients/carers, and consent/assent from patients as appropriate. Information sheets are required for the patient and families, who have as much time as required to consider whether they wish to participate in the study (Appendix B3). The information sheet was explained by an appropriate health professional that was able to answer any questions the child or family

had. Consent included access to patients' medical records, permission to approach patients in regard to other research and recording an email address for the parent/patient in order for contact for any appropriate follow up. Where English is not a family's native language, an interpreter was provided to facilitate complete understanding of what the study entailed.

Each patient recruited was allocated a study number to allow for anonymity of the patient, and the recording of each patient records, including clinical and demographic data and the number was locked on a site away from the database. The study number was used on all data collection and databases for the study. Paper forms for data collection were stored in a locked filling cabinet after being entered onto the study database. Patient identifiable data were not stored on the electronic database. The database was password protected.

Healthy adults were recruited from staff in the Department of Women's and Children's Health, University of Liverpool, following informed consent (Appendix B3) under regulatory approval from the University of Liverpool research ethics committee (Appendix A2).

2.3. Recruitment

2.3.1. Inclusion/exclusion criteria

All JSLE patients fulfilled four of more of the revised ACR classification criteria underpinning their diagnosis of SLE before the age of seventeen (Appendix B1) (14). Paediatric non-inflammatory controls were defined as children investigated for non-inflammatory musculoskeletal symptoms or attending for elective surgery where no inter-current infection was present. All paediatric patients were recruited from outpatient clinics and inpatient wards at Alder Hey Children's NHS Foundation Trust. Clinical data and laboratory results were blinded to all investigators until final experiments were completed. Healthy adult volunteers aged over 18 from The University of Liverpool were recruited on the basis of deeming themselves healthy.

2.3.2. Consent

All patients and adult volunteers were approached by either a research nurse/consultant (patients) or Good Clinical Practice (CGP) trained professional (adult volunteers) and presented with an information sheet about the study at time of approach (Appendix B3). Patients and families and adult donors were given time to process the information and were able to participate/not participate/withdraw without explanation at any time. Written patient assent and written parental consent was obtained for those under 16 years of age,

and written consent was obtained for those over 16 where appropriate (Appendix B3). All documentation was retained in a secure manner within the clinical sites (e.g. Alder Hey Children's NHS Foundation Trust) and anonymised data were held at the University of Liverpool, in accordance with regulatory permissions.

2.4. Clinical data and disease activity

Clinical data, using comprehensive paper-based data collection forms (Appendix B1), were recorded by the clinician and/or research nurse during routine clinical appointments. These were anonymised, given the patient's unique study number, and then transferred to be uploaded and stored on the UK JSLE Cohort Study database. Data forms are standardised for use across all the 21 centres, so a standardised data set was collected (Appendix B1). Baseline forms were submitted at first diagnosis, along with clinical data forms for each hospital visit and an annual review was submitted each year (Appendix B1).

The forms aimed to obtain comprehensive review of many important clinical and demographic aspects. These include; age, ethnicity and family history of SLE and/or autoimmune disorders (Appendix B2). Clinically, the ACR classification form was used to assess and confirm the diagnosis of JSLE (meeting four or more ACR criteria) (Appendix B1) (13, 14). The Systemic Lupus International Collaborating Clinics (SLICC) damage index was used to determine the extent of disease damage (Appendix B1) (17). An annual review was conducted, which consisted assessment of a range of clinical biomarkers and blood tests, a review of disease activity and damage, and specific review of organ domains (Appendix B1).

The paediatric adaptation of the British Isles Lupus Assessment Group (pBILAG) disease activity score was conducted at each appointment, which scores the severity of disease activity within each organ domain (Appendix B1) (9, 15, 204). The pBILAG has been adapted from the adult version of the scoring assessment to improve validity of the assessment in the paediatric population (9, 15, 204). The pBILAG is collected and submitted to the cohort database anonymously which is then used to calculate the pBILAG scores (9). At every appointment, the pBILAG is analysed by organ domain, which encompasses a series of questions to determine whether organ disease had improved or worsened. A rating is assigned to each organ domain based on how the questions are answered. A pBILAG score of A or B is deemed to reflect active disease in any given organ domain (such as renal or haematological; see Figure 1.1/Section 1.1.3), a score of C is deemed improving disease

activity in any given organ domain, a score of D is deemed inactive disease in any given organ domain; a score of E is deemed no previous disease involvement in that organ domain and is also deemed inactive disease. Overall inactive disease is defined as no A or B in any organ domain and overall active disease is defined as an A or B in any organ domain. However, within Chapter 4 (Section 4.4.2.3), JSLE patient sera were selected based on overall inactive disease and active disease in specific organ domains; renal and haematological domains. This was to fully investigate how sera from patients with active JSLE disease in different domains may influence neutrophil apoptosis.

2. Part B. Laboratory methods

Appendix C provides specific details of all laboratory reagents (Appendix C1) and equipment (Appendix C2) used in these assays.

2.5. Neutrophil isolation

Neutrophils were obtained by separating cells from whole blood using Polymorph Prep (Alere Ltd, UK) or HetaSep (Stemcell, UK) and Histopaque-1077 (Sigma, UK). These solutions separate blood cells using density gradients, either in a one-step approach (Polymorph Prep) or a two-step approach (HetaSep and Histopaque-1077). Neutrophils were drawn off, washed and any remaining red blood cells (RBCs) were lysed using ammonium chloride solution. Both protocols are described in more detail below (Sections 2.5.1 and 2.5.2).

2.5.1. Polymorph Prep

Initial neutrophil isolations were conducted using Polymorph Prep solution. Blood was layered onto an equal volume of Polymorph Prep and centrifuged at 1800 RPM for 30mins, with the brake off. This spin allows RBCs to separate from the white blood cells through sedimentation. The neutrophils also separate from the PBMCs via the gradient of the Polymorph Prep (Figure 2.1) (http://www.axis-shield-density-gradient-media.com/Polymorphprep%20package%20insert%202017.pdf).

The PBMCs and neutrophils were removed using a Pasteur pipette, taking care that other layers were not disrupted when removing them. The neutrophils were washed in Roswell Park Memorial Institute (RPMI) 1640 media with L-glutamine (Lonza, Switzerland/SLS, UK; subsequently known as ‘media’) and centrifuged at 2000 RPM for 10mins to pellet the cells.

The supernatant was discarded, and the cells were suspended in the residue. Neutrophils were re-suspended in 1ml of media and 9ml of ammonium chloride lysis buffer (155mM ammonium chloride [Sigma-Aldrich, UK], 10mM potassium bicarbonate [Sigma-Aldrich, UK], 127µM EDTA [Fisher, UK]) added to lyse any remaining RBCs, and the tube inverted several times, and incubated for 3mins at room temperature. The neutrophils were centrifuged at 2000 RPM for 5mins to pellet, and the supernatant decanted. The neutrophils were suspended in 1ml of media and purity was checked using flow cytometry or a Romanowsky stain (Section 2.5.1.1). An acceptable purity was defined as ≥95%. 10µl of the neutrophil suspension was removed and placed on haemocytometer to count.

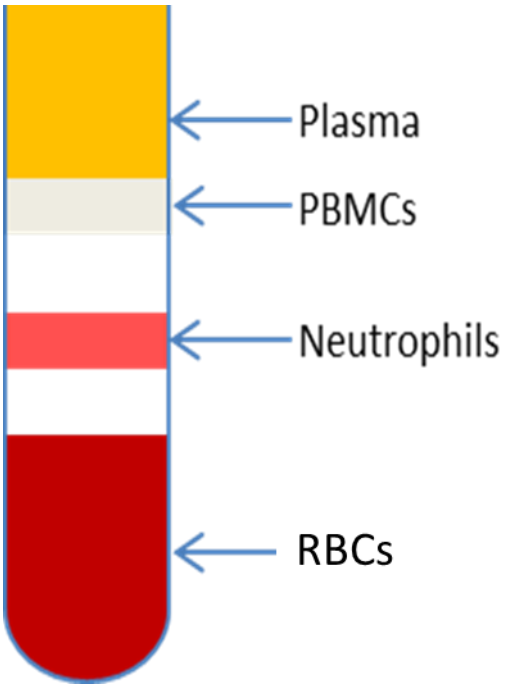


Figure 2.1. Separation of blood cells using Polymorph Prep and centrifugation. Blood was layered onto the Polymorph Prep, and centrifugation allowed separation of blood cells dependent on weight and the density gradient of the Polymorph Prep. Thus, the denser RBCs sink to the bottom and the intermediate neutrophils separate from the less dense PBMCs. This leaves the plasma layer at the top containing other blood substances.

2.5.1.1. Romanowsky stain

100µl (~1x10^5 cells) of neutrophils were centrifuged onto a microscope slide. The slide was centrifuged at 500xg for 5mins and left to air dry. The slide was placed in, in sequence, solution A (fixing solution, HD supplies, UK) for 30secs, solution B (acid dye, HD supplies, UK) for 20secs and solution C (base dye, HD supplies, UK) for 20secs, tapping on blue laboratory paper between dyes. Excess liquid was rinsed off and tapped dry on paper, taking care not to dislodge neutrophils. The slide was left to dry and neutrophils were analysed under the light microscope (Figure 2.2).

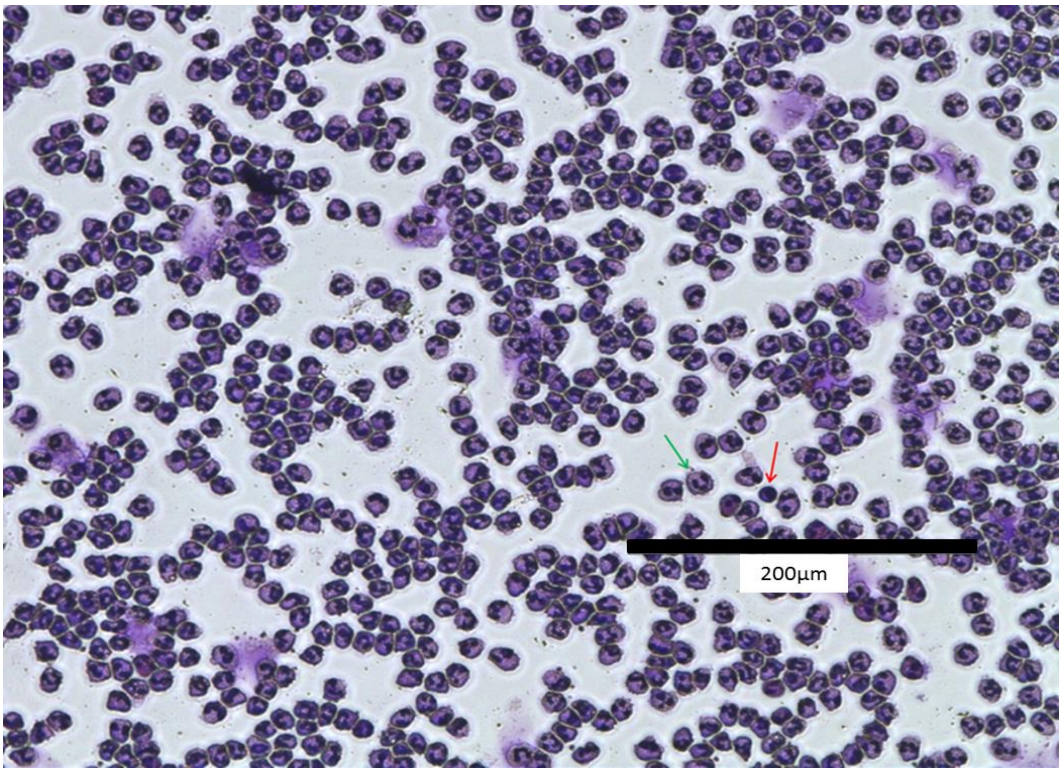


Figure 2.2. Romanowsky stain of healthy adult control population of neutrophils. Neutrophils were isolated using HetaSep and Histopaque-1077 method (below) and stained with Romanowsky stain. Picture taken at x20 objective on Evos inverted light microscope (ThermoFisherScientific, UK). Dark purple stain highlights the nucleus of the cell: cells with multilobular nuclei (main proportion of cell in image) are neutrophils (green arrow) and cells with lobular nuclei are PBMCs (red arrow). Light purple indicates the cytoplasm.

2.5.1.2. Limitations of the Polymorph Prep isolation method

A limitation of the Polymorph Prep isolation method was the final purity of the neutrophils. Although >95% purity was achievable, separating the RBCs and the PBMCs from the neutrophils in one stage using this isolation proved to cause inconsistency in the purity of the population of neutrophils. This was a particular problem with patient samples, in which particular the RBCs did not separate from the neutrophils properly and thus removing neutrophils without disrupting the RBCs was problematic. With adult healthy controls, the separation space between PBMCs and neutrophils was small, and PBMC layer was often indistinct and cause a cloudy like consistency, and thus decreasing the separation space further. Thus, PBMC contamination within the neutrophils was frequently higher than acceptable, with neutrophil populations with ≥5% PMBCs deemed a significant PBMC contamination and would need to be excluded. For example, PBMC significant contamination of neutrophil samples could affect protein analysis, protein constitution and stimulation experiments thereby limiting the reliability of data from such samples.

2.5.2. HetaSep and Histopaque-1077

The HetaSep and Histopaque-1077 method is a well-recognised and improved alternative way of isolating neutrophils. Specifically, the majority of the RBCs were removed in the first step, allowing the second step to separate mainly neutrophils from PBMCs. This helped particularly with patient samples in which the RBC layer did not always separate well. This method was therefore used throughout this thesis.

Whole blood was mixed with HetaSep at a ratio of 5:1 of blood:HetaSep. The suspension was left for approximately 30mins at room temperature to separate until the RBC layer and the leukocyte-rich plasma layer were equal (Figure 2.3). The leukocyte-rich plasma was drawn off and washed with 4x volume of phosphate buffered saline (PBS) (Sigma—Aldrich, UK; Fisher, UK). Leukocytes were centrifuged at 200xg for 10mins and suspended in 1ml of media. Suspension was layered onto 2ml of Histopaque-1077, taking care not to mix suspension and Histopaque-1077, and centrifuged at 2000 RPM for 20mins to separate the leukocytes into neutrophils and PBMCs (Figure 2.3). PBMCs and supernatant were removed using a Pasteur pipette, and neutrophils were suspended in 1ml of media. 9ml of ammonium chloride was added to neutrophil suspension and incubated for 3mins at room temperature to remove any remaining RBCs, inverting for the first 90 seconds. The neutrophils were centrifuged at 2000 RPM for 5mins, the supernatant decanted completely and the neutrophils were suspended in 1ml media with or without 10% foetal bovine serum (FBS) (Life Technologies, UK). An aliquot of neutrophils was diluted 1:10 to be counted using a haemocytometer, and

purity was checked either by flow cytometry (neutrophils were diluted 1:20 to a total volume of 200µl) or Turk's stain (neutrophils were diluted 1:10; Section 2.5.2.1). Neutrophils were initially suspended at $1x10^6$ in media with 10% FBS unless stated otherwise.

2.5.2.1. Turk's stain

Turk's stain was used to check purity of samples. 0.01% crystal violet (Sigma-Aldrich, UK) was mixed with 3% acetic acid (P&R Lab Supplies, UK) and 97% double distilled water, totalling 100 ml. 10µl of the final 1ml neutrophil suspension was mixed with 90µl of Turk's stain and 10µl was analysed on haemocytometer to check purity. This purity was defined by analysing the cells seen by the shape of the stained nuclei, neutrophils have granular nuclei and PBMCs have rounded nuclei, and determining the percentage of neutrophils seen. A population of ~95% neutrophils or higher was deemed a sufficient purity.

2.5.2.2. HetaSep and Histopaque-1077 optimisations

When possible, HetaSep was warmed to room temperature to optimise RBC sediment rate. Originally, media w/10% FBS was used in all stages. However, the manufacturers Sigma-Aldrich (http://www.sigmaaldrich.com/technical-documents/articles/biofiles/histopaque-troubleshooting-guide.html) suggest FBS can affect the Histopaque-1077 gradient and therefore subsequent final isolated neutrophil count. Thus, media w/10% FBS was used at the last stage only, once neutrophils were isolated from the PBMCs, and ready to be counted and checked for purity. All reagents worked most effectively when at room temperature. Thus, Histopaque-1077 and media were aliquoted, and PBS removed from the fridge immediately after the blood sample was obtained. Once the ammonium chloride lysis buffer was added to the neutrophils, the tube was inverted for the first 90secs to prevent neutrophils from settling and increasing lysis of RBCs.

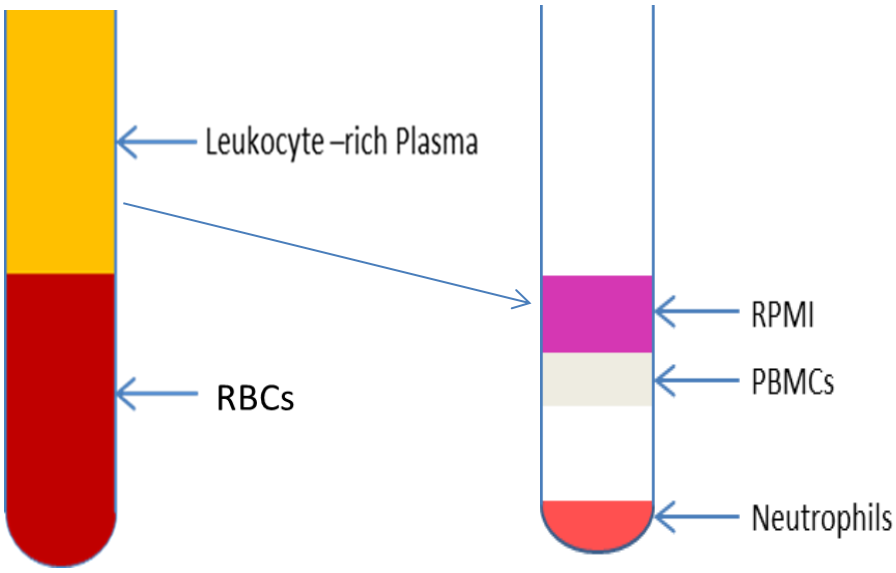


Figure 2.3. Separation of blood cells using HetaSep and Histopaque-1077. The majority of RBCs were sedimented by weight through a density gradient using HetaSep, leaving a leukocyte-rich plasma. The leukocytes were separated using Histopaque-1077, again using a density gradient to separate by weight.

2.6. Flow cytometry principle

Flow cytometry is a technique that is used to sort cells dependent on the properties detected on the cell membrane. Antibodies containing fluorophores bind to specific receptors or markers on the membrane that can determine whether the cell is a certain type (e.g. CD16 antibody for neutrophils) or undergoing a certain process (e.g. annexin V fluorescein isothiocyanate (FITC) for apoptosis). The cells are suspended in a liquid stream and are individually processed through the flow cytometer. The fluorophores are excited by a laser and are detected by different fluorescent channels (e.g. fluorescence channel 1 (FL1), etc.) depending on the wavelength: for example, FITC is detected at the 495nm wavelength in the FL1 channel. The size and shape of the cells can also be measured by analysing the forward scatter (FS) (size of cells) and side scatter (SS) (granularity of cells) of the lasers, which allow for selecting the right cell type to be analysed and checking the purity of the cell suspension. The percentage of cells that express the fluorophores, and hence express the marker, can be analysed using dot blots, and the amount of the marker per cell can be presented using histograms (Figure 2.4).

A key advantage of flow cytometry as a technique is that it is a quantitative method. However, flow cytometry equipment is expensive to buy and maintain, and requires extensive training. Flow cytometers are also prone to blockages and breakdowns that require time and specialist engineers to resolve. Incompetency, through lack of training, therefore, can result in not only breakage of the equipment but result in unreliable or inconsistent data. Through training, prior knowledge of how a flow cytometer works, how to set up an assay that produces reliable, quality data, and how to use flow cytometry analytical packages is required to save time, resources and minimise equipment malfunction. Other pitfalls of this technology include the requirement of cells being in suspension and lack of ability to conduct a comprehensive analysis of the distribution of intracellular proteins. This requires permeabilization, which would require confocal microscopy to analyse.

2.6.1. IFN dose response and subsequent time course

An IFN (PeproTech, UK) dose response (1, 5 and 10ng/ml) was conducted for each IFN subtype, whereby apoptosis was measured at 6hrs and 20hrs. No significant difference was seen in the rate apoptosis of at both time intervals between concentrations of IFNα, nor IFNγ; however, a concentration of 10ng/ml of IFNβ resulted in greater apoptosis than at the lower concentrations of IFNβ (6hrs: unstimulated = 14.5%, 1ng/ml = 15.5%, 5ng/ml = 13.0%, 10ng/ml = 10.0%, n=1; 20hrs: unstimulated = 74.9% 1ng/ml= 82.9%, 5ng/ml = 82.4%, 10ng/ml

= 76.8%, n=1) (Figure 2.5). Therefore, subsequently neutrophils were simulated with 10ng/ml for each of the IFN subtypes and 5ng/ml GMCSF (R&D Systems, UK) for 6hrs and 20hrs in 96 well plates at 37°C, 5% CO_2, $1x10^5$ neutrophils per condition. Apoptosis was analysed as below (Section 2.6.2) using both Beckman Coulter F500 and Guava EasyCyte flow cytometers and results analysed on FlowJo (v.10, USA).

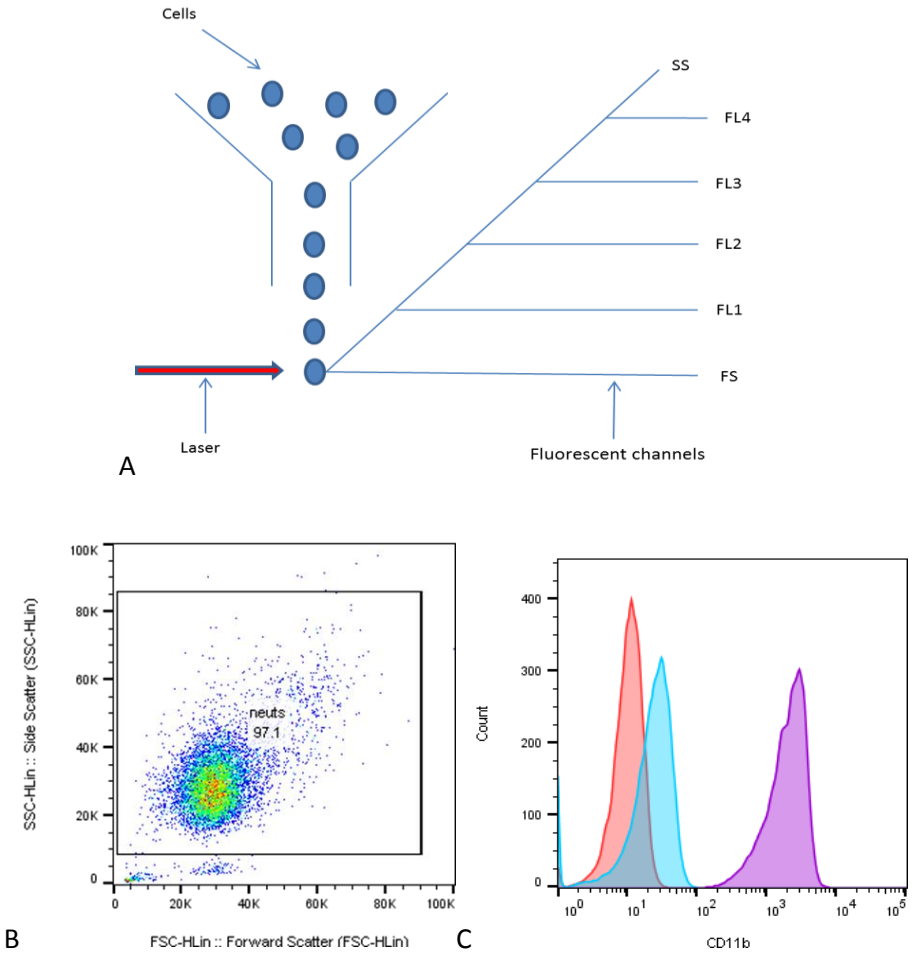
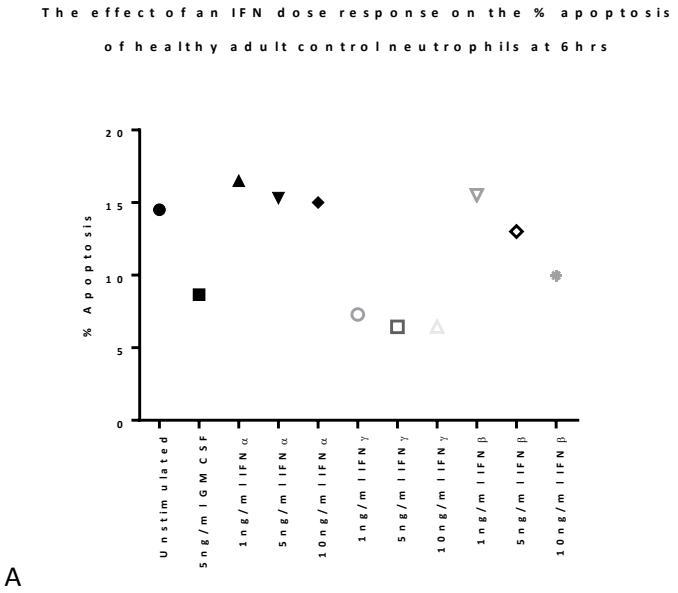
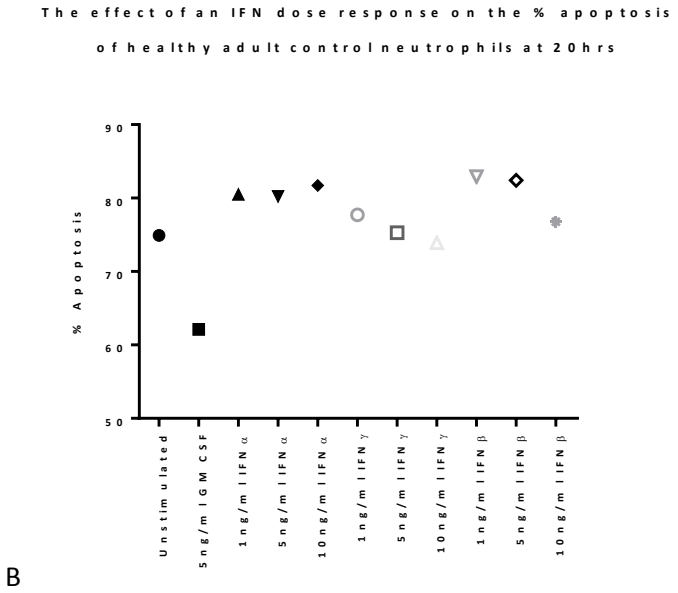


Figure 2.4. Depiction of the flow cytometry procedure and an example dot plot and histogram following flow cytometry analysis. Neutrophils were isolated from healthy adult donors and were stained with antibodies containing fluorophores. Neutrophils were processed through the flow cytometer and excited by a laser (shown in A). The fluorescence was detected by channels depending on wavelength (A), and dot blots (B, analysing the FS on X axis and SS on the Y axis) and histograms were produced (C). Within B, neutrophils were primed with TNFα for 30mins, and stained with CD11b. Unstained neutrophils (red peak), isotype control (blue peak) were used to set the protocol and the peak moves to the right when the neutrophils were positively stained (for example TNFα-primed neutrophils = purple peak).



A



B

Figure 2.5. Apoptosis of healthy adult neutrophils at 6hrs and 20hrs in response to different doses of IFNs. Neutrophils were isolated from healthy adult donors and were stimulated with 1, 5 and 10ng/ml IFNα, IFNγ or IFNβ, and 5ng/ml GMCSF for 6 and 20hrs. Apoptosis was measured at 6hrs (Figure A) and 20hrs (Figure B) respectively. Other than IFNβ, where 10ng/ml had a different effect (where it particularly delayed apoptosis at 6hrs) to other concentrations, there was no significant difference demonstrated between doses of IFNα or IFNγ. Therefore, 10ng/ml IFNs were subsequently used.

2.6.2. Annexin V FITC and PI staining

During early apoptosis, phosphatidylserines (PS) are expressed on the cell surface. Annexin V is able to bind to PS, and the subsequent fluorescence indicates that cells are undergoing apoptosis. During late stage apoptosis, annexin V is able to bind to cytosolic PS and propidium iodide (PI) is able to enter the cell and bind to DNA. PI is typically used to detect necrotic cells and in combination, annexin and PI are able to differentiate necrotic, apoptotic and viable cells within a heterogeneous population.

2.6.2.1. Original apoptosis staining assay

Neutrophils were lifted from the plate and placed in 5.4ml flow cytometry tubes for staining. The neutrophils were centrifuged at 1500 RPM for 5mins. The supernatant was decanted, being careful to not disrupt the pellet, and 1ml of Hanks' balanced salt solution (HBSS) (Sigma-Aldrich, UK) was added. The plate was centrifuged at 1500 RPM for 5mins. The supernatant was decanted, being careful not to disrupt the pellet, and the neutrophils were suspended in 100µl of HBSS. Annexin V FITC (Sigma-Aldrich, UK) was added a 1:100 and the tubes were incubated at 4°C for 15mins. 900µl of HBSS and 1:1000 PI (Sigma-Aldrich, UK) were added and the tubes were incubated at room temperature in the dark for 5mins. The tubes were centrifuged at 1500 RPM for 5mins, the supernatant was decanted and 600µl of HBSS was added and mixing the suspension well. Each sample was run using a pre-set annexin V FITC/PI program on the Beckman Coulter F500 (Beckman Coulter, UK), whereby events were allowed to reach up to 10,000 events. Analysis was conducted using the Beckman Coulter flow cytometry analysis software, Kaluza (v.1.3, USA).

2.6.2.2. Optimised apoptosis staining assay

Neutrophils were centrifuged at 1800 RPM for 5mins. The supernatant was decanted, being careful to not disrupt the pellet, and 100µl of HBSS was added. If not already in a 96 well plate, the neutrophils were transferred to one. The plate was centrifuged at 1500 RPM for 5mins. The supernatant was pipetted out, being careful not to disrupt the pellet, and the neutrophils were suspended in 100µl of HBSS. Annexin V FITC was added a 1:100 and the plate was incubated at 4°C for 15mins. PI was added at 1:1000 and the plate was incubated at room temperature in the dark for 2mins. 100µl of HBSS was added, and suspension was mixed well. Each sample was run using a pre-set annexin V FITC/PI program on the Beckman Coulter F500, or Guava EasyCyte (Merck Millipore), whereby events were allowed to reach up to 10,000 events. Analysis was conducted using flow cytometry analysis software, Kaluza or FlowJo (Figure 2.6).

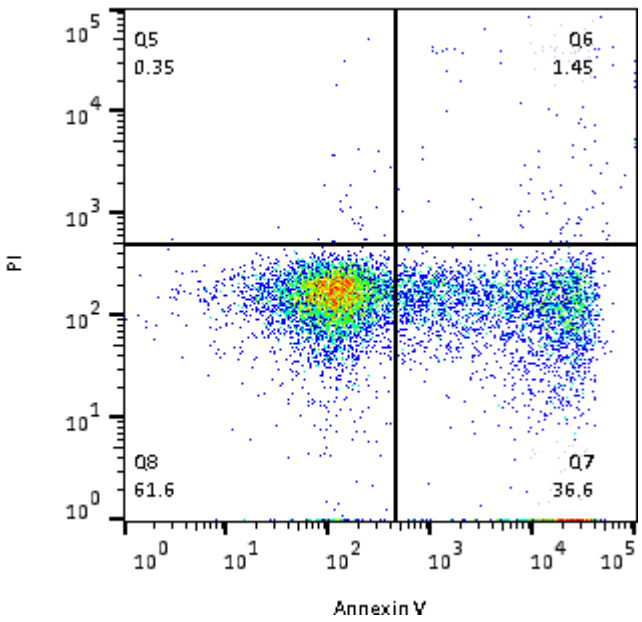


Figure 2.6. Annexin V FITC and PI staining of unstimulated neutrophils after 6hrs. Neutrophils were isolated from healthy volunteers and incubated for 6hrs. Neutrophils were stained with annexin V FITC and PI and the percentage of staining was analysed using flow cytometry. Cells in quadrant Q8 (bottom left) did not stain for annexin V FITC or PI, and thus were considered viable. Cells in quadrant Q7 (bottom right were stained for annexin V FITC only and were considered apoptotic. Cells in quadrant Q5 (top left) were stained for PI only and were considered necrotic. Cells in quadrant Q6 (top right) were stained for both annexin V FITC and PI and were considered secondary necrotic.

2.6.3. Activation assays

Flow cytometry assays were developed to analyse activation markers CD11b and CD62L on neutrophils. When primed for activation, CD11b expression has been shown to increase, and CD62L to decrease (95). TNFα can prime neutrophils to become activated, and this priming can be detected by analysing the expression markers CD11b and CD62L in naïve and TNFα-primed neutrophils (169). TNFα priming of neutrophils was used to model activated neutrophils that are found in inflammatory diseases, and which are found in SLE patients (83). Specific methods can be found in Chapters 4 and 5.

2.6.4. IFN receptor analysis

Flow cytometry assays were developed to analyse the individual IFN receptor chains (IFNAR1, IFNAR2, IFNGR1 and IFNGR2). IFNs signal through receptors on membrane surfaces to activate the JAK/STAT pathway. Specifically, it has been shown that IFNGR chain expression is differentiated depending on immune cell line and environment, and this contributes to whether IFNγ induces cell proliferation or apoptosis. (161). Thus, IFN receptor analysis was an important signalling element to investigate in relation to neutrophil apoptosis in JSLE. Specific methods can be found in Chapters 4 and 5.

2.6.5. Phagocytosis assay

Flow cytometry was used to analyse the phagocytic ability of neutrophils. Phagocytosis is a major function of neutrophils, and it thought to be dysregulated in JSLE (112, 115). PHrodo Red *E. coli* bioparticles, which fluoresce when phagocytosed by neutrophils, were incubated with neutrophils pre-treated with the different IFN subtypes, and phagocytosis was measured by fluorescence by flow cytometry. Specific methods can be found in Chapter 3.

2.7. Protein Analysis

Neutrophils were stimulated, and various intracellular proteins were extracted and analysed to understand the IFN signalling pathway further, and to supplement flow cytometry data already analysed. Protein concentration was analysed using a Pierce assay where appropriate, normalised and protein expression was analysed using Western blotting.

2.7.1. Protein extraction

Neutrophils were lifted from suspension and centrifuged at 1800 RPM for 5mins. The supernatant was poured off and the pellet washed in 100µl of media. The neutrophils were centrifuged at 1800 RPM, the supernatant was poured off and again washed in 100µl of

media. The neutrophils were then centrifuged at 8000 RPM for 5mins, or 1000g for 3-5mins. 25µl lysis buffer (89% sodium dodecyl sulphate (SDS) buffer [10% glycerol (BDH, UK), 125mM Tris buffer (pH 6.8, Fisher, UK), 3% SDS (BioRAD, UK) 0.2% bromophenol blue (Sigma-Aldrich, UK)], 10% dithiothreitol (DTT) (Sigma-Aldrich), 1% 100X protease and phosphoprotease inhibitor cocktail (ThermoFisherScientific, UK)) was added per $5x10^5$ cells, except for cells for MCL1, where 25µl per $2x10^6$ cells was added, and the samples heated for 3mins at 100°C vortexed every 60 secs to homogenise the samples.

2.7.2. Pierce assay

The Pierce assay measures protein concentration by light absorptions in reference to a standard curve of known protein concentrations.

A stock of fresh lysis buffer was made to use as the diluent for the assay (see above). Standards of between 62.5µg/ml and 2000µg/ml bovine serum albumin (BSA) (Sigma-Aldrich, UK) were made using a serial dilution, and placed at 4°C until needed. Protein samples were heated at 100°C for 3mins, and vortexed every 60secs to homogenise the samples. Aliquots of samples were diluted at 1:2 or 1:3 and stored at 4°C until needed. 20ml of Pierce 660nm protein assay reagent (ThermoFisherScientific, UK) was mixed with 1g of ionic detergent compatibility reagent (ThermoFisherScientific, UK) and was shaken until the powder was dissolved. The tubes were well covered with parafilm (StarLab, UK) and foil (Sainsbury's, UK) and kept stored at 4°C until needed.

10µl of standards and samples were aliquoted in duplicate in a 96 well plate and 150µl of Pierce 660nm protein assay reagent was added to each well. The plate was shaken for 60secs at room temperature and then incubated at room temperature for 5mins. The plate was read at 660nm on a Varioskan Flash plate reader (ThermoFisherScientific, UK). A standard curve was produced using absorptions of standards at known concentrations, and the equation of the curve was calculated (e.g. for shown example in Figure 2.7, absorbance = 0.0002(protein concentration) + 0.138, $R^2 = 0.9943$) (Figure 2.7).

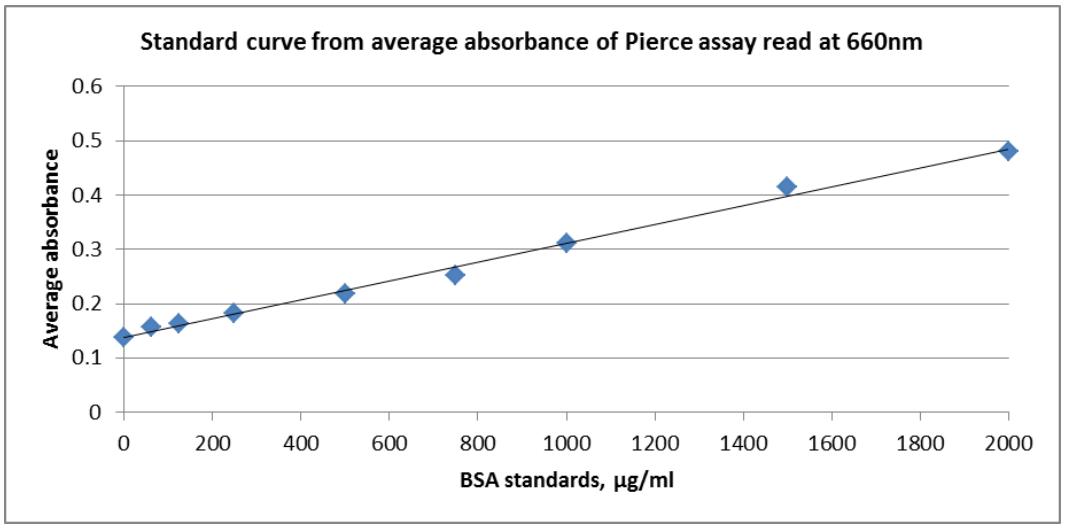


Figure 2.7. Pierce 660nm Assay standard curve. BSA was diluted at 2000µg/ml, and serial dilutions achieved other standards using protein lysis buffer (1500µg/ml, 1000µg/ml, 750µg/ml, 500µg/ml, 250µg/ml, 125µg/ml, 62.5µg/ml, and protein lysis buffer as 0µg/ml/blank). Standards and samples were loaded in duplicate into a 96 well plate, and the Pierce 660nm assay reagent was loaded. The plate was read, and the standard curve was plotted using average absorbance against the known standards.

2.7.3. Western Blot

Western blot is the technique by which proteins are detected and can be semi-quantified by the size of the protein and specific probing of antibodies. Proteins are reduced and denatured to their primary structure and SDS applies a negative charge to these proteins. Once placed into a polyacrylamide gel, proteins can be separated by molecular weight. An electrical field is induced in the gel, and proteins migrate towards a positive charge, with smaller proteins migrating further than larger proteins. Proteins are transferred to a membrane, and this membrane is incubated with antibodies grown against specific proteins (primary antibodies). Antibodies with broader specificities, bound with a fluorescent molecule (such as horse radish peroxidase [HRP]; secondary antibodies) are incubated with the membrane to bind to the primary antibodies. Chemiluminescent reagents are placed onto the membrane which allow fluorescent molecules to omit light, which allows proteins of interest to be detected and analysed. The Western blot process is depicted in Figure 2.8. Western blotting is cheaper than other techniques, and it is possible to analyse multiple proteins on one membrane. However, it is laborious, time consuming and semi-quantitative.

Assay optimisation is often required; optimum antibody dilutions and incubation protocols can vary depending on protein lysate and the protein of interest. Low expressed proteins may require higher concentrations of antibodies, and longer incubation with the antibodies. This increase in incubation time can cause increased background noise, which may then require different incubation conditions. For long incubations with antibodies, incubations at 4°C and appropriate washes in Tris base saline + (0.1%) tween 20 (TBST) after each antibody incubation can improve this. Time and voltage use for the sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) may need to differ depending on the size of protein of interest. Longer electrophoresis time, increased voltage or use of a gel with a lower polyacrylamide percentage can separate larger proteins better than standard protocols. However, this can result in loss of the proteins with the lowest molecular weights, so careful consideration of what proteins are to be analysed on the same gel is required.

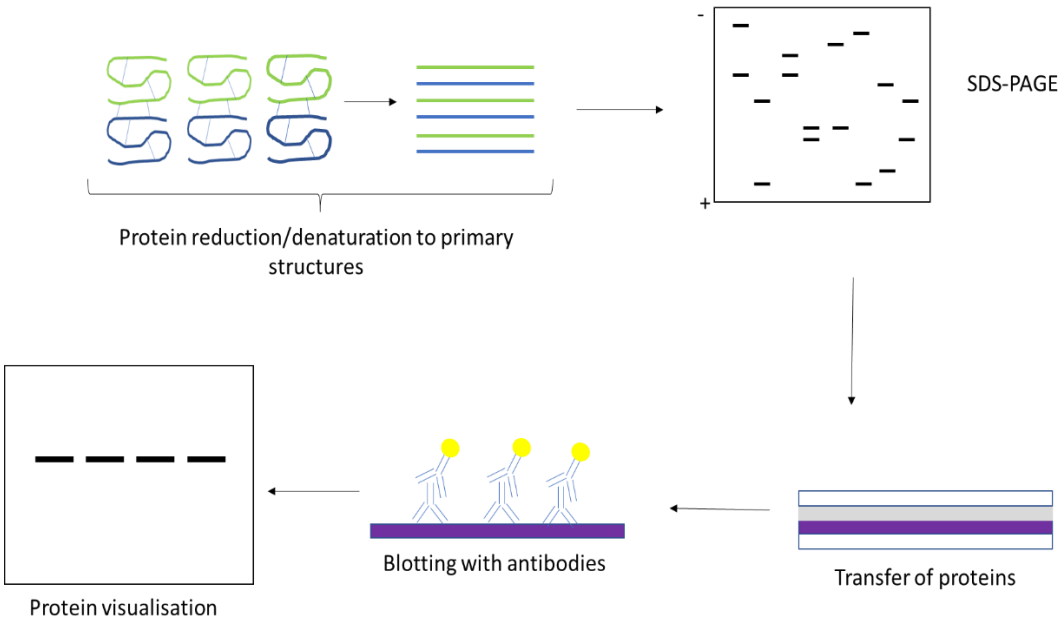


Figure 2.8. Detection of proteins of interest using Western blotting. Proteins were extracted and lysed to become their primary structures with a negative charge. Proteins were separated by electrophoresis and transferred to a membrane. The membrane was incubated with primary and secondary antibodies, and chemiluminescent reagents allows the secondary antibody fluorescent molecule to omit light. This allowed for protein detection, visualisation and analysis using specialist equipment and software.

2.7.3.1. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

15µl of sample was loaded into a 12% min-PROTEAN TGX precast protein gel (BioRAD, UK) placed in Western blot tank (BioRAD) surrounded in running buffer (3.5mM SDS, 192mM glycine, 25mM Tris base). 4µl of Precision Plus Protein™ WesternC™ standards ladder (BioRAD, UK) was loaded and an electrical current was applied to separate the proteins according to molecular weight. Standardly, the SDS-PAGE was run at 160 volts for 45mins, unless otherwise stated.

2.7.3.2. Transfer

The gel was removed from the plastic cassette and cut according to size of membrane and proteins being probed (e.g. if both pSTAT3 [79/86 kDa] and β actin [42 kDa] were being probed, the gel was cut just above 50kDa). The gel was placed on a PVDF membrane within Trans-Blot Turbo Mini PVDF transfer packs (BioRAD, UK), and the proteins transferred to be analysed by antibody blotting. The BioRAD TransBlot achieved this by creating a 'sandwich' with filter paper, the gel and membrane, the filter paper and membrane already having been pre-soaked in transfer buffer. The sandwich was placed in the TransBlot apparatus (BioRAD), and a mixed molecular weight pre-programmed protocol (1.3 ampere, 25 volts, 7mins) was applied to transfer the proteins from the gel to the membrane.

2.7.3.3. Blotting

The membranes were blocked in 5% milk (Marvel semi-skimmed milk, Sainsbury's, UK) in TBST (1mM Tris base, 15mM sodium chloride, 0.1% tween 20) for 1hr at room temperature, with gentle agitation. Any excess block was washed off using TBST, shaking 2x for 30secs.

Primary antibodies were diluted in 5% (w/v) milk TBST as per indicated by company data sheet (Table 2.1), and incubated with the membrane, either using plastic bags or plastic boats. The membranes were agitated at room temperature for 1hr or 4°C overnight to allow antibodies to bind. Primary antibodies were washed in TBST, initially 2x 30secs to wash off excess, 2x 5mins to wash off intermediate unspecific binding and background noise, and 1x 15mins to wash off tightly bound unspecific binding and background noise.

Secondary antibodies with chemiluminescent probe HRP were diluted in 5% milk TBST as per indicated by company data sheet (Table 2.1) and incubated with the membrane. The membranes were agitated at room temperature for 1hr to allow the binding to the secondary antibody. The antibody was washed off as with the primary antibody.

2.7.3.4. Imaging and analysis

WesternSure® PREMIUM chemiluminescent substrate (LI-COR, UK) was applied to the membrane and left to incubate at room temperature for 5mins. Multiple images were taken by the scanner and the densitometry was calculated by drawing round the bands using software Image Studio Digits V.3.1 (Figure 2.9). Ratios were then calculated by dividing the protein of interest densitometry by a control protein densitometry.

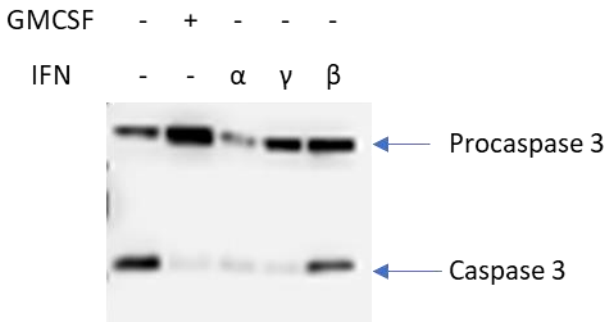


Figure 2.9. Western blot analysis of procaspase 3 and caspase 3 after 20hrs IFN incubation. An example Western blot showing neutrophils stimulated with 5ng/ml GMCSF, 10ng/ml IFNα, 10ng/ml IFNγ or 10ng/ml IFNβ for 20hrs. The protein was extracted and lysed into its primary structure with a negative charge. Proteins were loaded into a gel, according to condition (naïve, GMCSF stimulated, and stimulated with each IFN subtype) and separated based on molecular weight via electrical current towards a positive charge. The proteins were transferred to a membrane, cut around 50kDA and unwanted proteins blocked with 5% milk, and probed with caspase 3 antibody and its associated secondary antibody (Table 2.1). The expression of proteins was detected through the fluorescence of the secondary antibody through reaction with a chemiluminescent reagent. The caspase 3 antibody was stripped, re-blocked, and the membrane re-probed with β actin primary antibody, probed with its associated secondary antibody, and protein was detected through the fluorescence of the secondary antibody through reaction with a chemiluminescent reagent (not shown).

Table 2.1. Electrophoresis time course and antibody dilutions for western blots.

Protein of interest	Electrophoresis	Primary antibody type and dilution	Manufacturer	Secondary antibody type and dilution	Manufacturer
pSTAT1	160 volts; 60mins	rabbit; 1:1000	Cell Signalling (USA) New England Biolabs (UK	goat; 1:1000	R&D Systems, UK
pSTAT3	160 volts; 60mins	mouse; 1:1000	Cell Signalling (USA) New England Biolabs (UK	goat; 1:1000	R&D Systems, UK
STAT3	160 volts; 60mins	rabbit; 1:1000	Cell Signalling (USA) New England Biolabs (UK	goat; 1:1000	R&D Systems, UK
MCL1	160 volts; 45mins	rabbit; 1:1000	Cell Signalling (USA) New England Biolabs (UK	goat; 1:1000	R&D Systems, UK
caspase 3	160 volts; 45mins	rabbit; 1:1000	Cell Signalling (USA) New England Biolabs (UK	goat; 1:1000	R&D Systems, UK
β actin	Dependent on protein of interest above	mouse; 1:5000	Abcam, UK	goat; 1:1000	R&D Systems, UK

2.8. Chemotaxis assay

Chemotaxis assays, or Boyden Chamber Assays, are used to measure the random and directed migration of neutrophils (Figure 2.10). Neutrophils were plated within inserts that contain pores within the membrane. These pores expose neutrophils to media and stimulants (including chemoattractant fMLP and chemokine IL-8) and allow for migration of the neutrophils from the insert into the well underneath. After an incubation period, any migrated neutrophils are suspended within the media in the wells, and an aliquot from each well is removed to be counted using a haemocytometer.

These assays were quick, simple and relatively cheap, and allowed for data to be quickly acquired. However, the plates needed to be pre-coated with polyHEMA at least 24 hours before. Counting of neutrophils was conducted manually using a haemocytometer; this method is not as accurate as other methods such as automated cell counting and can introduce operator bias. Effort was made to consistently add the same number of neutrophils to the inserts for each experiment ($1x10^6$ neutrophils/200µl); however, after pre-treatment with IFNs, there was some neutrophil loss from centrifugation. Therefore, for these experiments, neutrophils in each condition were normalised to the lowest concentration of neutrophils for each donor. This was to try to minimise any variation in the trend of migration between priming conditions due to differences in number of neutrophils seeded.

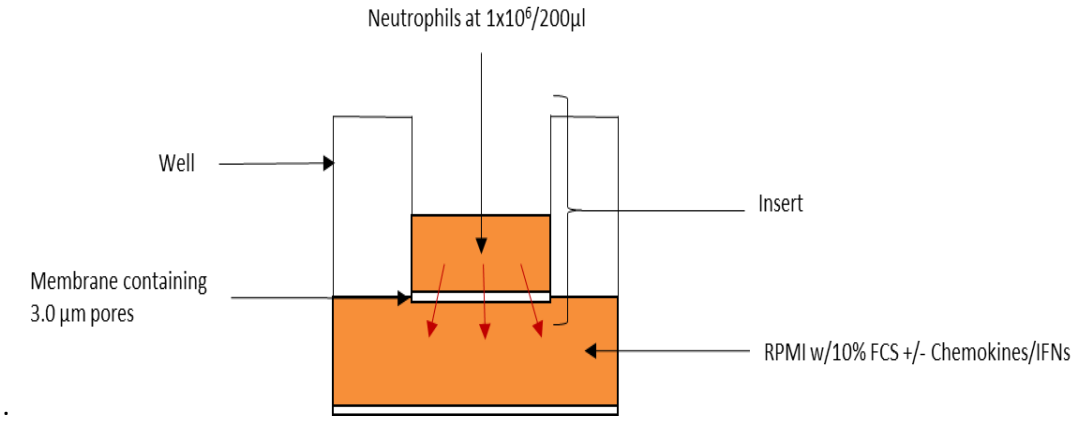


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Figure 2.10. Neutrophil migration through pores into lower well containing chemokines. Neutrophils were either primed or left unstimulated and suspended up to 1x10^6/200µl media w/10% FBS to be placed in a hanging insert. Neutrophils underwent migration, either directed or random into the well below and counted after 90mins.

2.9. NETosis assay

Confocal microscopy and DNA quantification was used to analyse NETosis in response to IFN priming or IFN stimulation. Isolated Neutrophils (Section 2.5.2) settle onto coverslips, stimulated, and mounted onto microscope slides. The mounting medium contains 4', 6-diamidino-2-phenylindole (DAPI), which stains DNA, which can be visualised using confocal microscopy; NETs are detected through the exposure of DNA fibres. Additionally, DNA is cleaved into the supernatant using a nuclease, and the concentration can be measured via fluorescence using a PicoGreen reagent.

2.9.1. Confocal microscopy principle

Confocal microscopy uses lasers to stimulate florescent emission from fluorophore bound antibodies, which can be detected by a computer. Cells are fixed onto microscope cover slips using formaldehyde, permeabilised and blocked in Tris base saline (TBS) containing 2% BSA. The cells are subsequently stained for specific proteins using antibodies attached to fluorophores, and also stained with DAPI, a commonly used DNA stain. The coverslips are mounted (in this case using a mounting medium containing DAPI) and left to set. As with flow cytometry, the fluorophores on the antibodies are excited by a laser, and are detected by computer depending on the wavelength, and all proteins can be visualised individually, and can be merged to create a comprehensive image (Figure 2.11).

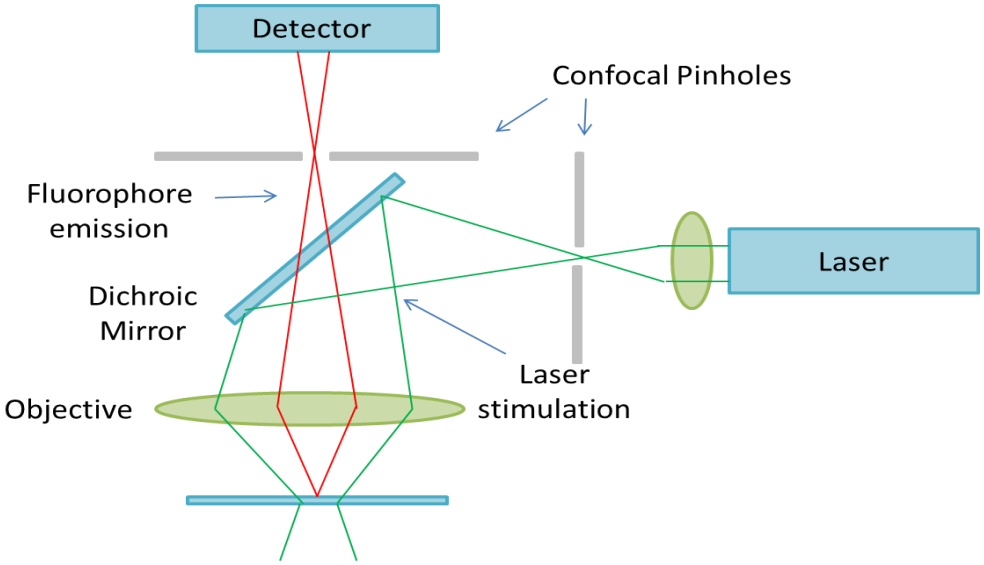


Figure 2.11. Diagram depicting confocal microscopy function. Samples were fixed, stained with DAPI, and mounted onto microscope slides. Fluorophores were stimulated with a laser and emissions were detected on a computer. Multiple emissions can be analysed and merged to create a comprehensive image.

2.9.2. DNA quantification analysis

DNA is a major component of NETs (37). Therefore, to complement the confocal images, and give a consistently quantifiable analysis, DNA concentration can be measured in the supernatants from neutrophils analysed for NETs. Specific methods are described in chapter 3.

2.10. Statistical analysis

Statistical analyses were conducted using GraphPad Prism 6. Non-parametric tests were conducted for all analyses due to the low sample sizes (majority n=5, one control analysis n=6), and thus does not make assumption about the samples being normally distributed. Paired analyses were used for the majority of statistical tests, as multiple conditions were conducted on dependent/related samples. One-tailed Wilcoxon matched-pairs signed rank test was used for activation assays (two related conditions for each marker), where it is established in that CD11b increases upon activation, and CD62L decreases upon TNFα priming, and thus the direction of observations were already known (169). The Friedman test was conducted for experiments on related sample with more than two conditions, with a *post hoc* Dunn's multiple comparison test to specify which condition is significantly different and to correct for multiple comparisons. A Kruskal-Wallis test was conducted to compare between paediatric control and JSLE neutrophil conditions when analyzing the IFN receptor, activation marker and apoptosis data, which compared multiple conditions between two independent sample sets (e.g. unstimulated paediatric control neutrophils vs unstimulated JSLE neutrophils) with a *post hoc* Dunn's multiple comparison test to specify which condition is significantly different and correct for multiple comparisons.

2.11. Summary

Here, it was shown how patients with JSLE were identified, diagnosed, and phenotypically determined samples acquired through processes governed by appropriate regulatory approvals. Anonymised patient data, including demographics and clinical data were also collected and stored, in accordance with regulatory approvals, which were analysed to accompany experimental data where appropriate. Major experimental processes and background theory is discussed, allowing for specific methods and any optimisations to be outlined in results chapters. Appropriate statistical analysis was selected and described,

based on experimental data output, following consultation of a statistician at the Department of Biostatistics, University of Liverpool.

Chapter 3: The role of IFNs in neutrophil functions in JSLE

3.1. Introduction

Neutrophils are among the first immune cells to defend against infection and therefore many of their functions are essential in killing invading pathogens, dysregulation in these functions can lead to inflammatory disease. Neutrophils and IFNs have been indicated to be important in JSLE pathogenesis (Section 1.5). The concentration of IFNs has been shown to be increased in the serum of patients with SLE and JSLE, and there is a pronounced granulocyte and IFN signature found in JSLE patients (Section 1.5.4.1). It thus seems possible that any neutrophil functions that may be dysregulated in JSLE may be influenced by IFNs (41, 68, 171, 172). Here, it was investigated whether IFNs influence neutrophil phagocytosis, chemotaxis and NETosis, which have been shown to be dysregulated in SLE and JSLE (112, 116-118) (Sections 1.3, 1.4.3, 1.5.3).

Chemotaxis is the process by which neutrophils migrate towards certain stimuli, such as chemokines and bacterial products. It has been shown that these stimuli have an attracting hierarchy, which allows neutrophils to be finely directed to the site of infection (47). It has been suggested that other cytokines, such as TNFα and GMCSF, can influence neutrophil chemotaxis, and thus that the neutrophil environment is very important for this process (205, 206). Importantly, it has been demonstrated that IFNγ can influence expression of chemokine receptors on neutrophils, and thus IFNs may have an influential role in neutrophil chemotaxis in JSLE (179).

Within a North American population of SLE patients, neutrophils reduced random migration and directed migration towards C5a compared to healthy controls (116). Chemotactic activity within SLE serum was also reduced compared to that of healthy controls, and the level of dsDNA negatively correlated with chemotactic activity (117). The reduced chemotactic activity was found in patients who were younger at onset of disease and onset of renal involvement, and these patients tended to have increased incidence of infection (117). However, another study in a Taiwanese population of JSLE patients found that infection, disease activity or medication had no influence on chemotaxis, although there was a suggestion that neutrophil chemotaxis was reduced (112). Thus, the pro-inflammatory environment within SLE patients, which includes infection, medication and disease activity,

may reduce neutrophil chemotaxis. However, this effect may be very dependent on ethnicity and age of onset of SLE.

In contrast to the above studies, growth regulated oncogene (GRO)α, a neutrophil chemokine, has been shown to be higher in a Chinese population of SLE patients than in healthy controls, and thus suggesting increased chemotaxis in these patients, highlighting that ethnicity may be important in how chemotaxis is dysregulated in SLE (118). Interestingly, there were no observed differences in IL-8 plasma levels between SLE and healthy control patients (118). This is interesting as IL-8 is a potent neutrophil chemokine, and thus it is thought that blood levels of IL-8 would correlate with the degree of dysregulated chemotaxis already outlined (116, 117, 207). Other cytokines, such as IP-10, RANTES, monokine induced-by-γ-IFN (MIG) and MCP1, which have chemotactic properties, were shown to be higher in SLE patient plasma than healthy control patients (118). MCP1 and IL-8 plasma concentrations correlated with Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) scores in SLE patients, and plasma concentrations of IP-10 and IL-8 correlated with SLEDAI scores of SLE patients with renal involvement (118). This elevation in chemokine concentration, and thus possible increase in neutrophil chemotaxis within SLE, contradicts other published studies that found a reduced chemotactic activity within SLE patients (116-118).

Although it has been shown in adults with SLE that a history of infection reduces neutrophil's phagocytic ability compared to patients without infection, this isn't the case in JSLE. A history of infection does not influence neutrophil phagocytosis in JSLE patients compared to neutrophils of healthy control patients (112, 115). Of interest, neutrophil phagocytosis in JSLE patients is also not affected by medication or disease activity (112). Interestingly, in a previous study, only the phagocytosis of *salmonella* by JSLE neutrophils is reduced, whilst the phagocytosis of *E.coli* and *S.aureus* is comparable to healthy control neutrophils (112). Phagocytosis may be affected by the presence of autoantigens, as demonstrated by the fact that 38% of adult SLE patients positive for anti-Sjögren's syndrome type B (SSB)/La antibodies had reduced levels of neutrophil phagocytosis (115, 189). Thus, the ability of neutrophils to phagocytose may be affected by many factors, which may include expression of autoantigens, and the type of bacteria undergoing phagocytosis.

IFNs have been shown to have an effect on phagocytosis, although this effect is dependent on the sub-type of IFN, the immune cell type and the bacteria being phagocytised. IFNα and IFNβ both can increase the attachment and phagocytosis of non-opsonised and opsonised *E.coli* by mouse peritoneal macrophages, whereas IFNγ reduces these functions (183).

Additionally, both IFNα and IFNβ increase both the Fc receptor mediated and C3b receptor mediated phagocytosis of opsonised sheep erythrocytes, by mouse peritoneal macrophages, whereas IFNγ only slightly reduces these types of phagocytosis (183). Another study showed IFNα and IFNγ up-regulate neutrophil phagocytosis of *S. aureus*, and additionally IFNα stimulated bacterial killing by the neutrophils (184). IFNs may have a role on neutrophil phagocytosis, but this may be very dependent on the receptors that mediate the process and the type of infection that neutrophils are responding to.

NETosis is a unique type of death in which the neutrophils release their contents to trap bacteria (neutrophil extracellular traps, or NETs) – Section 1.3.3. It has been shown that there is an increase of NETosis in adult-onset SLE and this leads to increased expression of autoantigens in the form of exposed chromatin and DNA material, which strongly suggests that NETs are important in SLE development (208). Similar to the clearance of apoptotic debris, NET clearance is dysregulated in SLE patients via the inhibition of DNase 1 within the serum, which then may allow autoantigens to persist and thus lead to organ damage such as lupus nephritis (208).

Anti-RNP has been shown to induce NETs in JSLE neutrophils, which resulted in increased secretion of leucine leucine (LL)37 and HMGB1 which localised with globular structures in NETs from SLE patients (163). LL37 is the only human cathelicidin-derived antimicrobial peptide and can be produced by epithelial cells and neutrophils (209, 210). LL37 activates immune cells by increasing the secretion of cytokines and chemokines from a variety of cells (210). LL37 can also enhance innate immune cell responses through multiple pathways (210). HMGB1 is a nuclear protein that acts as a DNA chaperone, and if HMGB1 is released, it can stimulate cytokine production through the NFκB pathway (211). Supernatants from JSLE neutrophils cultured with anti-RNP antibodies were able to induce pDC activation, resulting in IFNα production (163). It was shown that TLR7 is needed for NETosis in response to anti-RNP (163). IFNα up-regulated TLR7, and anti-RNP IgG was able to induce NETosis in IFNα-primed healthy neutrophils. The supernatants from these cells were able to activate pDCs in a DNA-dependent manner (163). IFNα and IFNγ have been indicated to prime neutrophils for NETosis, and NETing neutrophils in turn have been indicated to promote IFNα production from pDCs in JSLE (163, 175). This creates a positive feedback loop which may increase the autoantigens produced by NETs (212). Although IFNs are unlikely to induce NETs on their own, they seem to be important in priming neutrophils to produce NETs.

3.1.1. Summary

Cytokines are important in regulating many important neutrophil functions. There is no clear evidence that IFNs are directly involved in neutrophil chemotaxis, NETosis or neutrophil phagocytosis in the pathogenesis of JSLE. However, the increased level of IFNs in JSLE and SLE sera and the pronounced granulocyte and IFN signature found in JSLE patients suggests some potential and important interaction, and thus IFNs may have some direct or indirect role in these functions. It has been shown that priming neutrophils with exposure to IFN does influence neutrophil functions, as seen in JSLE, and thus may contribute to JSLE pathogenesis.

3.2. Chapter hypothesis

There is an increase of IFNs within the serum of patients with adult-onset SLE and JSLE and an increased IFN and neutrophil signature in JSLE PBMCs, suggesting an interaction of IFNs and neutrophils within JSLE pathogenesis. IFNs may have a role in dysregulated neutrophil function that contributes to JSLE pathogenesis.

3.3. Aims and Objectives

Aim: To investigate the effect of IFNs on chemotaxis, phagocytosis and NETosis in combination with other factors.

- Objective 1: Using transwells and manual counting using a haemocytometer and light microscopy, measure the rate of a) naïve healthy adult neutrophil migration towards IFNs and known neutrophil chemoattractants, IL-8 and fMLP; and b) IFN-primed healthy adult neutrophils towards known neutrophil chemoattractants, IL-8 and fMLP.

- Objective 2: Using flow cytometry, measure the rate of healthy adult neutrophil phagocytosis of *E.coli* bioparticles after IFN priming.

- Objective 3: Using confocal microscopy and DNA quantification (via Quant-iT™ PicoGreen™ dsDNA assay kit), measure NETosis after a) IFN-priming and subsequent LPS stimulation of healthy adult neutrophils and b) stimulation of IFNs on naïve and TNFα-primed healthy adult neutrophils.

3.4. Methods

3.4.1. Chemotaxis assays

3.4.1.1. Plate coating

12mg/ml of Poly (2-hydroxyethyl methacrylate) (polyHEMA) (Sigma-Aldrich, UK) was diluted in warmed ethanol (Chemistry Department, University of Liverpool, UK) and 400µl was used to coat wells of a 24 well plate. The ethanol was allowed to evaporate in a 37°C incubator for 24 hours. The plates were used immediately or stored at room temperature.

3.4.1.2. The effect of IFNs on neutrophil migration

PolyHEMA coated plates were washed with media 2-3 times to remove any remaining ethanol. 800µl of media w/10% FBS was added to the wells. Chemokines (100ng/ml IL-8 [PeproTech, UK] and 10nM fMLP [Sigma-Aldrich, UK] as positive controls) or 10ng/ml IFNα, 10ng/ml IFNγ or 10ng/ml IFNβ were added to the wells. Polyethylene terephthalate (PET) hanging inserts with 3.0 µm pore size (Merck Millipore, UK) were added on top of wells, and were left to soak in the media for at least 10mins.

Neutrophils were isolated from healthy adults as described in the technical methods chapter (Section 2.5.2). Neutrophils were either primed with 10ng/ml IFNα, 10ng/ml IFNγ or 10ng/ml IFNβ for 30mins before chemotaxis analysis (Section 3.4.1.3) or left unstimulated and immediately analysed for chemotaxis. Neutrophils were suspended at up to $1x10^6$/200µl and 200µl of neutrophil suspension was added to each insert. The plate was incubated at 37°C, 5% CO_2 for 90mins to allow migration to occur. The inserts were lifted out and the media in the wells was mixed by pipetting to make sure the neutrophils were evenly suspended. A 10µl aliquot was removed and counted on a haemocytometer. The count was then extrapolated to calculate the number of neutrophils per 800µl, which represents the number of migrated neutrophils.

3.4.1.3. IFN priming of neutrophils for chemotaxis analysis

Neutrophils were isolated from healthy adults as described in the technical methods chapter (Section 2.5.2). Neutrophils were aliquoted into four Falcon tubes at $3\text{-}4x10^6$ per Falcon (3-4mls). One aliquot of neutrophils was left unstimulated, and 10ng/ml IFNα, 10ng/ml IFNγ or 10ng/ml IFNβ were added to the other three aliquots. The neutrophils were placed on a rocker at 37°C, 5% CO_2 for 30mins. The neutrophils were centrifuged at 1500 RPM for 5mins, washed in 1ml media w/10% FBS and centrifuged at 1500 RPM for 5mins. The supernatant was decanted, and any remaining was removed using a pipette. 400-600µl of media was

added to the neutrophil pellet and neutrophils were recounted to account for any loss in neutrophils during centrifugation. Neutrophils in each condition were normalised to the lowest concentration of neutrophils for each donor ($4.8x10^5$-$1x10^6$/200µl), and chemotaxis assay was conducted as described above (Section 3.4.1.2).

3.4.2. Phagocytosis assay

Neutrophils were isolated from healthy adults (Section 2.5.2). 1ml of extra blood was also taken from each healthy volunteer and centrifuged at 2000 RPM for 10mins to obtain autologous serum. Neutrophils were plated in a 96 well plate at $1.5x10^5$/150µl per condition, with one extra well empty for the 'bioparticles only' condition. Either 10ng/ml IFNα, 10ng/ml IFNγ or 10ng/ml IFNβ were subsequently added to neutrophils and incubated for 30mins at 37°C, 5% CO_2. PHrodo Red *E. coli* bioparticles (Life Technologies, UK) were initially suspended in 2ml of HBSS/20mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (pH 7.4, Sigma-Aldrich, UK), vortexed and subsequently diluted further: 4.5µl of bioparticles plus 55.5µl of buffer per condition. For treatment conditions, in which bioparticles needed to be opsonised, 60µl of bioparticles was suspended in 15µl of autologous serum (which equates to 10% serum in final assay set up) and left for at least 15mins at room temperature. Neutrophils were centrifuged at 1800 RPM for 5mins, and the supernatant was decanted. For each 'treatment' condition (including unstimulated cells), 75µl of media was added to the neutrophils, plus 75µl of bioparticles/serum. For cells only, 150µl of media was added. For bioparticles only, cells + bioparticles, and no serum conditions, 90µl media plus 60µl bioparticles (in buffer only) were added to the neutrophils. The neutrophils were incubated at 37°C, 5% CO_2 for 10mins, and centrifuged at 1800 RPM for 3mins. The supernatant was decanted and 200µl of HBSS/20mM was added. Neutrophils were transferred to tubes and run on a pre-programmed phagocytosis protocol on the Guava EasyCyte. Percentage of bioparticles phagocytosed was analysed using FlowJo.

3.4.3. NETosis assays

3.4.3.1. Cytokine priming and stimulation for NETosis assay

Neutrophils were isolated and suspended as protocol (Section 2.5.2) and suspended at $1x10^6$/ml in media w/2% FBS. Neutrophils were plated at $2x10^5$ in 2x24 well plates, one with sterile cover slips (for confocal microscopy) and one without (for DNA quantification). 500µl of media w/2% FBS was added, for a final neutrophil concentration of $2x10^5$/500µl.

Neutrophils were left to settle on coverslips for 30mins at 37°C, 5% CO_2. Neutrophils were left unstimulated or primed with 1µg/ml TNFα (PeproTech, UK), 10ng/ml IFNα, 10ng/ml IFNγ or 10ng/ml IFNβ for 30mins at 37°C, 5% CO_2. Naïve neutrophils were stimulated with 320nM PMA (Sigma-Aldrich, UK), 10ng/ml IFNα, 10ng/ml IFNγ or 10ng/ml IFNβ, TNFα-primed neutrophils were stimulated with 10ng/ml IFNα, 10ng/ml IFNγ or 10ng/ml IFNβ and IFN primed neutrophils were stimulated with 1µg/ml of LPS (Sigma-Aldrich, UK). Neutrophils were incubated at 37°C, 5% CO_2 for 3hrs, and then processed for confocal and DNA quantification analysis.

3.4.3.2. Confocal analysis

Medium was removed, and coverslips were washed twice with 300µl PBS. 300µl 4% formaldehyde (Sigma-Aldrich, UK) was added to coverslips for 10mins at RT to fix the neutrophils. The 4% formaldehyde was removed and coverslips were washed twice with 300µl PBS. Coverslips were mounted onto microscope slides using Prolong Gold Antifade Reagent with DAPI (Molecular Probes by Life Technologies, UK). Slides were wrapped in foil and left to dry at RT for at minimum overnight, or until ready to analyse. Analysis was carried out using a DM2500 confocal microscope (Leica, UK) with a pre-programmed protocol on Leica Application Suite, Advanced Fluorescence, whereby 5-6 images were taken per condition.

3.4.3.3. Quantification of NETs from confocal microscopy

Quantification of NETs from confocal images was trialled. From all the images taken during the confocal analysis, a representative sample of confocal images was produced. This included an image from each donor (n=5) for each condition (n=13) which in total was 65 images to be analysed for each user. To help define NETosis as visualised using confocal microscopy, an example of a viable neutrophil (no NETosis) and a NETing neutrophil was provided. Three independent operators calculated the percentage of NETs in each image, using the calculation:

$$\%\ of\ NETs = \left(\frac{No f NETing\ neutrophils}{No f NETing\ neutrophils + no. of\ viable\ nuetrophils}\right) X100$$

From each operators' image analysis, an average percentage for each condition across the five donors was calculated per operator; these three operator averages were plotted for each condition and analysed for inter-operator variability.

3.4.3.4. DNA quantification analysis

After stimulation and incubation of neutrophils, DNA was cleaved into the supernatant, which was centrifuged to remove cellular debris. The DNA concentration was measured by a plate reader using fluorescence from Quant-iT™ PicoGreen™ reagent and comparison to a standard curve.

250 micro (m)Units of micrococcal nuclease (Sigma-Aldrich, UK) was added to the neutrophil culture with 5µl of 0.1M of calcium chloride (Sigma-Aldrich, UK) to cleave NET material. The neutrophils were incubated for 10mins at 37°C, 5% CO_2. 5mM EDTA was added to terminate the enzyme digestion, and the supernatant was centrifuged at 200xg for 8mins to remove remaining cells. Care was taken to avoid disrupting any cellular pellet and the supernatant was stored at -80°C until analysis.

The NET material was analysed using Quant-iT™ PicoGreen™ dsDNA Assay Kit (Invitrogen/ThermoFisherScientific, UK). λDNA was diluted to 2000ng/ml, 200ng/ml, 20ng/ml and 2ng/ml in 1x Tris EDTA (TE) buffer, and 100µl of both standards and samples were loaded into a black, flat-bottomed 96 well plate, with 1xTE buffer used as a blank. 100µl of 1x Quant-iT™ PicoGreen™ was added to all samples and standards and the plate was incubated at room temperature for 4mins. The fluorescence was measured at 485nm excitation and 535nm emission on a TECAN GENios Plus plate reader (Tecan, UK).

A standard curve was generated, plotting standards against absorbance, and the concentration of each sample was extrapolated from the line of best fit equation using the average of the absorbance obtained (Figure 3.1).

3.4.4. Statistical analysis

Statistical analyses undertaken for data arising from these assays were analysed using Friedman's test with Dunn's *post hoc* multiple comparison test in GraphPad Prism 6. For detailed justification, Section 2.10.

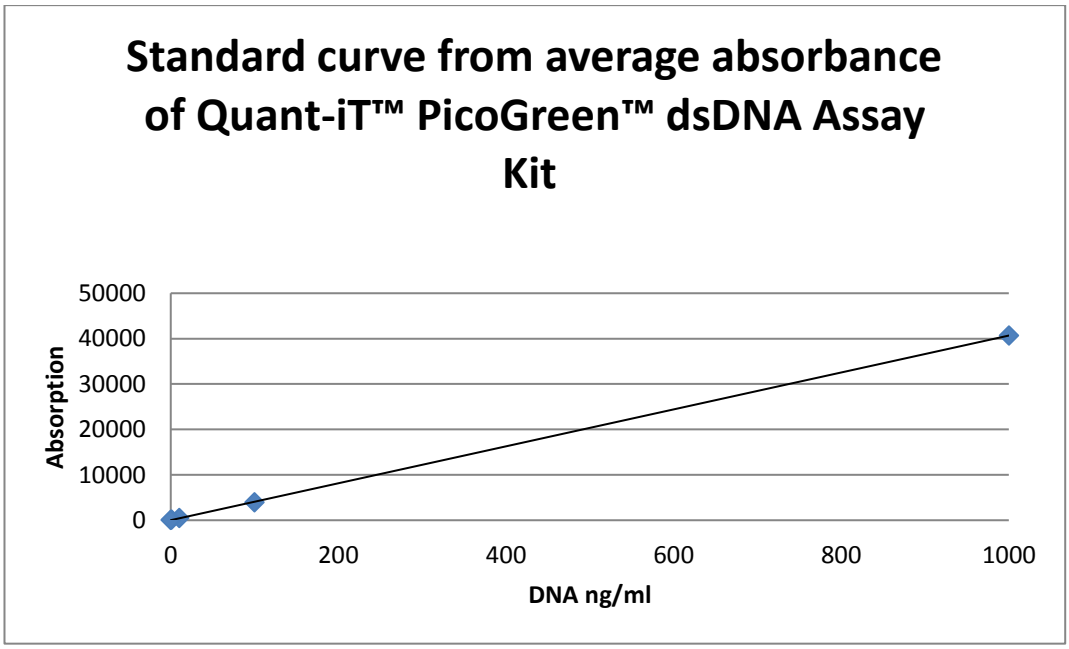


Figure 3.1. Quant-iT™ PicoGreen™ dsDNA Assay Kit standard curve. λDNA was diluted at 200ng/ml, and dilutions using 1xTE buffer and PicoGreen was conducted to achieved other standards. Standards and samples were loaded in dulicate into a 96 well plate, and the Quant-iT™ PicoGreen™ reagent was loaded. The plate was read, and the standard curve was plotted using average absorbance against the known standards.

3.5. Results

3.5.1 Investigating the effect of IFNs as 'neutrophil chemokines'

The first neutrophil function to be investigated was chemotaxis. Although there was no significant published evidence to confirm the role of IFNs as chemokines for neutrophils, this function was investigated to confirm or disprove this role.

Compared to random migration occurring in control conditions (media w/10% FBS only; $1.7x10^5 \pm 0.3x10^5$ n=5), both IL-8 ($2.7x10^5 \pm 0.4x10^5$ n=5) and fMLP ($3.5x10^5 \pm 0.8x10^5$, n=5, p=0.020) (Figure 3.2) were shown to increase the migration of neutrophils in this model, this being more pronounced with fMLP-induced migration (see Figure 3.2). These data correlate well with the published literature (47) and indicate the model was working effectively. Notably, none of the IFN subtypes demonstrated any additional effect on neutrophil migration (IFNα = $1.7x10^5 \pm 0.2x10^5$; IFNγ = $1.6x10^5 \pm 0.3x10^5$; IFNβ = $1.8x10^5 \pm 0.2x10^5$; n=5) compared to the neutrophil random migration occurring in controlled conditions.

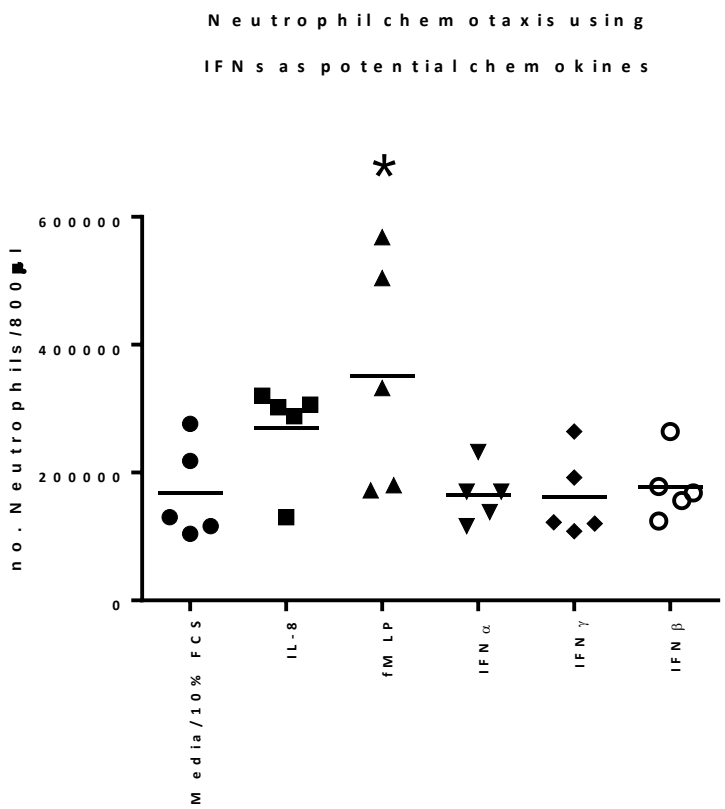


Figure 3.2. Neutrophil chemotaxis towards media (random migration) and chemokines/cytokines after 90mins. Plates were coated with PolyHEMA and media w/10% FBS was added, and was left un-supplemented, or supplemented with 100ng/ml IL-8, 10nM fMLP, or 10ng/ml IFNα, 10ng/ml IFNγ or 10ng/ml IFNβ. Inserts were left to soak for 10mins. Neutrophils were isolated from healthy adult donors and were plated in inserts and left to migrate for 90mins. Neutrophils were suspended in wells, counted and calculated as number of neutrophils/800µl. fMLP significantly attracted neutrophils compared to random migration (*, n=5, p=0.020), and IL-8 has increased migration compared to random migration, although this was not significant (n=5, p=0.14). None of the IFN subtypes had any demonstrable chemokine effect on neutrophils.

3.5.2. Investigating the effect of IFN priming on neutrophil chemotaxis

Based on published data (179, 205, 206), the next step was to investigate whether IFN priming could affect random neutrophil migration (towards control medium) or directed migration (towards IL-8 and fMLP) neutrophil chemotaxis. Chemotactic conditions were therefore replicated as above (Section 3.5.1) with the addition of a 30mins pre-treatment of the neutrophils with each of the IFN subtypes.

With naïve (un-primed) neutrophils, both IL-8 and fMLP were shown to have an increased migration effect compared to control media alone, although in these experiments this was not shown to be statistically significant (media w/FBS = $1.2x10^5 \pm 0.5x10^5$, n=5; IL-8 = $4.0x10^5 \pm 1.0x10^5$, p=0.22, n=5; fMLP = $3.4x10^5 \pm 0.8x10^5$, p=0.93, n=5) (see Figure 3.3).

IFNα priming (Section 3.4.1.2) had no demonstrable additional influence over random or directed migration compared to naïve neutrophils (IFNα primed neutrophils: media w/FBS = $0.8x10^5 \pm 0.4x10^5$; IL-8 = $2.4x10^5 \pm 0.5x10^5$; fMLP = $2.9x10^5 \pm 0.6x10^5$; n=5) (Figure 3.3). IFNγ priming also had no additional demonstrable influence over random or directed migration compared to naïve neutrophils (IFNγ primed neutrophils: media w/FBS = $1.1x10^5 \pm 0.8x10^5$; IL-8 = $3.8x10^5 \pm 1.3x10^5$ fMLP = $3.0x10^5 \pm 0.8x10^5$, n=5) (Figure 3.3). Finally, IFNβ also had no demonstrable additional effect on random or directed migration compared to naïve neutrophils (IFNβ primed neutrophils: media w/FBS = $0.8x10^5 \pm 0.3x10^5$; IL-8 = $2.2x10^5 \pm 0.6x10\%$; fMLP = $2.1x10^5 \pm 0.4x10^5$, n=5) (Figure 3.3). Thus, these experiments demonstrated that IFN priming had little effect on chemotaxis.

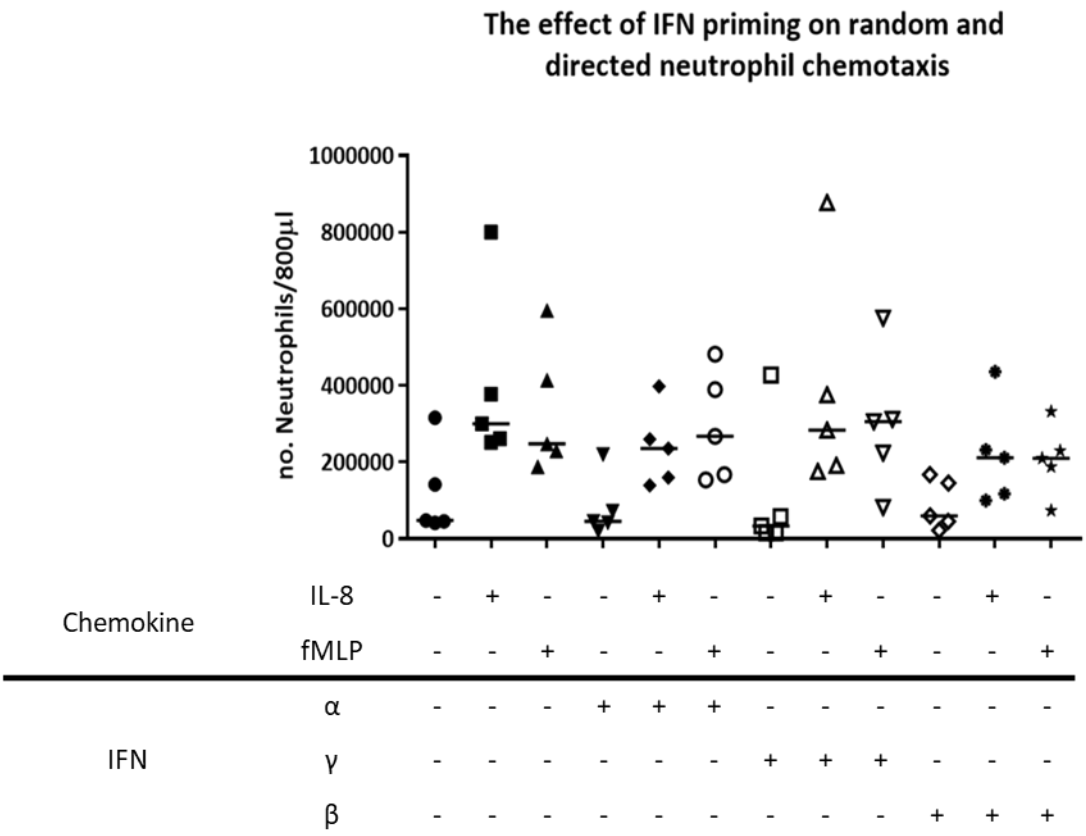


Figure 3.3. Neutrophil chemotaxis towards media (random migration) and known neutrophil chemokines (IL-8 and fMLP) following priming with 10ng/ml IFNα, 10ng/ml IFNγ, or 10ng/ml IFNβ for 30mins. Plates were coated with polyHEMA and media w/10%FBS was added to the wells. Media was left un-supplemented or supplemented with 100ng/ml IL-8 or 10nM fMLP. Inserts were added and left to soak for 10mins. Neutrophils were isolated from healthy adult donors and were left naïve or were primed with 10ng/ml IFNα, 10ng/ml IFNγ or 10ng/ml IFNβ for 30mins, then plated into the inserts and left to migrate for 90mins. Neutrophils were suspended in wells, counted and calculated as number of neutrophils/800µl (n=5). IL-8 and fMLP had an increased migration effect. None of the IFN subtypes showed a demonstrably significant effect on either random or migrated chemotaxis.

3.5.3. Investigating the IFN priming effect on phagocytosis

The next neutrophil function assay to be investigated was phagocytosis. It has been shown previously that neutrophil phagocytosis is significantly reduced in both SLE and JSLE patients (112, 115) (Section 1.4.3). Thus, it may be that the high levels of IFNs found in the sera of patients with SLE and JSLE are involved in this reduced phagocytosis (68, 112, 115, 171, 172). For these reasons, IFN primed neutrophils were investigated on their subsequent phagocytosis capability.

Overall, compared to naïve neutrophils (analysed as fluorescence within the neutrophil population; 66.6 ± 10.2%, n=4) the data showed there was no demonstrable priming effect of any of the IFN subtypes on neutrophil phagocytosis of *E. coli* bioparticles (IFNα 68.2 ± 9.4%, IFNγ 64.7 ± 10.8%; IFNβ 67.8 ± 10.0%, n=4)(Figure 3.4). It was notable that there was great variation in phagocytosis in neutrophils between donors (n=4) (Figure 3.4). Therefore, IFNs do not affect neutrophil phagocytosis of E.coli bioparticles.

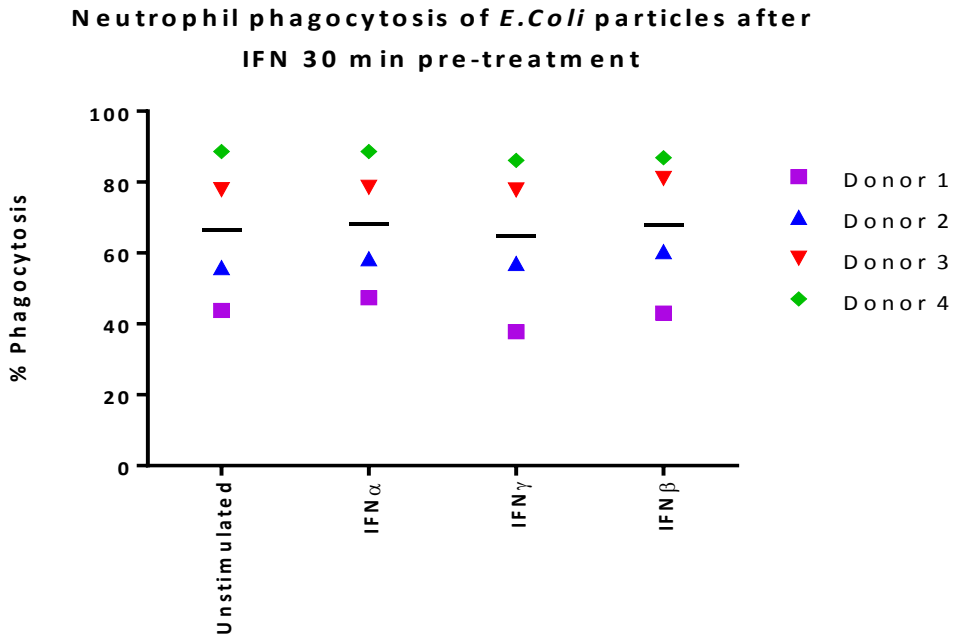


Figure 3.4. Healthy adult neutrophil phagocytosis of E.coli bioparticles after priming with 10ng/ml IFNα, 10ng/ml IFNγ or 10ng/ml IFNβ for 30mins. Neutrophils were isolated from healthy adult donors and were either left naïve or primed with 10ng/ml IFNα, 10ng/ml IFNγ or 10ng/ml IFNβ for 30mins, and subsequently stimulated with *E.coli* bioparticles for 10mins. The percentage of neutrophil phagocytosis of *E. coli* bioparticles was analysed using flow cytometry (n=4).

3.5.4. Investigating the effect of IFN subtypes on NETosis

The next function of neutrophils to be investigated was NETosis, which has been shown to potentially be important in the pathogenesis of SLE and JSLE (176, 208, 212) (Section 1.5.3.2). To do this, a series of experiments were performed. These included determining the effects of stimulation by each of the IFN subtypes on both naïve and TNFα-primed neutrophils. Additionally, the effect of LPS stimulation on IFN-primed neutrophils was investigated. LPS is a bacterial product and has been shown to induce NETosis in canines (213, 214). LPS was therefore used to try model NETosis during infections. Together, these experiments were undertaken to determine if, and in what way, the IFN subtypes may either together or individually have a role in NETosis.

3.5.4.1. Investigating the role of IFN subtypes on NETosis using confocal microscopy

Initially, a confocal analysis was conducted to investigate how the different IFN subtypes effect NETosis, either through stimulation or priming. Firstly, neutrophils isolated from healthy adult donors were left to settle on coverslips for 1hr, and then either unstimulated or stimulated with 320nM PMA and incubated for 3hrs, and subsequently fixed and stained with DAPI within mounting media before being assessed using confocal microscopy. As expected, there were minimal NETs demonstrated in the unstimulated conditions (9.7 ± 7.3%; average of three operators' analysis, Sections 3.4.3.3 and 3.5.4.2) (Figure 3.5, Figure 3.10 and Appendix E, Figure 1) whilst NETs were clearly induced on stimulation with PMA 53.6 ± 26.6%; average of three operators' analysis, Sections 3.4.3.3 and 3.5.4.2) (Figure 3.5, Figure 3.10 and Appendix E, Figure 2). These results confirmed that the assay was working as expected.

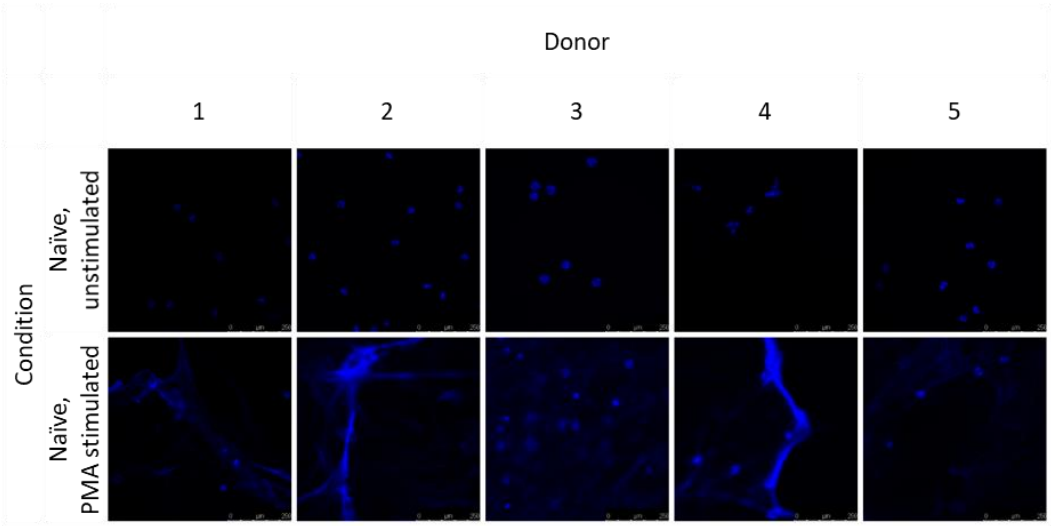


Figure 3.5. NET visualisation at 4hrs of unstimulated healthy adult neutrophils or healthy adult neutrophils stimulated with 320nM PMA, as shown by DAPI staining. Neutrophils were isolated from healthy adult donors (n=5) and were left to settle on coverslips for 1hr. Neutrophils were unstimulated or stimulated with 320nM PMA, and incubated for 3hrs, and subsequently fixed and stained with DAPI within mounting media. Each image is representative of 5 images taken for each donor. Unstimulated neutrophils did not undergo NETosis. However, PMA, was shown to be a strong inducer of NETosis.

As the assay has been confirmed to work as expected as shown above, the next stage was to investigate how IFN stimulation effects NETosis in naïve neutrophils. Naïve neutrophils were stimulated for three hours by adding fixed concentrations of each of the IFN subtypes, namely: 10ng/ml IFNα, 10ng/ml IFNγ or 10ng/ml IFNβ, and subsequently fixed and stained with DAPI within mounting media before being assessed using confocal microscopy. IFNα-stimulated neutrophils did demonstrate evidence of DNA release as seen by confocal microscopy (IFNα = 26.3 ± 2.7%, IFNγ = 12.2 ± 1.4%, IFNβ = 14.6 ± 2.2%; average of three operators' analysis, Sections 3.4.3.3 and 3.5.4.2) (Figure 3.6, Figure 3.10 and Appendix E, Figures 3-5), compared to naïve, unstimulated neutrophils (9.7 ± 7.3%; average of three operators' analysis; Sections 3.4.3.3 and 3.5.4.2) (Figure 3.6, and Appendix E, Figure 1) indicating evidence of NETosis. However, this was limited and not seen in all images (Figure 3.6 and Appendix E, Figures 1, 3-5), so whilst some induction occurred, this was modest and less striking than that occurring with PMA, and therefore it remains inconclusive whether IFNs have a true effect on NETosis in naïve neutrophils.

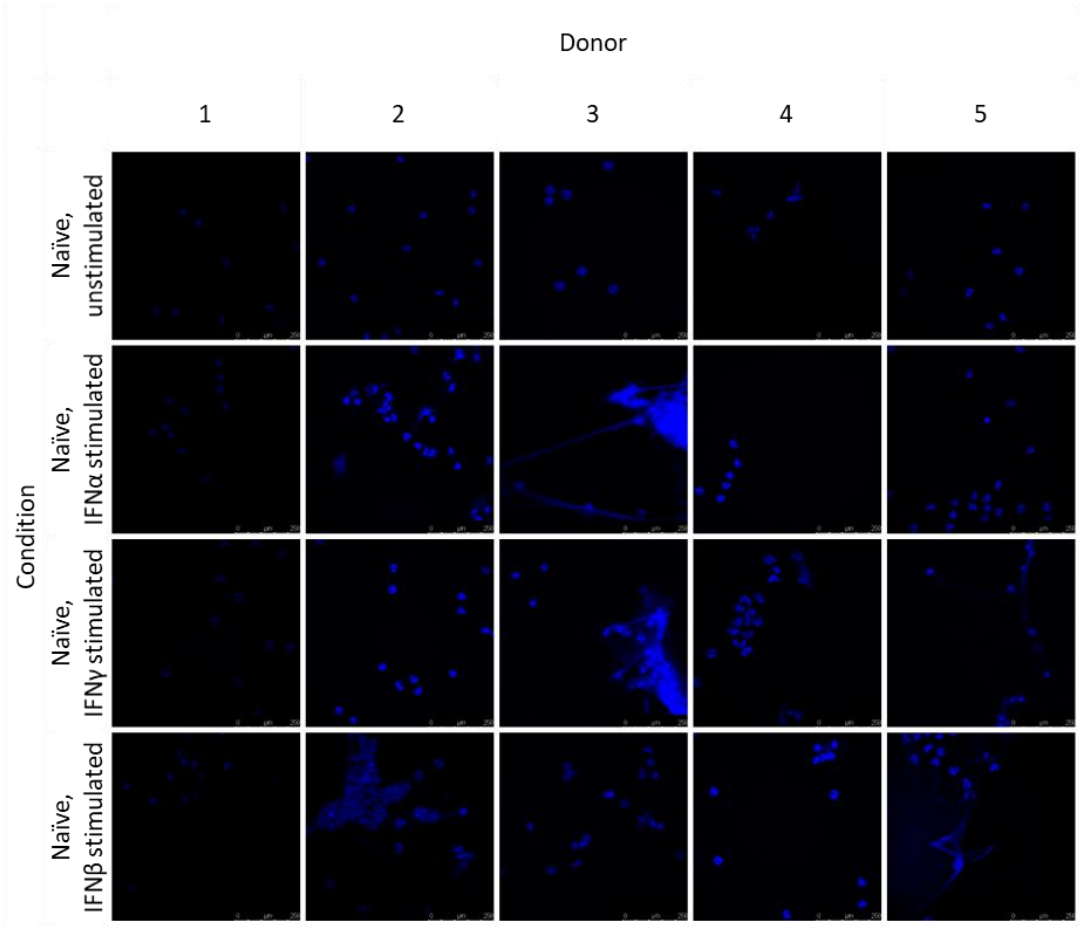


Figure 3.6. NET visualisation at 4hrs of naïve, unstimulated healthy adult neutrophils, or healthy adult neutrophils stimulated with 10ng/ml IFNα, 10ng/ml IFNγ or 10ng/ml IFNβ, as shown by DAPI staining. Neutrophils were isolated from healthy adult donors (n=5) and were left to settle on cover slips for 1hr. Neutrophils left unstimulated or were stimulated with 10ng/ml IFNα, 10ng/ml IFNγ or 10ng/ml IFNβ for 3hrs, and subsequently fixed and stained with DAPI within mounting media. Each image is representative of 5-6 images taken for each donor. IFN-stimulated neutrophils did undergo DNA release and thus did undergo NETosis in naïve neutrophils, although this was limited, and not seen in all images.

The next set of experiments explored the effect of TNFα-priming on NETosis (without any further neutrophil stimulation) and the effect of the different IFN subtypes on NETosis in TNFα-primed neutrophils, as assessed by confocal microscopy (Figure 3.7). Neutrophils were primed with 1μg/ml TNFα and were left unstimulated or stimulated with 10ng/ml IFNα, 10ng/ml IFNγ or 10ng/ml IFNβ for 3 hours. These data gave a suggestion of increased NETosis in TNFα-primed neutrophils (17.3 ± 1.5%; average of three operators' analysis; Sections 3.4.3.3 and 3.5.4.2) (Figure 3.7, Figure 3.10 and Appendix E, Figure 1) compared to that of naïve, unstimulated neutrophils (9.7 ± 7.3%; average of three operators' analysis; Sections 3.4.3.3 and 3.5.4.2) (Figure 3.7, Figure 3.10 and Appendix E, Figure 6), as shown through some DNA release on the confocal images. Those healthy neutrophils primed with TNFα and then stimulated with IFNγ and IFNβ, but not IFNα, did demonstrate evidence of DNA release as seen by confocal microscopy indicating evidence of NETosis did give an indication of some NET formation (IFNα = 12.6 ± 1.4%, IFNγ = 23.9 ± 5.2%, IFNβ = 30.9 ± 11.4%; average of three operators' analysis, Sections 3.4.3.3 and 3.5.4.2) (Figure 3.7, Figure 3.10 and Appendix E, Figures 7-9). However, this was limited and not seen in all images (Figure 3.7 and Appendix E, Figures 7-9) and was therefore modest and less striking than that occurring with PMA. Therefore, it remains inconclusive whether either TNFα-priming or IFN stimulation on these primed neutrophils have a true effect on NETosis, although according to the operator analysis (Section 3.5.4.2).

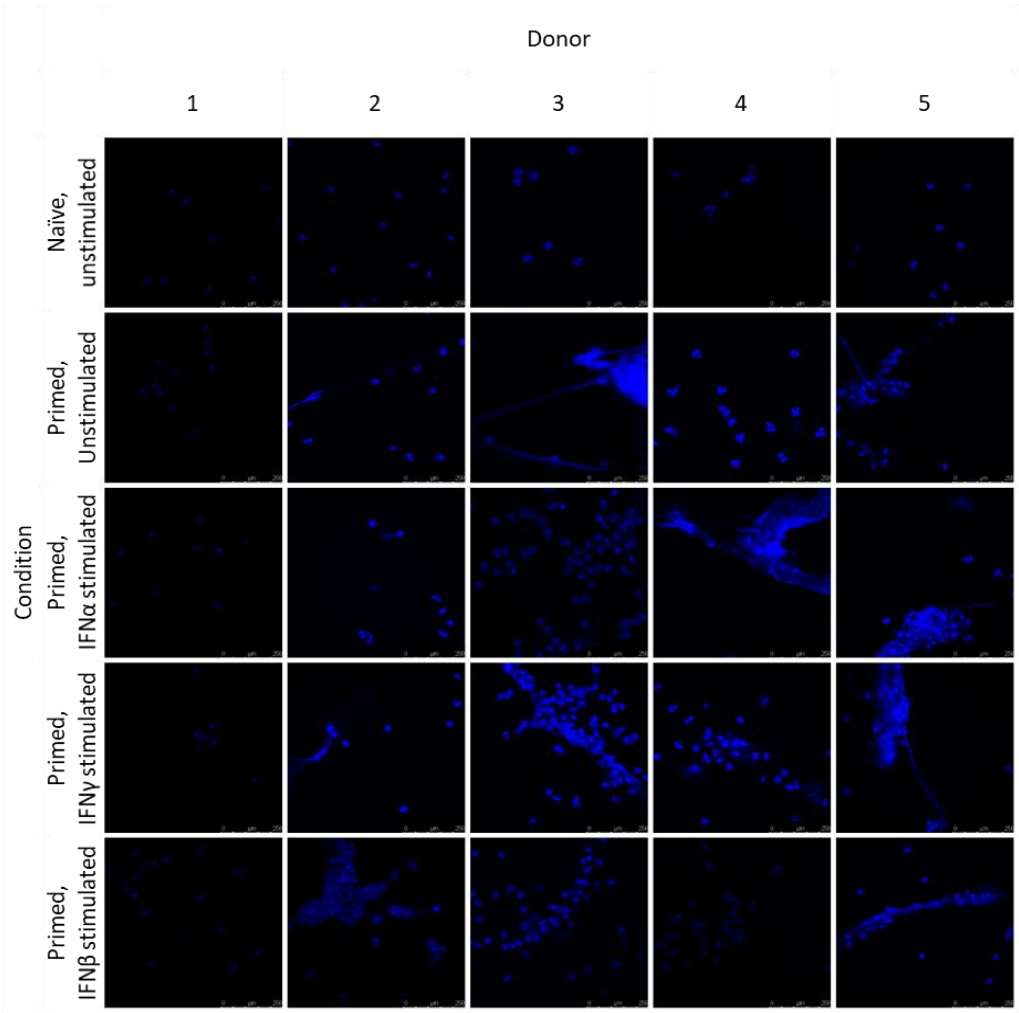


Figure 3.7. NET visualisation at 4hrs of naïve, unstimulated healthy adult neutrophils, TNFα-primed, unstimulated healthy adult neutrophils and TNFα-primed, healthy adult neutrophils stimulated with 10ng/ml IFNα, 10ng/ml IFNγ or 10ng/ml IFNβ, as shown by DAPI staining. Neutrophils were isolated from healthy adult donors (n=5) and were left to settle on cover slips for 30mins. Neutrophils were left naïve or primed with 1µg/ml TNFα for 30mins, and stimulated with 10ng/ml IFNα, 10ng/ml IFNγ or 10ng/ml IFNβ for 3hrs. Neutrophils were fixed and stained with DAPI within mounting media. Each image is representative of 5-6 images taken for each donor. IFN stimulated neutrophils did undergo DNA release and thus did undergo NETosis in TNFα-primed neutrophils, although this was limited, and not seen in all images.

Lastly, it was important to investigate whether priming with the different IFN subtypes has an effect on NETosis. Therefore, healthy adult neutrophils were left naïve, or primed with 10ng/ml IFNα, 10ng/ml IFNγ or 10ng/ml IFNβ, subsequently stimulated with 1μg/ml LPS, and assessed for NETosis using confocal microscopy. LPS stimulation of naïve neutrophils did not result in DNA release and therefore did not induce any NETs (15.1 ± 4.5%; average of three operators' analysis; Sections 3.4.3.3 and 3.5.4.2) (Figure 3.8, Figure 3.10 and Appendix E, Figure 10). LPS stimulation of IFNα-primed neutrophils (but not IFNγ or IFNβ), however did demonstrate evidence of DNA release as seen by confocal microscopy indicating evidence of NETosis formation (IFNα = 21.5 ± 7.1%, IFNγ = 13.3 ± 3.9%, IFNβ = 16.3 ± 6.3%; average of 2-three operators' analysis, Sections 3.4.3.3 and 3.5.4.2) (Figure 3.8, Figure 3.10 and Appendix E, Figures 11-13). However, this was limited and not seen in all images (Figure 3.8 and Appendix E, Figure 11-13) and was therefore modest and less striking than that occurring with PMA. Therefore, it remains inconclusive whether priming with any of the IFN subtypes has a true effect on NETosis.

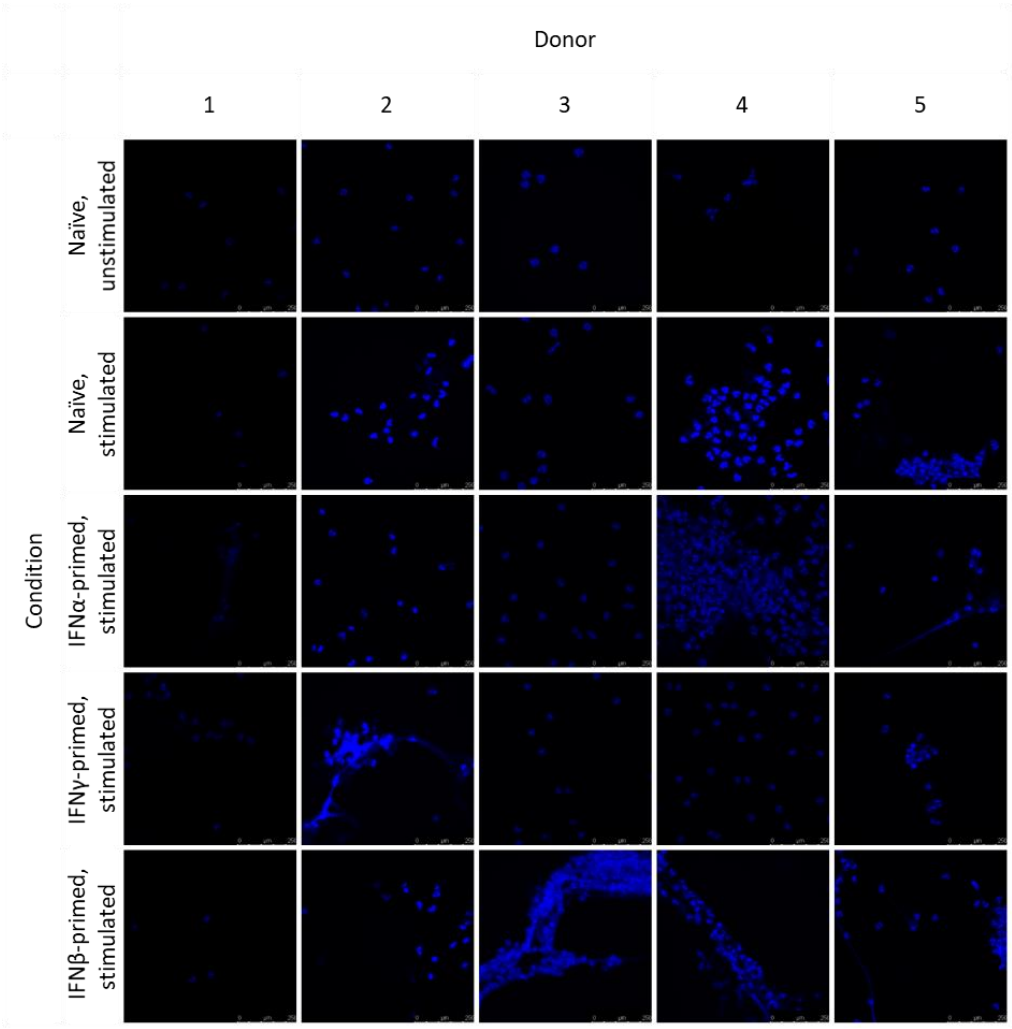


Figure 3.8. NET visualisation at 4hrs of naïve, unstimulated healthy adult neutrophils, naïve healthy adult neutrophils stimulated with 1μg/ml of LPS, or healthy adult neutrophils primed with 10ng/ml IFNα, 10ng/ml IFNγ or 10ng/ml IFNβ and stimulated with 1μg/ml of LPS, as shown by DAPI staining. Neutrophils were isolated from healthy adult donors (n=5) and were left to settle on cover slips for 30mins. Neutrophils were left naïve or primed with 10ng/ml IFNα, 10ng/ml IFNγ or 10ng/ml IFNβ for 30mins and stimulated with 1μg/ml LPS for 3hrs. They were subsequently fixed and stained with DAPI within mounting media. Each image is representative of 5-6 images taken for each donor. LPS-stimulated, IFN-primed neutrophils did release DNA and thus did undergo NETosis, although this was limited, and not seen in all images. Additionally, no differences in NETosis were seen upon priming with the different IFN subtypes.

It should be noted that NETs were not always visualised in all images for each of the set of experiments described above. It was evident that there was a great deal of variability between donors in the ability of their healthy neutrophils to undergo NETosis in each of the various sets of experimental conditions explored, irrespective of whether they were being primed or stimulated or left without either taking place (unstimulated, naïve). Many factors such as donor medical history and demographics may have contributed to this. An example set of images is shown in Figure 3.9, in which for a given set of conditions, all of the images associated with each of the five donors is shown. They show intra-donor and inter-donor variability in the demonstration of NETosis between images. Typical or illustrative examples have been given in the sets of experiments above. For all image sets, please see Appendix E.

Another methodological challenge noted during these experiments was that clumping of neutrophils occurred within the visualised images taken. This made categorising (for some images) what was a NET and what was a viable neutrophil difficult to determine. It was recognised therefore that this introduces a potential level of subjectivity to this method of assessment of the effect of the IFN subtypes on healthy neutrophil NETosis. Whilst this can be overcome in part by inter-observer assessment of all the images by a second or more investigator (Section 3.5.4.2, Figure 3.10), the extent and number of images needing to be assessed make this very difficult in practical terms for a large study. These images highlight an unavoidable inconsistency in NETosis visualisation both within and between donors, which makes making firm conclusions of findings difficult. In view of this difficulty in analysing consistently the level of NETosis in these images using confocal microscopy, a quantifying analytical approach was undertaken to determine if this gave a more objective assessment (Section 3.5.4.2; Figure 3.10).

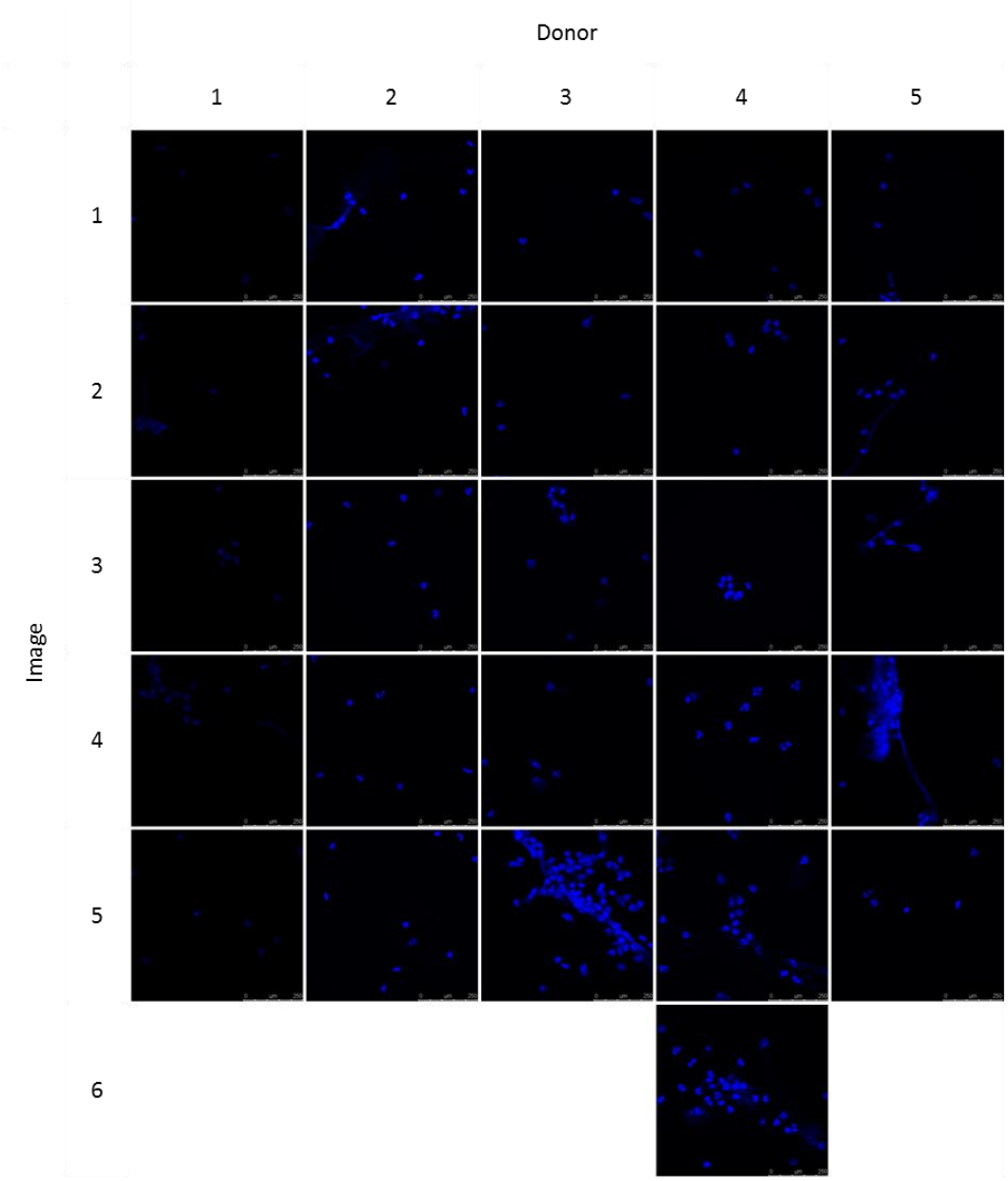


Figure 3.9. NET visualisation at 4hrs of healthy adult neutrophils, primed with TNFα-primed, stimulated with IFNγ and stained with DAPI. Neutrophils were isolated from healthy adult donors (n=5) and were left to settle on cover slips for 30mins. Neutrophils were primed with 1µg/ml TNFα for 30mins and stimulated with 10ng/ml IFNγ for 3hrs. They were subsequently fixed and stained with DAPI within mounting media. 5-6 images were taken per donor.

3.5.4.2. Quantification of NETs from confocal microscopy

Although estimation of the relative extent of NETosis taking place under different experimental conditions can indicate major differences between groups, assessment by confocal microscopy, as illustrated and discussed above (Section 3.5.4.1) is a subjective method dependent largely upon operator interpretation of fixed 2D images. The differentiation between a viable neutrophil from a neutrophil undergoing NETosis may therefore be dependent in part upon individual operator experience, expertise and their interpretation of any image. This is likely to introduce significant variability into an estimate of NETosis using this method, compounded by the inter-donor variability effect also previously noted above. There are no published data on inter-operator reliability of confocal microscopy as a method to estimate neutrophil NETosis. In order to gain insight into inter-operator variability using this method, three independent operators were asked to estimate the extent of NETosis taking place. They were asked to do this using the equation to estimate the percentage of NETosis taking place in each of the images (Section 3.4.3.3), using the same set of confocal microscopy images. Each operator was blinded from the other operators' estimates. Prior to carrying out their assessments, each were given instruction and examples of images of viable neutrophils and of NETs., Each of the three operators calculated a percentage of NETs within each of the five images (five separate donors) presented for each condition (five percentages/condition/operator). For each condition, an average percentage from the five images was calculated for each operator (one average percentage/condition/operator); these three operator averages was plotted for each condition and analysed for inter-operator variability (Figure 3.10).

It was shown that although some conditions there was reasonable inter-observer consistency in percentage NETosis estimates (e.g. all three IFN subtype stimulated naïve neutrophils). However, for some conditions (e.g. PMA and LPS stimulated neutrophils), there was marked inconsistency, particularly in regards to PMA and LPS stimulated neutrophils (Figure 3.10). Additionally, one operator found analysing what was a viable neutrophil and what was a NETing neutrophil difficult due to neutrophil clumping (PMA stimulation and IFNβ priming plus LPS stimulation had no percentage calculated and were omitted for this operator). Due to the difficulty and subjectivity of quantifying the percentages of NETs in the confocal images, another, more quantifiable assay, was therefore needed to supplement the important information and contribution that the confocal images could make to these assessments

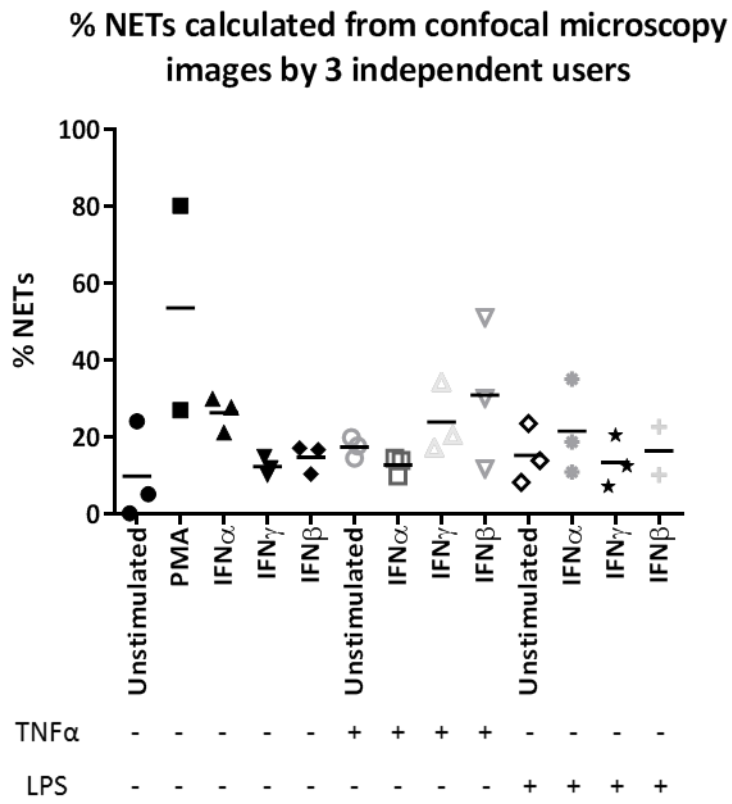


Figure 3.10. NET quantification analysis of confocal microscopy images by three independent operators. One confocal image from each donor (n=5) was selected for each experimental condition undertaken and were assessed by each of the operators to determine the extent (percentage) NETosis present. Results were compiled for analysis. Scoring rules was established and each operator was instructed to count the both number of viable and number of NETing neutrophils and calculate the percentage of NETs within the population seen in each image. Each operator's percentages were averaged for each condition, and this was graphically presented above. Of note, one operator found the PMA and IFNβ-primed, LPS-stimulated images too difficult to quantify accurately, and thus no percentages were calculated for these from this operator.

3.5.4.3. Investigating the role of IFNs on NETosis-related DNA release

In order to seek to overcome the subjective nature of confocal microscopy assessment of NETosis, and the inter-observer variability of this approach (due to clumping of the cells within certain conditions making it difficult to analyse), an alternative strategy was explored. DNA is a major component of NETs, and is released into neutrophil culture upon NETosis induction (37). Therefore, investigation into whether quantification of DNA release could rigorously quantify NETosis within this study was explored. The amount of DNA released during the process of NETosis was quantified from supernatants processed from neutrophil cultures (Section 3.4.3.1 and 3.4.3.4) using Quant-iT™ PicoGreen™ dsDNA Assay Kit (Section 3.4.3.4).

Using the experimental conditions previously carried out (Sections 3.4.3.1. and 3.4.3.4), the quantity of DNA released within supernatants was measured and compared. Of note, although effort was made to collect matched supernatants to the confocal analysis conditions, one confocal analysis repeat did not have a matched supernatant to be analysed. PMA increased neutrophil DNA released into the supernatant compared to naïve unstimulated neutrophils, and this was presumed to relate to the amount of NETs formed (PMA-induced: 438 ± 120 ng/ml versus unstimulated: 91 ± 16 ng/ml, n=5) (see Figure 3.11). No other experimental conditions used in this study (Section 3.4.3.1) with or without each of the IFN subtype stimulating or priming the neutrophils, demonstrated any clear increase in DNA release compared to the unstimulated controls (naïve neutrophils: IFNα = 77 ± 14 ng/ml, IFNγ = 81 ± 6 ng/ml, IFNβ =79 ± 7 ng/ml, n=5; TNFα-primed neutrophils: unstimulated = 84 ± 9 ng/ml, IFNα = 72 ± 12 ng/ml, IFNγ = 80 ± 8 ng/ml, IFNβ =110 ± 18 ng/ml, n=5; IFN primed neutrophils with LPS stimulation: naïve = 180 ± 97 ng/ml, IFNα = 71 ± 8 ng/ml, IFNγ = 80 ± 9 ng/ml, IFNβ =98 ± 34 ng/ml, n=5) (Figure 3.11).

These data alone would indicate that, except for PMA, NETs were not actually formed in these conditions. The increase in release of DNA into the matched supernatant by NETosis-positive control PMA indicates that the DNA quantification technique was working. Therefore, it is likely that although confocal microscopy did show that some NETs were produced visually, any differences seen in the confocal images may be superficial, and that IFNs may not have a big role in NETosis. The number of cells used in the NETosis assay was used limited, and an increase in cell number may improve the number of NETs visualised and increase the rate of DNA released into the supernatant (below). Additionally, many confocal images did not show NETs in each donor; NETs may be present but not seen in the images

taken. Therefore, a more accurate and inclusive analysis may be more reflective of the role of IFNs on NETosis than the confocal analysis used here.

It is of note that DNA quantification did allow for more rigorous and consistent analysis of a NET material and eliminated operator subjectivity that was a major problem in quantifying NETs from confocal images. However, the method of assessing the amount or relative extent of NETosis by quantifying the amount of DNA in the supernatant may not be appropriate. This may be due to DNA concentrations within supernatants isolated from neutrophil cultures was below the sensitivity of the DNA quantification kit, possibly partly due to potential DNA loss during the supernatant processing. Additionally, any DNA detected may not be exclusively from NETs. Therefore, other techniques, including ROS analysis and additional staining of confocal slides for release of NET-associated proteins, such as MPO and NE, may strengthen this analytical approach and confirm that, contrary to published literature, no differences are seen in NETosis upon IFN stimulation (175).

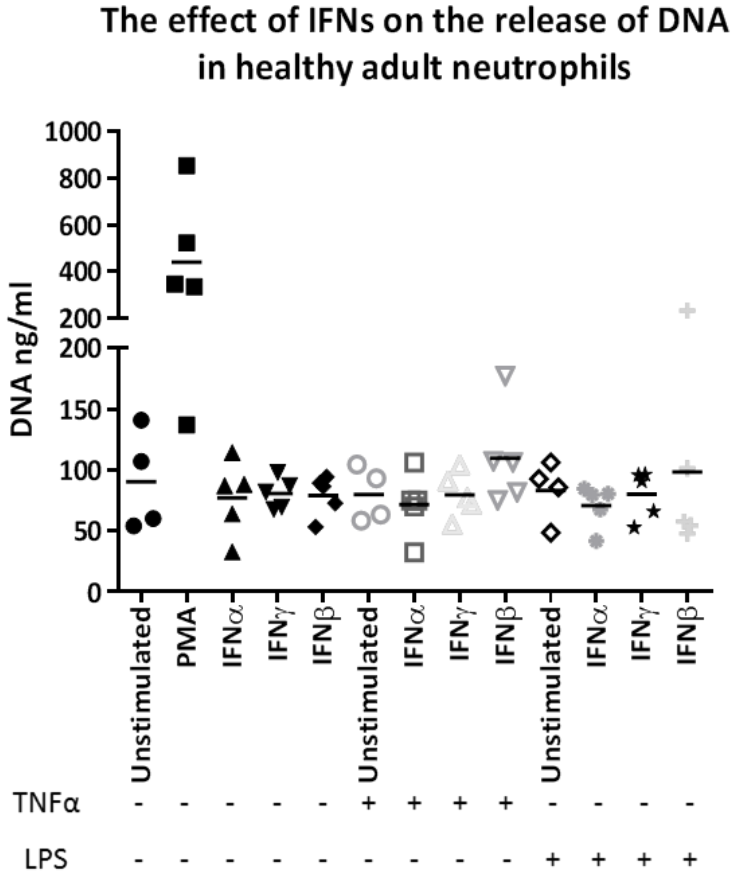


Figure 3.11. Quantification of DNA released by neutrophils at 4hs after cytokine priming and stimulation. Neutrophils were isolated healthy adult donors (n=5) and left to settle on coverslips for 30mins. Neutrophils were left unstimulated or primed with 1μg/ml TNFα or 10ng/ml IFNα, 10ng/ml IFNγ or 10ng/ml IFNβ for 30mins, subsequently stimulated with 10ng/ml IFNα, 10ng/ml IFNγ or 10ng/ml IFNβ (naïve and TNFα-primed neutrophils) or 1μg/ml LPS (IFN primed neutrophils) for 3hrs, and the DNA was cleaved using nuclease. Supernatants were lifted and any remaining cells were removed by centrifugation. Supernatants were analysed for DNA using Quant-iT™ PicoGreen™ dsDNA Assay Kit (n=4-5). PMA stimulated neutrophils released DNA, which correlates well with confocal images. However, no other experimental conditions resulted in an overall measurable increase in the amount of DNA released.

3.6. Discussion

IFNs are anti-viral cytokines that have been found to influence many immune functions, including neutrophils (Section 1.5). Neutrophil function is dysregulated in SLE and JSLE, and thus factors influencing neutrophil function and how they are dysregulated is an important mechanism to understand in order to improve the understanding of the processes and pathways underpinning the pathogenesis of JSLE (112, 115-117, 176, 208) (Sections 1.3, 1.4.3). It has been noted already that some IFN subtypes are increased in the sera of patients with SLE and JSLE. In addition, there is an increased IFN and neutrophil gene signature in JSLE PBMCs (Section 1.5.4.1). This chapter therefore sought to explore the potential role that each of the IFN subtypes may have on influencing some key roles and function of neutrophils (41).

3.6.1. IFNs and neutrophil chemotaxis

Experiments presented here confirmed that both IL-8 and fMLP are strong chemoattractants for neutrophils, in line with other published literature (47, 205, 206) (Section 3.5.1). However, in contrast, it was shown that each of the IFN subtypes did not demonstrate a chemokine role in the model of chemotaxis studied here (Figure 3.3, Section 3.5.2). An alternative *in vitro* model may have been able to elucidate a specific function, but of note, as no published literature has been found to suggest otherwise, it is likely that none of the IFN subtypes have a key role directly as a chemokine for neutrophils. Additionally, priming with all three IFN subtypes did not have any effect on random or IL-8 and fMLP directed chemotaxis.

However, it is known that some of the IFN subtypes have been shown to affect other types of cell migration. A study by Stüve *et al* that demonstrated increased PBMCs migration towards chemokines RANTES, macrophage inflammatory protein 1 α (MIP1α) and MCP1 (177) showed that 2hrs pre-treatment with IFNβ, plus addition of IFNβ to PBMC suspension before migration commencement, resulted in a reduction of migration compared to unstimulated PBMCs (177). This reduction of migration is likely due to the IFNβ related inhibition of the matrix metallopeptidase (MMP)9 mRNA expression and protein secretion, which would be otherwise enhanced in the presence of chemokines such as MCP1 (177). MMP9 is a matrix metalloproteinase which facilitates T lymphocyte migration across extracellular matrix likely through the interaction with fibronectin (178). This process was confirmed in another study by the same group, which showed that T cells migrated via the degradation of fibronectin (178). IFNβ reduced T cell migration and reduced MM9 activity (178). The study concluded that T cells migrate through interaction of MMP9 and fibronectin, and IFNβ reduces migration by dysregulating this interaction (178).

IFNγ has been shown in other studies to up-regulate neutrophil chemotaxis through the up-regulation of receptors CCR1 and CCR3, whereas in T lymphocytes, IFNγ has no effect on chemotaxis (178, 179). In contrast to the experimental conditions conducted in this present study, the incubation time of the neutrophils with the three IFN subtypes is different in this published study; neutrophils were primed for 6hrs with IFNγ before a 60mins migration assay (179). Time of exposure may therefore be a key factor. It is also possible that the chemokines used here to analyse the effect of directly induced migration may affect the results obtained. Although IL-8 and fMLP are potent and effective neutrophil chemokines, they are not the only neutrophil chemokines known (Section 1.5.3.3). It may be that the IFN subtypes up- or down-regulate other chemokine or chemoattractant receptors and that they may be more influential on chemotaxis and be key chemokines not used in this study. This is supported by a study that showed IFNγ not only up-regulated CCR1, CCR3 (both interact with MCPs and RANTES) and the MIP1α receptor on neutrophils, but also induced an increase in neutrophil migration towards MCP3, MIP1α, MIP5 and RANTES (179-182). As previously indicated, IFNβ reduced MMP9 in PBMCs, and also reduced migration of these cells towards RANTES, MIP1α and MCP1 (177).

It should be noted that in the chemotaxis assays, IFN priming resulted in a reduced and thus limited number of cells. This is due to loss of cells initially when lifting from the plate (through neutrophil adhesion to the wells, which were difficult to remove) and through subsequent centrifugation during washing steps. However, the cells were re-counted and each donor set was standardised to the lowest neutrophil concentration to minimise any effect this experimental issue may have on subsequent results. Additionally, a haemocytometer was used, and although it is not the most reproducible method of counting, every effort was made to keep counting as accurate as possible.

In conclusion, in this model of neutrophil chemotaxis and experimental conditions investigated, there was no evidence to support the role of any of the IFN subtypes in having a direct effect or post stimulation effect on neutrophil chemotaxis investigate. They may however have a role in neutrophil migration under the direction of other chemokines not studied here.

3.6.2. IFNs and neutrophil phagocytosis

It has been established that neutrophil phagocytosis is dysregulated in JSLE and that there are various factors that affect phagocytosis (112, 115, 189) (Sections 1.3.2 and 1.4.3). IFN subtypes have previously been shown to affect phagocytosis in various ways. However, this

effect is dependent upon a variety of factors, including type of immune cell, type of bacteria and the receptors used to undergo phagocytosis (183, 184).

In this study, it was shown that IFN priming using any of the IFN subtypes had no overall effect on neutrophil phagocytosis of *E.coli* bioparticles, although there was a large donor variation in the results obtained. This is interesting as it has been suggested that IFNs can up-regulate the neutrophil phagocytosis of *S. aureus* bacteria and in particular IFNγ can up-regulate phagocytosis of *S.aureus* bioparticles (184, 185). This suggests that the mechanism of action of IFNs on phagocytosis is reliant on the cell types and bacteria being studied. The importance in the type of bacteria or particle being phagocytised has previously been noted. IFNα increases macrophage phagocytosis of both *E.coli* and erythrocytes, whereas IFNγ only decreases macrophage phagocytosis of *E.coli* (183). Additionally, the differential effect of IFNγ on phagocytosis is dependent on the type of immune cell being used; IFNγ up-regulates neutrophil phagocytosis but down-regulates macrophage phagocytosis (183, 184).

It is of note that LDGs have reduced phagocytosis compared to normal density neutrophils (Section 1.2.2.2). It was observed that SLE and JSLE patients have increased LDGs, which correlate with disease activity; therefore these LDGs may be contributing to any reduced phagocytosis seen in SLE and JSLE. Therefore, it would be of interest to investigate the role of IFNs on LDG phagocytosis, which may result in a different observation than shown within this thesis.

In summary, although there is some indication from mouse studies that IFNγ may have a role in the reduced phagocytosis seen in JSLE, this was not supported for the role of any of the IFN subtypes in specifically reducing neutrophil phagocytosis by data arising from this present study (112, 115, 183) (Section 3.5.3). Most previous literature indicates an IFN-induced increase in phagocytosis and receptors in healthy immune cells (184, 185). Previous literature suggests that generally, immune cell phagocytosis increases in the presence of IFNs (183-185, 215). Therefore, as IFNs are increased in JSLE, decreased phagocytosis noted in JSLE is unlikely due to be directly under the influence of IFN involvement (41, 68, 112, 113, 115, 171, 172). However, further investigation would be beneficial to fully elucidate IFNs influence on JSLE neutrophil phagocytosis, with an additional investigation of IFNs on LDG phagocytosis.

3.6.3. IFNs and NETosis

NETosis has been shown to be up-regulated in both SLE and JSLE patients (163, 208) (Section 1.4.3). It has been proposed that IFNs have a role in increased NET formation through

priming, and NETing neutrophils in turn have been indicated to promote IFNα production from pDCs in JSLE (163, 175).

In this study, using confocal microscopy, the results showed that unstimulated neutrophils did not undergo NETosis, and IFN stimulated neutrophils had minimal subsequent NET formation irrespective of IFN subtype (Section 3.5.4). Priming of neutrophils with TNFα caused a slight increase in NET production, with IFN stimulation on occasion having an additional increasing effect with each of the IFN subtypes; however, this effect varied between donors (Section 3.5.4). IFN priming also slightly induced more NET formation when LPS stimulation was used. However, again this varied between donors. Additionally, LPS on its own had no effect on NETosis compared to unstimulated naïve neutrophils, suggesting priming with cytokines is an important step in NETosis (Section 3.5.4). This suggestion that IFN priming may increase NETosis following subsequent stimulation correlates well with published data (175). However, it was shown that IFN stimulation alone is not enough and that IFN priming with additional subsequent stimuli are needed to induce NETosis (163, 175).

It is noted that the staining for confocal microscopy as conducted in these experiments only targeted DAPI. Thus, although the 'NETs' seen were likely to be neutrophil DNA material, quantification of the images could be very subjective. Additionally, there was a great deal of intra and inter donor variation between images and thus may be too inconsistent to fully conclude analysis. DNA quantification was used as an additional method in an attempt to provide a quantitative measure of NETosis (Section 3.4.3.4). The DNA quantification method showed that only PMA, the positive control, induced a quantifiable increase in DNA release from NETs, albeit that this also was the only experimental condition that consistently showed NETs in the confocal images (Sections 3.5.4.1, 3.5.4.3). All the other conditions did not have any quantifiable additional DNA compared to unstimulated neutrophils. This correlates somewhat with the confocal images, that although generally there was not huge overall induction of NETs in any condition other than PMA, some images did show some increase in NETosis, through release of DNA. However, this increase in DNA release in some donors was not detected by the DNA quantification (Section 3.5.4.3). This may be due to the concentration of the DNA in the supernatants was below the sensitivity of the DNA quantification kit, or the DNA was lost in the supernatant processing.

Although these data do not show any significant involvement of IFN or its various subtypes in NETosis, either as stimulants or priming agents, a published study indicated a IFN priming role (175). It is likely that the high level of IFNs within JSLE serum may be priming neutrophils

to undergo NETosis upon stimulus from infections (163, 175). JSLE neutrophils have also been suggested to have dysregulated signalling pathways (particularly shown through the increased pro-apoptotic proteins, and decreased anti-apoptotic proteins) and thus their intracellular components may cause the JSLE neutrophils to become more susceptible to induced NETosis than the healthy adult neutrophils used in this study (170).

Additionally, literature has stated that LDGs have enhanced ability to undergo NETosis compared to normal density neutrophils (40) (Section 1.2.2.2). These LDGs may contribute more so to any increased NETosis seen with SLE and JSLE patients, and therefore priming or stimulating LDGs with IFNs may result in more NETs than what is observed here. Investigation of IFNs and LDG NETosis may be paramount in future work.

Of note, effort was made within this study to quantify the number of NETs seen in the confocal images by multiple operators within the laboratory. This *post hoc* analysis was conducted so that some quantification comparison could be used to: a) accurately and statistically compare the conditions observed and b) to compare the confocal images to the DNA assays conducted. However, this quantification method from confocal images was very limited in that categorising viable and NETing neutrophils from confocal images was very subjective between operators. This limitation was particularly problematic in images that contained clumps of neutrophils; there was a difficulty in concluding what proportion of the clump was viable neutrophils and what portion was NET material.

Quantification of confocal images can be conducted using computer software. This requires programming the software to differentiate between viable and NETing neutrophils and remove viable cell fluorescence from the analysis to give an accurate NET fluorescence measurement. However, this method may not have been possible in the more problematic clumps of neutrophils seen in the confocal images as NETs and viable neutrophils were not easily distinguishable from each other. However, a study has successfully used ImageJ in another way to quantify NETs; the area of Sytox green, which detects extracellular DNA, was measured and normalised to the mean area of PKH26, which detects all neutrophils (216). This may be a more sensitive way to distinguish NETs and viable neutrophils and allow for a more rigorous and objective quantification through ImageJ. Automated/computational quantification of microscopy images have been also been reported (217). The study used a series of images that contain DNA and histone fluorescent labelling, and manually label NET regions (217). This allows for a model to be produced that can be applied to other images to

estimate the area of NETs (217). This automation correlated well with human labelling, and thus may be useful to use in future experiments (217).

It should be noted that the scoring system used for analysing NETs on confocal images in this study has been used successfully by another group (218). The group added LL37 and NE staining, which visualised NETs more clearly and thus allowed more consistent quantification than was possible in this study (218). Thus, the images, and scoring, showed here may be improved using additional NET-associated protein staining. This additional staining may be particularly beneficial in regards to images containing clumps where distinguishing viable cells from NETs was difficult; the additional staining may improve the quality of the images, which may improve ease of scoring and allow for more consistent quantification of the images.

Measuring certain components of NET material using quantitative methods (such as fluorescent emission using a plate reader) could be another useful way to accurately and objectively analyse NET release. Although DNA was quantifiably measured from neutrophil supernatants, a question of the kit's sensitivity did arise; there were some images that showed an increase in NET material in the form of DNA in some of the conditions, and this was not obviously detected in the supernatant by the DNA quantification assay. Thus, other components may be easier to retain a large enough quantity in the supernatant processing, and thus easier to quantify (maybe by more sensitive assays), such as the release of proteins associated with NETs, such as histones, MPO and NE.

3.7. Summary

Functionally, in this study, the IFN subtypes demonstrated little influence on neutrophil chemotaxis and phagocytosis, although this may be due to many factors including the choice of chemokines and bacteria used in this study. However, from these data, it seems that if IFNs do have a role, especially with phagocytosis, it does not explain the marked dysregulation of phagocytosis seen in JSLE. This study suggested that IFNs have a priming role on NETosis but showed no marked NET-inducing role in naïve or TNFα-primed neutrophils. The role of IFN may be purely priming and that it may be other factors such as pathogens having a greater role in inducing NETosis.

Chapter 4: The role IFNs in neutrophil apoptosis in JSLE

4.1. Introduction

Apoptosis, the process of programmed cell death, is a crucial physiological function, and is important in the resolution of inflammation (73). It takes place through the cleavage of caspases, with active caspase 3 (cleaved from the inactive caspase 3 during apoptosis) being the ultimate apoptotic effector protein (73) (Section 1.3.4). However, the observed increase in neutrophil apoptosis and subsequent dysregulation of clearance of apoptotic debris is thought to contribute to JSLE pathogenesis, leading to the increased production of autoantigens directed against nuclear material (72, 110, 113) (Section 1.1.4).

The increase in neutrophil apoptosis within JSLE may be due to both environmental factors and intrinsic signalling factors (72, 170). SLE neutrophils have been shown to be activated through the up-regulation of activation marker CD11b, which may be a result of the pro-inflammatory environment, and activation studies have shown this can alter activation and expression of intrinsic signalling proteins, such as increased activation of ERK and p38 MAPK proteins, and increase expression of IL-1 receptor (R)a and pro-IL-1β within neutrophils (83-85). There is an imbalance of apoptotic factors within the serum of JSLE patients, with an increase concentration of pro-apoptotic factors such as Fas and TRAIL, and decrease of anti-apoptotic factors such as GMCSF (72). It has also been shown that JSLE neutrophils express increased mRNA of pro-apoptotic proteins such as caspase 7, 8 and 9 and a decrease in anti-apoptotic proteins such as IAP1 and 2 and xIAP (170).

IFNs are important cytokines in innate immunity and are released in response to viral infection (Section 1.5). Type 1 and 2 IFNs, including IFNα and IFNβ, and IFNγ respectively, have been shown to be increased in serum from adults with SLE (171) (Section 1.5). However, to date, within the serum of individuals with JSLE, only IFNα was has been shown to be elevated (68, 172) (Section 1.5). Genome wide studies have described a granulocyte and IFN genetic signature, with increased expression of genes associated with neutrophils and the IFN signalling pathway, within PBMCs isolated from JSLE patients, (41). Therefore, this indicates a potential interaction between IFNs and neutrophils in JSLE. Type 1 and 2 IFNs have a pro- and anti-apoptotic effect, which can depend on cell type (138, 141, 164, 165, 219). IFNs signal through the JAK/STAT pathway, as shown in Figure 1.7 (Section 1.5.1). This is dependent on the downstream signalling pathway, whereby STAT1 is pro-apoptotic and

STAT3 is anti-apoptotic (138, 141, 164, 165, 219). It is possible that within JSLE, different IFN subtypes differentially effect the induction of anti-apoptotic effect through STAT3, or a pro-apoptotic effect via STAT1 (Section 1.5.1).

TNFα has been shown to prime neutrophil functions and this priming activates neutrophils, which reflects of that of neutrophils in SLE (81-83). Through priming, TNFα can also alter the expression of proteins in neutrophils (84). Therefore, to fully investigate IFNs effect on apoptosis, TNFα priming of neutrophils can be used to model that of JSLE neutrophils and elucidate whether activation of neutrophils is an important step that leads to increased apoptosis in JSLE.

GMCSF has been shown to reduce neutrophil apoptosis induced by JSLE serum, and the GMCSF anti-apoptotic mechanism is through the stability of MCL1, which inhibits the pro-apoptotic Bak dimerization and activity (72, 77, 110, 220). Of note, GMCSF has been shown to also reduce the activation of pro-apoptotic proteins caspase 3, caspase 7 and caspase 8 within healthy neutrophils incubated with JSLE serum (110). Therefore, in this study, GMCSF is used as a positive control for a reduction in apoptosis in JSLE, and its downstream effect on anti-apoptotic MCL1.

4.1.1. Summary

Neutrophils are more apoptotic in JSLE. This may be due to both a pro-inflammatory and apoptotic environment which activates neutrophils, and a potential up-regulation of intrinsic pro-apoptotic proteins compared to healthy neutrophils. Type 1 and 2 IFNs are both increased in SLE serum and in particular IFNα concentrations are increased in JSLE serum. The increased IFN and granulocyte genetic signatures present in JSLE indicates a potential interaction. Thus, further understanding on whether the IFN subtypes may be involved in regulating increased neutrophil apoptosis is required, which could indicate a potentially relevant signalling pathway in JSLE pathogenesis and therefore a potential therapeutic target.

4.2. Chapter hypothesis

Type 1 and Type 2 IFNs have both a pro- and anti-apoptotic effect, and this is dependent on differential activation of the JAK/STAT pathway (discussed in Chapter 5). This differential apoptotic effect may be due to activation of neutrophils by either priming agents such as TNFα or the pro-inflammatory environment found in SLE and JSLE patients. It is therefore

hypothesised that one or more IFNs may have an anti-apoptotic effect on naïve/healthy neutrophils and a pro-apoptotic effect on primed or JSLE neutrophils.

4.3. Chapter aim and objectives

Aim: To investigate the role of IFNs on neutrophil apoptosis.

- Objective 1: Using flow cytometry, quantify the rate of apoptosis on naïve and TNFα-primed healthy adult neutrophils following a 6hr incubation with a high concentration of IFNs.
- Objective 2: Using flow cytometry, quantify the rate of apoptosis on naïve and TNFα-primed healthy adult neutrophils following a 6hr incubation with a lower concentration IFNs, and measure the downstream expression of anti-apoptotic MCL1 (at 6hrs) and caspase 3 (at 20hrs) using Western blotting.
- Objective 3: Using flow cytometry, quantify the rate of apoptosis on naïve and patient sera-primed healthy adult neutrophils following a 6hr incubation with a lower concentration IFNs.
- Objective 4: Using flow cytometry, quantify the rate of apoptosis on paediatric control patient and JSLE patient neutrophils following a 6hr incubation with a lower concentration IFNs.

4.4 Methods

4.4.1. Flow cytometry analysis

4.4.1.1. Neutrophil priming and stimulation with a high concentration of IFNs

Neutrophils were isolated from healthy adults (Section 2.5.2) Neutrophils were either unstimulated or primed with 1µg/ml TNFα for 30mins at 37°C, 5% CO_2, $5x10^5$ neutrophils per condition. Neutrophils were either analysed for evidence of their activation (Section 4.4.2.6) and/or subsequently stimulated for 6hrs with 10µg/ml IFNα, 1µg/ml, IFNγ or 1µg/ml IFNβ for 6hrs at 37°C, 5% CO_2, using $5x10^5$ neutrophils per condition. Apoptosis was analysed (Section 2.6.2.2) and results analysed on Kaluza.

4.4.1.2. Lower concentrations of IFNs on naïve neutrophils at 6hrs and 20hrs

An IFN dose response was conducted (Section 2.6.2.1) for each of the IFN subtypes, whereby only 10ng/ml IFNβ had any differential effect on apoptosis compared to other doses of IFNβ; there were no differences in the rate of apoptosis with each of the different doses of IFNα or IFNγ investigated, and thus a concentration of 10ng/ml for each of the IFN subtypes was chosen. Neutrophils were isolated from healthy adults (Section 2.5.2) and were either unstimulated or incubated for 6hrs with 10ng/ml IFNα, 10ng/ml IFNγ or 10ng/ml IFNβ for 6hrs and 20hrs at 37°C, 5% CO_2, using $1x10^5$ neutrophils per condition. Apoptosis was analysed (Section 2.6.2.2) and results analysed on FlowJo.

4.4.1.3. Lower concentrations of IFNs on TNFα-primed neutrophils

Neutrophils were isolated from healthy adults (Section 2.5.2) and were either unstimulated or primed with 1μg/ml TNFα for 30mins at 37°C, 5% CO_2, $1x10^5$ neutrophils per condition. Neutrophils were analysed for activation (Section 4.4.2.7) and subsequently incubated for 6hrs with 10ng/ml IFNα, 10ng/ml IFNγ or 10ng/ml IFNβ for 6hrs at 37°C, 5% CO_2, using $1x10^5$ neutrophils per condition. Apoptosis was analysed (Section 2.6.2.2) and results analysed on FlowJo.

4.4.2.3. Patient sera selection

The patients' pBILAG score (Sections 1.1.3 and 2.4) at the time of sample collection was used to select serum samples from JSLE patients with active or inactive disease. Samples were characterised as inactive, active in the renal domain or active in the haematological domain of pBILAG – Section 2.4. Inactive JSLE sera were selected based on having a score of D or E in all organ domains of the pBILAG with a resulting overall pBILAG score of 0. JSLE sera that were active in the haematological domain were chosen based on an A or B in the haematological domain of the pBILAG, with an overall pBILAG score of >6. JSLE sera that were active in the renal domain were chosen based on an A or B in the renal domain of the pBILAG, an overall pBILAG score of >6. Sera from patients who were dually active in the haematological and renal domains were not selected due to overlap and thus may affect result analysis. Demographics were analysed; the age at sampling and gender were analysed first to select approximately aged matched and gender matched paediatric control patient sera. Other demographic variables analysed included: duration of disease, ethnicity, family history, medication and clinical biomarkers such as dsDNA, C3 and erythrocyte sediment rate (ESR).

4.4.2.4. Lower concentrations of IFNs on patient sera-primed neutrophils

JSLE patient sera samples were selected based on clinical conditions at sampling (Section 4.4.2.3 and Section 2.4), and paediatric control patient were selected from age- and gender-matched patients (Section 4.4.2.3 and Section 2.4). Neutrophils were isolated from healthy adults (Section 2.5.2) and were either incubated in 10% FBS or primed with 10% patient sera for 30mins at 37°C, 5% CO2, $9x10^4$ per condition. Neutrophils were either analysed for activation (Section 4.4.2.7) or subsequently stimulated with 5ng/ml of GMCSF, 10ng/ml IFNα, 10ng/ml IFNγ or 10ng/ml IFNβ for 6hrs at 37°C, 5% CO_2. Apoptosis was analysed (Section 2.6.2.2) and results analysed on FlowJo.

4.4.2.5. IFN effect on apoptosis in paediatric control and JSLE patient neutrophils

Neutrophils were isolated from healthy adults (Section 2.5.2) and were unstimulated or stimulated with 10ng/ml IFNα, 10ng/ml IFNγ or 10ng/ml IFNβ for 6hrs at 37°C, 5% CO_2, using $1x10^5$ neutrophils per condition. Apoptosis was analysed (Section 2.6.2.2) and results analysed on FlowJo. Demographic information was also analysed, including age at sampling, disease duration, ethnicity, family history, medication and clinical markers such as dsDNA, C3 and ESR.

4.4.2.6. Activation assay for high concentrations of IFNs

Initially, we aimed to analyse the effect of a high, saturating concentration of IFNs on naïve and TNFα-primed neutrophils. An initial dose response of 1, 5 and 10µg/ml TNFα (data not shown), showed no significant difference in concentrations on the priming of neutrophils after 30mins. Subsequently, neutrophils were isolated from healthy adults (Section 2.5.2) and were either unstimulated or treated with 1µg/ml TNFα for 30mins at 37°C, 5% CO_2, using $1x10^6$ neutrophils per condition. Neutrophils were centrifuged at 1800 RPM for 5mins, and the supernatant was poured off. Neutrophils were washed in 1ml PBS/0.5% BSA (Sigma-Aldrich, UK), and centrifuged at 1500 RPM for 5mins. Neutrophils were suspended in residue, and stained with 100ng/ml CD11b (eBioscience, UK), 2.5µg/ml CD62L (eBioscience, UK), 100ng/ml phycoerythrin (PE) isotype control (Beckman Coulter, UK) and 2.5µg/ml allophycocyanin (APC) isotype control (Beckman Coulter, UK). Neutrophils were incubated at 4°C in the dark for 30mins, and then centrifuged at 1500 RPM for 5mins. The supernatant was poured off and neutrophils were suspended in 600µl PBS/0.5% BSA, and samples run through the flow cytometer using pre-programmed activation assay. Data were analysed using Kaluza.

4.4.2.7. Activation assay for lower concentrations of IFNs

As the activation assay above did not consistently show an increase in CD11b upon TNFα priming, a new activation assay was set up to analyses the activation of neutrophils before stimulation with a lower concentration of IFNs. This lower concentration was more akin to cytokine stimulation published in other studies (77, 110, 221).

Neutrophils were isolated from healthy adults (section 2.5.2). Neutrophils were treated with 1µg/ml TNFα for 30mins at 37°C, 5% CO_2, using $1x10^5$ neutrophils per condition or 10µl of a patient serum for 30mins at 37°C, 5% CO_2, $9x10^4$ neutrophils per condition. The plate was centrifuged at 1800 RPM for 5mins, and the supernatant was decanted. Neutrophils were washed in 100µl of PBS/0.5% BSA, and the plate was centrifuged at 1500 RPM for 5mins. The supernatant was decanted and 100µl of PBS/0.5% BSA was added. PE and APC isotype controls (eBioscience, UK), and CD11b and CD62L were added individually to unstimulated neutrophils to a final concentration of 1µg/ml. Dual stain CD11b and CD62L were added to unstimulated and TNFα-stimulated neutrophils at a final concentration of 1µg/ml. Neutrophils were incubated at 4°C for 30mins, and the plate centrifuged at 1500 RPM for 5mins. The supernatant was decanted and 200µl PBS/0.5% BSA was added. The neutrophils were transferred to 5.4ml flow cytometry tubes and run through Guava EasyCyte, using a pre-set protocol.

4.4.2.8. Activation assay for patient neutrophils

Neutrophils were isolated from paediatric control and JSLE patients (Section 2.5.2) and seeded at $1x10^5$. The plate was centrifuged at 1800 RPM for 5mins, and the supernatant decanted. Neutrophils were washed in 100µl of PBS/0.5% BSA, and the plate was centrifuged at 1500 RPM for 5mins. The supernatant was decanted and 100µl of PBS/0.5% BSA was added. PE and APC isotype controls, and CD11b and CD62L were added individually to unstimulated neutrophils to a final concentration of 1µg/ml. Dual stain CD11b and CD62L were added to unstimulated and TNFα-primed neutrophils at a final concentration of 1µg/ml. Neutrophils were incubated at 4°C for 30mins, and the plate centrifuged at 1500 RPM for 5mins. The supernatant was decanted and 200µl PBS/0.5% BSA was added. The neutrophils were transferred to 5.4ml flow cytometry tubes and run through Guava EasyCyte, using a pre-set protocol.

4.4.2. Western blot analysis

Neutrophils were isolated from healthy adults (Section 2.5.2) and seeded at $5x10^5$ – $2x10^6$ per condition. Neutrophils were stimulated with 5ng/ml GMCSF, 10ng/ml IFNα, 10ng/ml IFNγ

or 10ng/ml IFNβ for 6hrs or 20hrs 37°C, 5% CO_2. Proteins were extracted and analysed for MCL1 (6hrs IFN stimulation) and caspase 3 (20hrs IFN stimulation) using Western blotting (Section 2.7).

4.4.3. Statistical analysis

The raw data obtained from the experiments carried out here were analysed in GraphPad Prism 6. Friedman's test with Dunn's *post hoc* multiple comparison tests were conducted for analysis of the majority of data sets, to analyse between multiple paired conditions within a sample set. One-tailed Wilcoxon matched-pairs signed rank test was used for analysis of activation marker expression. Kruskal-Wallis test with a *post hoc* Dunn's multiple comparison test was conducted for analysis of comparing multiple comparisons between JSLE and paediatric control patient data sets. For full justification, see Section 2.10.

4.5. Results

4.5.1. Optimisation of the apoptosis assay

Initial experiments were conducted to optimise the neutrophil apoptosis assays. Using the assay outlined previously (Section 2.6.7.1), preliminary experiments of rates of apoptosis showed variability at 4hrs and a higher than anticipated rate at 6hrs (see Figure 4.1, A). GMCSF did not always reduce apoptosis, and the observed reductions were variable. The event rates achieved on the flow cytometer were inconsistent and often too low to analyse, and thus optimisation of the assay was needed.

It was found that plating/incubating neutrophils and staining the neutrophils in a 96 well plate increased the rate of cells going through the flow cytometer compared to using a 24 well plate to incubate cells and staining in flow cytometry tubes. The PI stain incubation was reduced to 2mins, as 5mins was deemed excessive due to the swift rate it was able to stain the neutrophils. The last centrifugation that was originally 1500 RPM for 5mins was omitted to reduce the amount of assay-induced apoptosis before the flow cytometry analysis and to retain an appropriate yield of as many viable neutrophils as possible (Figure 4.1, B). These steps improved the quality of the data set, via event rate and viability of cells, and the data was more consistent with that of published data.

An IFN dose response on inducing apoptosis was conducted, using doses of: 5 and 10µg/ml IFNα, 1, 5 and 10µg/ml IFNγ and 1 and 5µg/ml IFNβ. The dose response showed that 10µg/ml IFNα had the greatest reducing effect on apoptosis (data not shown), whilst little differences

were seen in the various doses of IFNγ and IFNβ. It was therefore agreed that 10µg/ml of IFNα, 1µg/ml IFNγ or 1µg/ml of IFNβ were the most appropriate concentrations to maximise the effect seen (Figure 4.1, C and D). Thus, subsequent experiments were conducted using these concentrations.

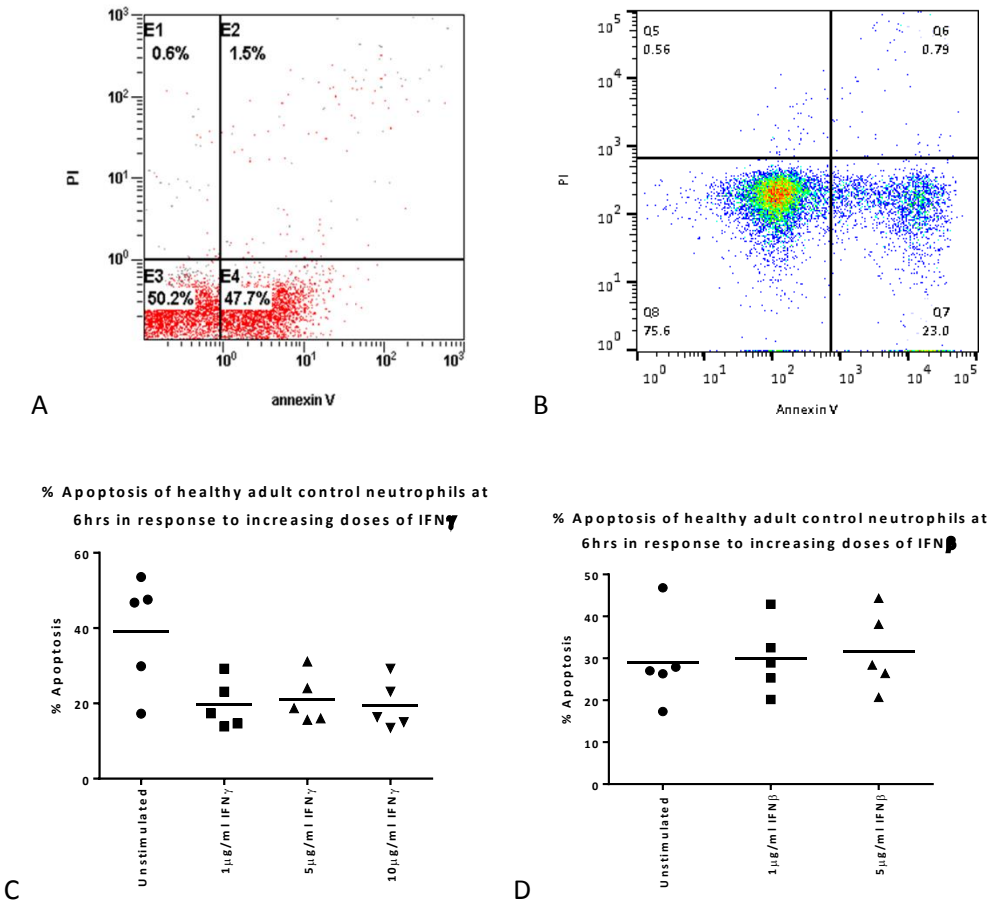


Figure 4.1. Apoptosis analysis of healthy adult neutrophils at 6hrs. Neutrophils were isolated from healthy adult donors and were left unstimulated or stimulated with a dose response of IFNs and incubated for 6hrs. Unstimulated neutrophils were stained with Annexin V FITC and PI using the original protocol and measured using Beckman Coulter F500 and analysed using Kaluza software (A) or stained with optimised protocol and measured using Guava EasyCyte and analysed using FlowJo software (B). The initial apoptosis experiments had inconsistent results, and as shown, had abnormally increased annexin V FITC staining (dot blot A, quadrant E4, 47.7% annexin V FITC staining), indicating increased apoptosis. With the optimised apoptosis assay, the annexin V FITC was reduced (dot blot B, quadrant Q7, 23% annexin V FITC staining), indicating less apoptosis, which is more consistent with published data. A dose response of IFNγ and IFNβ were conducted (C and D respectively), and apoptosis was measured at 6hrs using Beckman Coulter F500 and this showed little differences in IFNγ and IFNβ dose responses and thus 1µg/ml was used in subsequent experiments.

4.5.2. Activation assay optimisations

It has previously been shown that TNFα, amongst other cytokines, is a potent neutrophil priming agent (81, 84, 85, 90, 167) (Section 1.3.5). It has been demonstrated that in individuals with SLE, neutrophils are activated and this may be true of neutrophils in individuals with JSLE (83) (Section 1.3.5). Therefore, neutrophil activation, through TNFα priming, was used to model neutrophils that may be present in inflammatory conditions such as those occurring in JSLE patients. This model was used frequently in this study, as an activated neutrophil phenotype was considered important in the investigation of how the different IFN subtypes may subsequently affect JSLE neutrophil apoptosis. However, it is notable that data need to be carefully analysed an interpreted in this scenario, as TNFα can have both pro- and anti-apoptotic effects on neutrophils (23).

An activation assay was therefore set up on the Beckman Coulter F500 flow cytometer, and a dose response of 1, 5 and 10μg/ml TNFα was investigated. A 1:100 – 1:1000 dilution of CD11b, and a 1:40 dilution of CD62L were used to stain cells within the flow cytometry tubes. Little difference was observed in the activation effects of the different TNFα concentrations (data not shown), and thus a TNFα concentration of 1μg/ml was used in subsequent experiments. Although an increase in CD11b was not consistently seen, a significant decrease in CD62L was seen following stimulation with TNFα (Figure 4.2) (see also Section 4.5.3.1 for actual results of assays). This was therefore used to analyse initial TNFα-induced priming for subsequent incubation of neutrophils with the high concentration of IFNs.

Another activation assay was developed in order to analyse both CD11b and CD62L in a more rigorous and consistent way using the Guava EasyCyte. Additionally, neutrophils were stimulated and stained in a 96 well plate to improve cell viability, event count and quality of staining. Neutrophils were primed with 1μg/ml TNFα and stained with 1:100 diluted CD11b and CD62L. Comparative isotype controls were used to confirm specific binding. There was an increase in CD11b and decrease in CD62L in TNFα-primed neutrophils compared to unstimulated neutrophils, confirming activation (Figure 4.3). This assay was used for all activation assays using the lower concentration of IFNs.

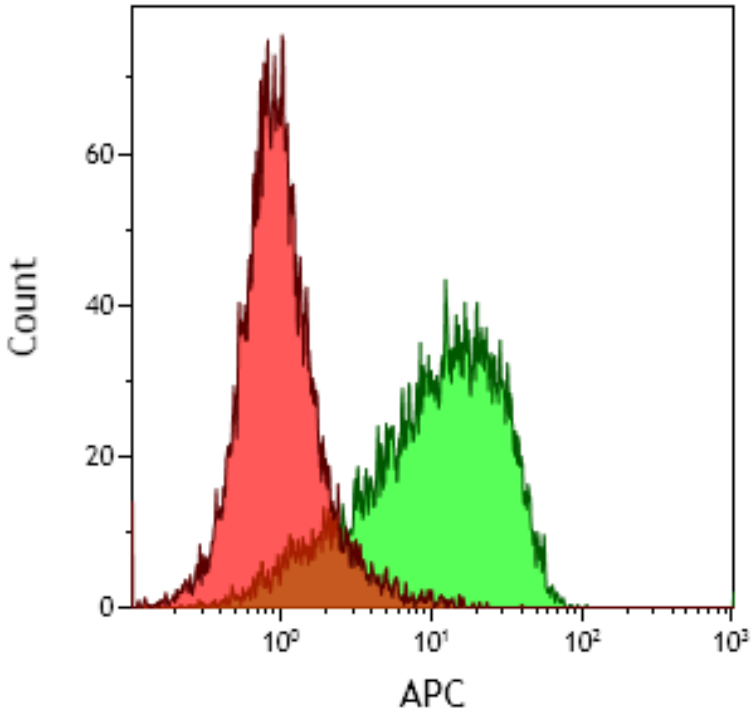


Figure 4.2. TNFα priming of healthy adult neutrophils resulted in a reduction in expression of CD62L, assessed using the Beckman coulter flow cytometry assay. Neutrophils were isolated from healthy adult donors and were left naïve or primed with 1µg/ml TNFα for 30mins. Neutrophils were stained with CD62L antibody and the relative expression via geometric mean of CD62L was measured using flow cytometry. A reduction of CD62L expression after priming with TNFα (red) was noted compared to unstimulated neutrophils (green peak) indicating successful neutrophil priming.

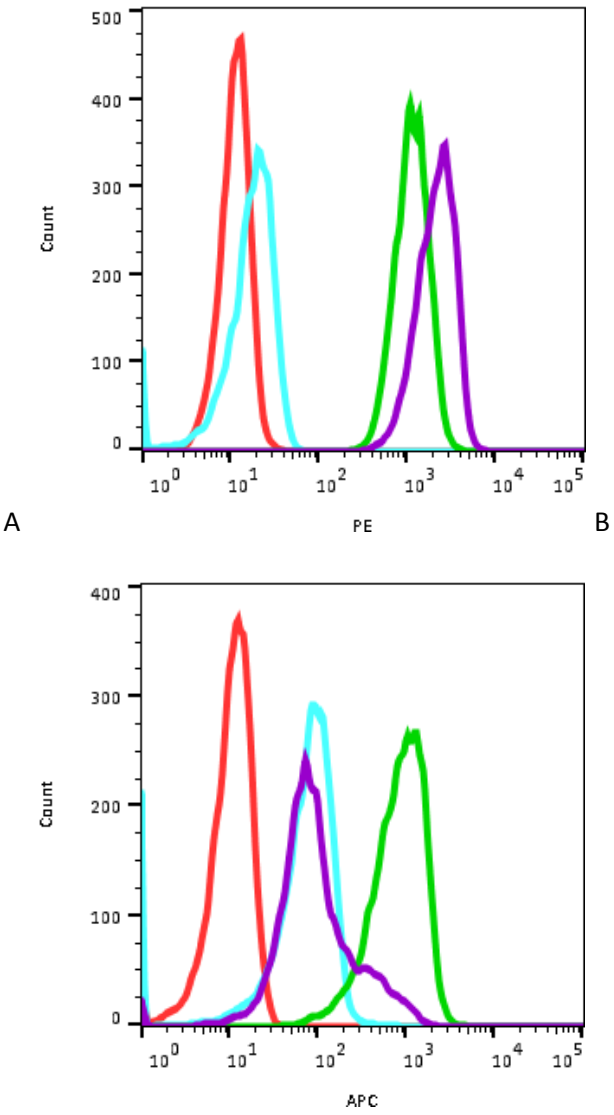


Figure 4.3. TNFα priming of healthy adult neutrophils resulted in neutrophil activation, assessed using the Guava EasyCyte flow cytometry assay. Neutrophils were isolated from healthy adult donors and were left naïve or primed with 1µg/ml TNFα for 30mins. Neutrophils were stained with CD11b and CD62L antibodies and the relative expression assessed by determining the geometric mean expression for CD11b (A) and CD62L (B) using flow cytometry. An increase in CD11b expression (purple, Figure A) and a decrease in CD62L expression (purple, Figure B) compared to unstimulated neutrophils (green, both figures) confirms neutrophil activation. Isotype controls (blue, both figures) and unstained neutrophils (red, both figures) were used to confirm specific binding.

4.5.3. Investigating the rate of neutrophil apoptosis in naïve and primed healthy adult neutrophils following incubation with a high concentration of IFNs

Published data suggests that IFNs have both an anti- and pro- apoptotic function (138, 141) (Section 1.5.3.1), and thus this dual function may be dependent on the activation state of neutrophils. Therefore, the effect of a high concentration of IFNs on apoptosis in naïve and primed neutrophils was investigated.

4.5.3.1. The effect of TNFα on neutrophil priming

The first objective was to confirm that TNFα was able to prime neutrophils, which is shown here through the shedding of CD62L. Initial 30 min priming of healthy adult control neutrophils with 1μg/ml of TNFα showed significant reduction in CD62L (1.61 ± 0.3, n=5, p=0.031) compared to unstimulated neutrophils (11.4 ± 1.4, n=5) (Figure 4.4). This confirmed neutrophil priming for subsequent incubation of these neutrophils with the different IFNs.

4.5.3.2. The effect of a high concentration of IFNs on naïve and primed neutrophil apoptosis

Naïve and primed neutrophils were stimulated with 10μg/ml of IFNα and 1μg/ml of IFNγ and IFNβ for 6hrs, and apoptosis was measured (section 2.6.2.2). In naïve neutrophils, IFNα (6.6 ± 1 .2%, n=5) and IFNγ (10.8 ± 1.9%, n=5) both reduced apoptosis in compared to unstimulated neutrophils (22.0 ± 3.2%, n=5) although the reduction was not statistically significant (p>0.5), whereas IFNβ had no demonstrable effect (20.3 ± 5.1%, n=5) (Figure 4.5). However, in primed neutrophils, IFNα (48.9 ± 4.8%, n=5), IFNγ (43.1 ± 3.0%, n=5) and IFNβ (39.4 ± 3.2%, n=5) all induced apoptosis compared to unstimulated naïve neutrophils (22.0 ± 3.2%, n=5) although these differences did not meet statistical significance (p>0.05) (Figure 4.5). This increase in neutrophil apoptosis was not due to TNFα in the neutrophil suspension as priming with TNFα had no effect on apoptosis in unstimulated neutrophils (26.4 ± 4.3%, n=5) (Figure 4.5).

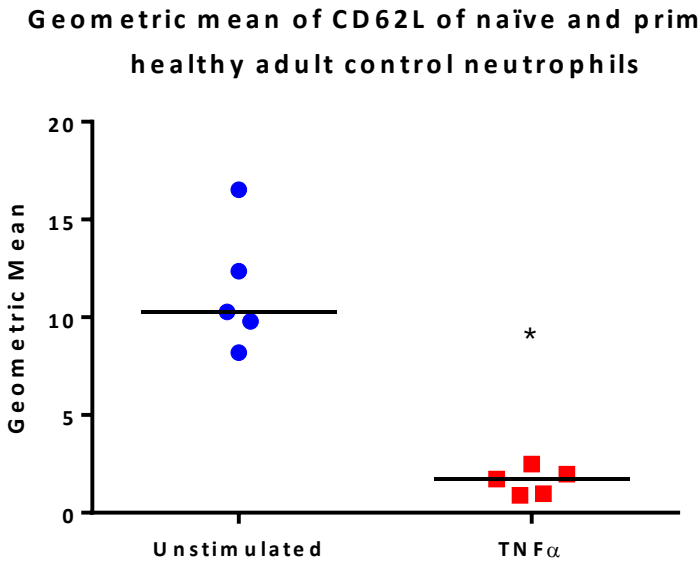


Figure 4.4. Neutrophils were isolated from healthy adult donors and were left naïve or were with 1μg/ml TNFα for 30mins. Neutrophils were stained with CD62L antibodies and the relative expression via geometric mean of CD62L was measured using flow cytometry. Neutrophils were deemed primed when CD62L was shed, as measured by the geometric mean (n=5, p=0.031).

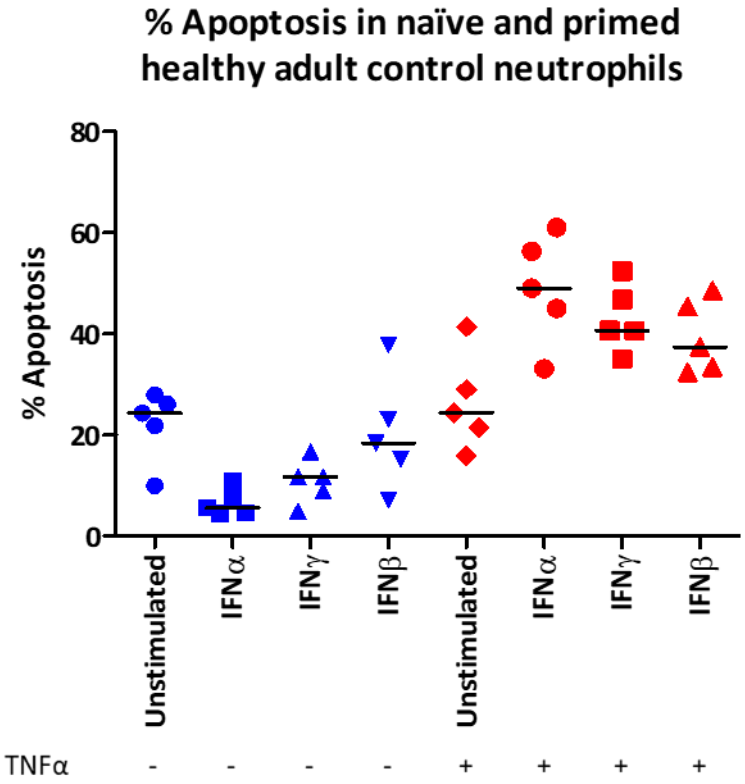
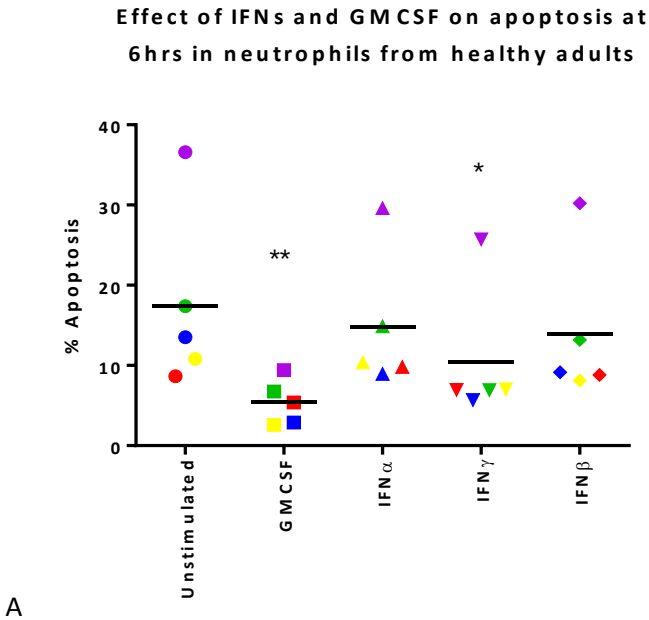


Figure 4.5. Apoptosis analysis of naïve and 1µg/ml TNFα-primed adult healthy neutrophils after 6hr incubation with 10µg/ml IFNα, 1µg/ml IFNγ or 1µg/ml IFNβ. Neutrophils were isolated from healthy adult donors and were left naïve (blue) or were primed with 1µg/ml TNFα for 30mins (red), and subsequently stimulated for with 10µg/ml IFNα, 1µg/ml IFNγ or 1µg/ml IFNβ for 6hrs. Neutrophils were stained with annexin V FITC and PI, and the percentage of apoptosis was measured using flow cytometry (n=5).

4.5.4. Investigating the role of lower concentration of IFNs on apoptosis in naïve and primed neutrophil at 6hr and 20hrs

Following on the observations that high concentrations of IFNs have a dual effect on neutrophil apoptosis depending on whether the neutrophils are naïve or primed (Section 4.5.3), the next objective was to investigate how a lower concentration of (10ng/ml) IFNs (which are more typically used in *in vitro* models (77, 110)) affected neutrophil apoptosis. An initial investigation of IFNs at 6hrs and at 20hrs was aimed to explore any effect on either the immediate or prolonged neutrophil lifespan*.*

Healthy adult neutrophils were stimulated with lower concentrations of IFNs (10ng/ml) and GMCSF (5ng/ml) for 6 and 20hrs, and apoptosis was measured (Section 2.6.2.2). At 6hrs it was shown that compared to unstimulated neutrophils (17.4 ± 5.0%, n=5), both IFNγ (10.4 ± 3.8%, n=5, p=0.037) and positive control GMCSF (5.4 ± 1.2%, n=5, p=0.0013) significantly reduced the level of apoptosis (Figure 4.6A). There was a suggestion that IFNα (14.7 ± 3.9%, n=5) and IFNβ (14.0 ± 4.2%, n=5) had some reducing effect on apoptosis at 6hrs, however this difference was not statistically significant (p>0.5). This reducing effect on apoptosis was also seen at 20hrs, where compared to unstimulated neutrophils (74.1 ± 4.1%, n=5), IFNα (62.1 ± 3.4%, n=5), IFNγ (61.8 ± 6.3%, n=5) and IFNβ (63.4 ± 4.5%, n=5) had a smaller reduction in the level of apoptosis but GMCSF significantly reduced the level of apoptosis (53.2 ± 3.5%, n=5, p=0.0055) (Figure 4.6B). These data suggest that the optimal effect of IFNs on neutrophil apoptosis is at 6hrs rather than at 20hrs.



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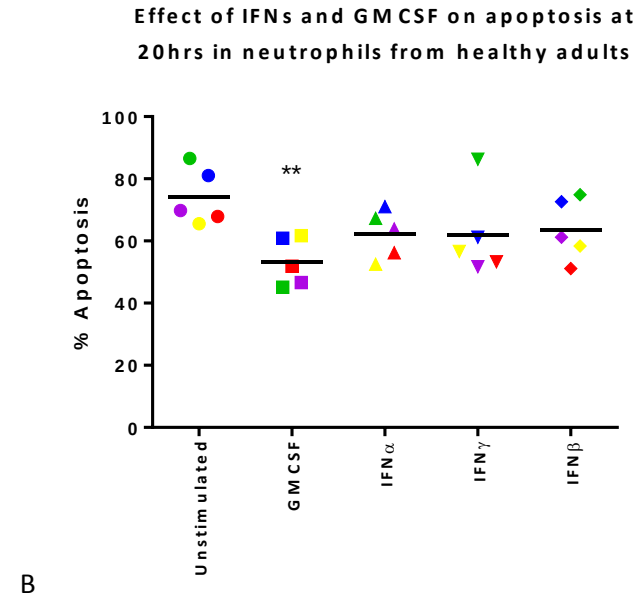


B

Figure 4.6. Apoptosis analysis of healthy adult neutrophils after 6hr and 20hr incubations with 5ng/ml GMCSF, 10ng/ml IFNα, 10ng/ml IFNγ or 10ng/ml IFNβ. Neutrophils were isolated from healthy adult donors and were left unstimulated or were stimulated with 5ng/ml GMCSF, 10ng/ml IFNα, 10ng/ml IFNγ or 10ng/ml IFNβ for 6hrs (A) or 20hrs (B). Neutrophils were stained with annexin V FITC and PI, and the percentage of apoptosis was measured using flow cytometry. GMCSF and IFNγ significantly reduced apoptosis at 6hrs (A) (GMCSF – **, n=5, p=0.0013; IFNγ - *, n=5, p=0.037) and GMCSF, significantly reduced apoptosis at 20hrs (B) (**, n=5, p=0.0055).

4.5.5. Downstream apoptosis signalling pathway analysis at 6hrs and 20hrs

In view of both the pro- and anti-apoptotic effects seen in IFN-stimulated neutrophils, it is important to investigate the influence of the different IFNs on stability of anti-apoptotic MCL1 and cleavage of pro-apoptotic caspase 3 from procaspase 3. As a control, GMCSF was used, which has been shown to reduce apoptosis through the stability of MCL1 (77) (Section 1.3.4).

Downstream proteins important in apoptosis were measured by Western blotting (Section 2.7). MCL1 was measured after 6hrs 10ng/ml IFNα 10ng/ml IFNγ 10ng/ml IFNβ and 5ng/ml GMCSF incubation and procaspase 3/caspase 3 was measured after 20hrs 10ng/ml IFNα, 10ng/ml IFNγ, 10ng/ml IFNβ and 5ng/ml GMCSF incubation.

At 6hrs, only GMCSF stabilised MCL1 expression in neutrophils (1.4 ± 0.6, n=5) compared to unstimulated neutrophils (0.6 ± 0.2, n=5, p=0.29) (Figure 4.7). This suggests that compared to unstimulated neutrophils, the IFN subtypes each had little effect on MCL1 stability (IFNα = 0.6 ± 0.1, n=5; IFNγ = 1.2 ± 0.75, n=5; IFNβ = 0.6 ± 0.2, n=5) (Figure 4.7). Thus, using flow cytometry, overall, the results show that although IFNs may have an anti-apoptotic effect at both a high and lower concentration (Figures 4.5 and 4.6), it is unlikely to be through MCL1 stability (Figure 4.7).

At 20hrs, neutrophils stimulated with all cytokines had a higher procaspase 3/caspase 3 expression ratio (GMCSF = 5.6 ± 2.6, IFNα = 2.4 ± 0.6, IFNγ = 4.4 ± 0.9, IFNβ 3.1 ± 0.9, n=5) compared to unstimulated neutrophils (1.0 ± 0.3, n=5) indicating reduced cleavage of caspase 3 (Figure 4.8). However, only the difference induced by IFNγ was statistically significant (n=5, p=0.0013) (Figure 4.8). This reduced caspase 3 cleavage generally at 20hrs reflects the reduction in apoptosis at this time point (see Figure 4.6B). It indicates that the IFN subtypes, specifically IFNγ, may reduce apoptosis through the reduction of cleaved caspase 3.

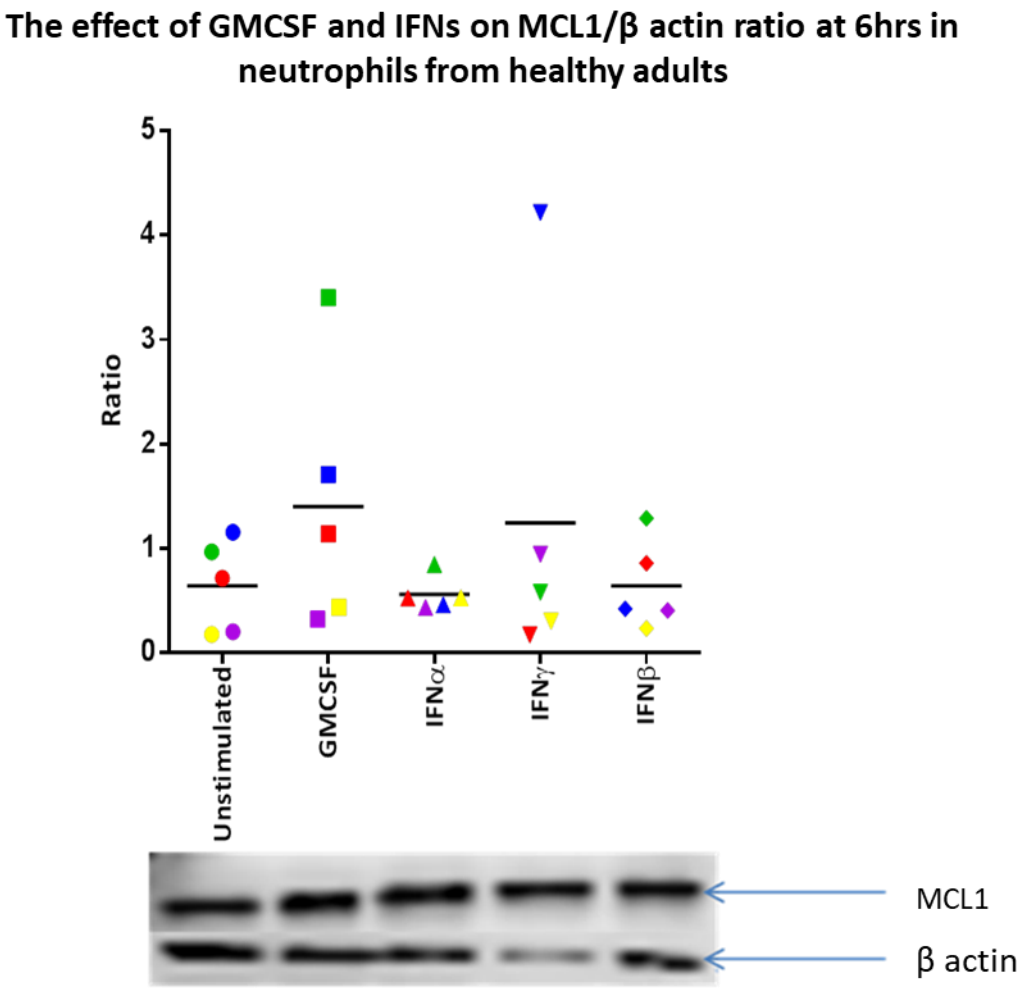


Figure 4.7. The expression ratio of MCL1/B actin in healthy adult neutrophils after 6hr incubation with 5ng/ml GMCSF, 10ng/ml IFNα, 10ng/ml IFNγ or 10ng/ml IFNβ. Neutrophils were isolated from healthy adult donors and were left unstimulated or were stimulated with 5ng/ml GMCSF, 10ng/ml IFNα, 10ng/ml IFNγ or 10ng/ml IFNβ respectively for 6hrs. Proteins were extracted, and the MCL1/B actin expression ratios were measured using Western blotting. Analysis showed that GMCSF stabilises MCL1, whereas the different IFN subtypes had little effect (n=5).

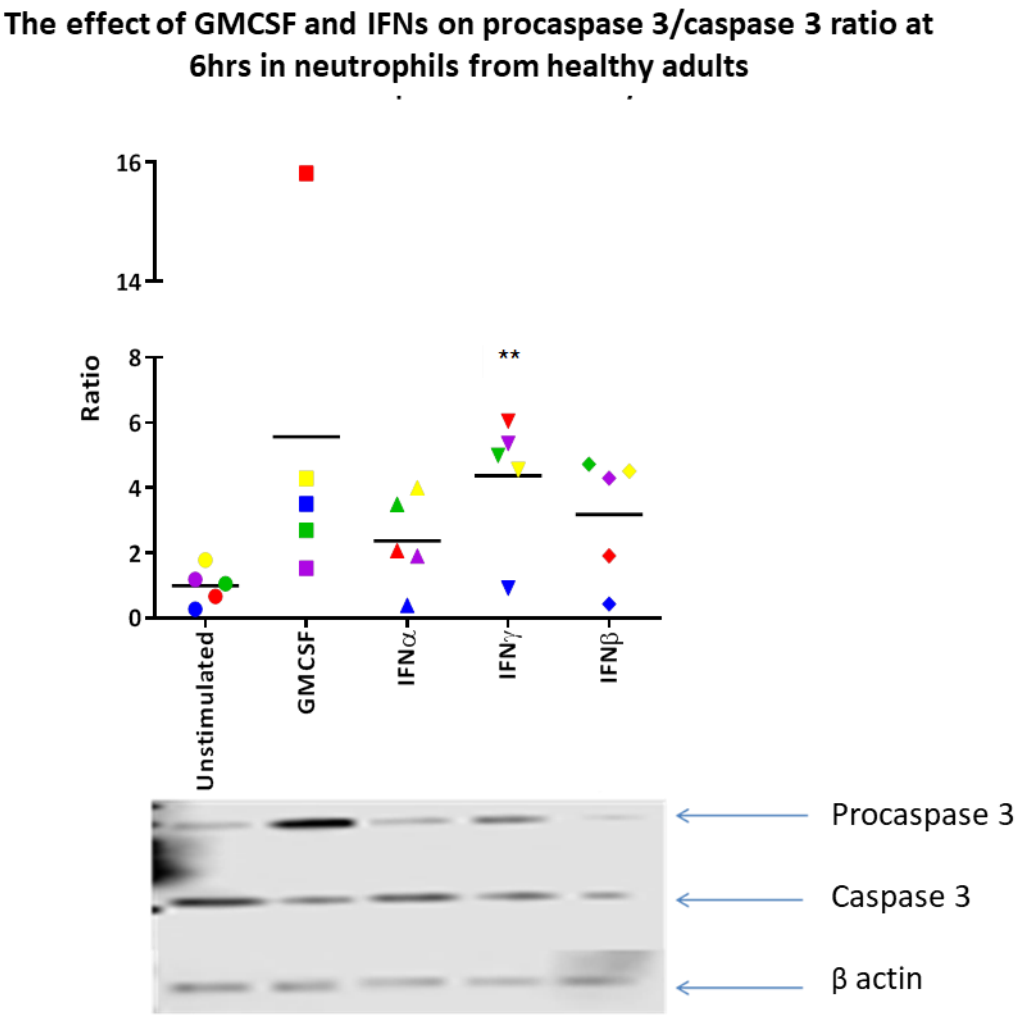


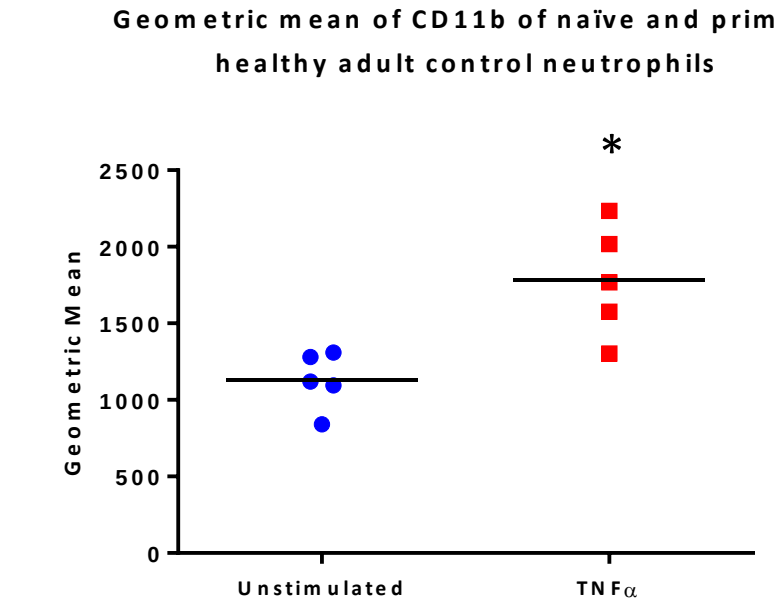
Figure 4.8. The expression ratio of procaspase 3/caspase 3 in healthy adult neutrophils from after 20hr incubation with 5ng/ml GMCSF, 10ng/ml IFNα, 10ng/ml IFNγ or 10ng/ml IFNβ. Neutrophils were isolated from healthy adult donors and were left unstimulated, or were stimulated with 5ng/ml GMCSF, 10ng/ml IFNα, 10ng/ml IFNγ or 10ng/ml IFNβ for 20hrs. Proteins were extracted, and procaspase 3/ caspase 3 expression ratios were measured using Western blotting**.** Analysis showed a decrease in caspase 3 cleavage (shown by a high procaspase 3/caspase 3 ratio) in all conditions. IFNγ significantly reduced the cleavage of caspase 3 (*, n=5, p=0.0013). The outlier within the GMCSF condition, although seems extreme, may be due to the variability of Western blotting protocol, although there were no obvious discrepancies within the raw data of that particular experiment compared to other blots.

4.5.6. Investigation of the effect of lower concentrations of IFNs on naïve and primed neutrophil apoptosis

From data using a lower concentration of IFNs on apoptosis (Section 4.5.4), an important objective was to investigate how the different subtypes of IFNs (at 10ng/ml) effects apoptosis in naïve and primed neutrophils from healthy adults compared to that of the higher concentrations (1-10μg/ml, Section 4.5.3).

The activation state of healthy adult control neutrophils was tested by measuring the expression of CD11b and CD62L on the neutrophil surface using flow cytometry after 30mins priming with 1μg/ml TNFα. There was a significant increase in CD11b (1779.8 ± 163.3 vs 1128.8 ± 83.5, n=5, p=0.031) and a significant decrease in CD62L (92.6 ± 13.4 vs 460.0 ± 87.0, n=5, p=0.031) in TNFα-primed neutrophils compared to unstimulated neutrophils respectively, confirming neutrophil activation (Figure 4.9).

Naïve and TNFα-primed neutrophils were subsequently stimulated with 10ng/ml, IFNα 10ng/ml, IFNγ, 10ng/ml IFNβ and 5ng/ml GMCSF for 6hrs. Compared to naïve unstimulated neutrophils (25.3 ± 6.7%, n=5), IFNα (19.8 ± 6.5%, n=5), IFNγ (13.4 ± 4.9%. n=5) and IFNβ (17.9 ± 5.7%, n=5) all reduced apoptosis in naïve neutrophils (Figure 4.10), although these differences were not statistically significant. GMCSF significantly reduced the level of apoptosis occurring (6.9 ± 1.3%, n=5, p=0.026). This effect was abrogated in TNFα-primed neutrophils (IFNα = 21.7 ± 2.1%, IFNγ = 23.66 ± 4.4%, IFNβ = 27.4 ± 1.3%, n=5), although these differences were not statistically significant (Figure 4.10).



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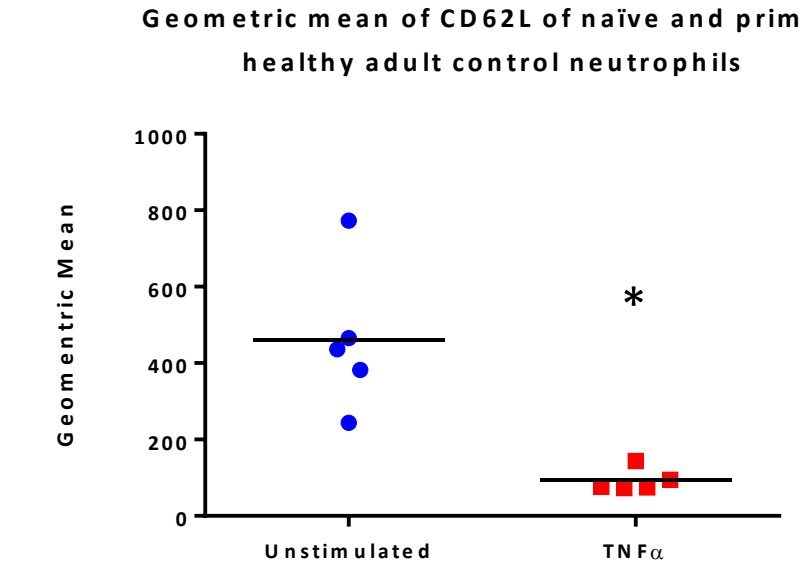


B

Figure 4.9. CD11b and CD62L expression analysis of healthy adult neutrophils primed with 1µg/ml TNFα for 30mins. Neutrophils were isolated from healthy adult donors and were left naïve or were primed with 1µg/ml TNFα for 30mins. Neutrophils were stained with antibodies to CD11b (A) or CD62L (B) and the relative expression via geometric mean was measured using flow cytometry. Increase in CD11b and decrease in CD62L after TNFα priming confirms neutrophil activation (*, n=5, p=0.031).

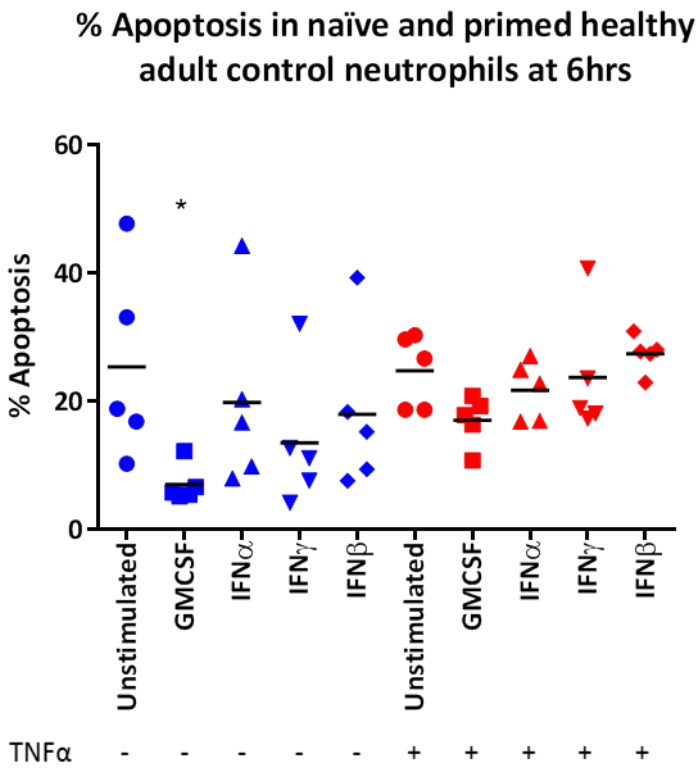


Figure 4.10. Apoptosis analysis of naïve and 1µg/ml TNFα-primed adult healthy neutrophils after 6hrs incubation with 10ng/ml IFNα, 10ng/ml IFNγ or 10ng/ml IFNβ. Neutrophils were isolated from healthy adult donors and were left naïve (blue) or were primed with 1µg/ml TNFα for 30mins (red), and subsequently stimulated with 5ng/ml GMCSF, 10ng/ml IFNα, 10ng/ml IFNγ or 10ng/ml IFNβ for 6hrs. Neutrophils were stained with annexin V FITC and PI, and the percentage of apoptosis was measured using flow cytometry. GMCSF significantly reduced apoptosis in naïve neutrophils compared to naïve unstimulated neutrophils (*, n=5, p=0.026). IFNs reduced apoptosis in naïve neutrophils, and this effect was abrogated in primed neutrophils (n=5).

4.5.7. Investigating the role of IFNs on adult neutrophil apoptosis in the presence of patient sera

Following on from investigating the role of IFNs on apoptosis TNFα-primed neutrophils (which was a good, but simplistic in vitro model; Sections 4.5.3 -4.5.6), the next stage was to investigate how the complex JSLE environment affects IFN-related neutrophil apoptosis. Published data demonstrate that JSLE serum can increase the rate of neutrophil apoptosis and this increase may be due to the imbalance of apoptotic factors shown within the JSLE serum (72, 110) (Sections 1.4.3, 1.5.3.1, 4.1). It has been shown that adult-onset SLE neutrophils are more active than neutrophils from healthy adults, and that the serum may contain factors that cause this increase in activation (83) (Section 1.3.5). The following experiments aimed to investigate the influence of paediatric control and JSLE patient sera on: a) the activation state of healthy neutrophils; b) the subsequent influence on neutrophil apoptosis; and c) the influence on IFN-mediated apoptotic effect.

4.5.7.1. Patient sera selections

Patient sera were selected from the UK JSLE Cohort Study biobank based on their disease activity status, as determined by the patients' pBILAG a time of sample collection. Samples were selected that were deemed inactive, active in the renal domain and active in the haematological domain of pBILAGs, (Section 4.4.2.3). In addition, age- and gender- matched paediatric control sera were also selected. Demographics, medication and laboratory variables were recorded (Table 4.1).

Table 4.1. Clinical and demographic information of individuals providing serum for experimental analysis at time of blood collection.

Variables	Active Haematological pBILAG[a] (n=3)	Active Renal pBILAG[a] (n=5)	Inactive pBILAG[a] (n=5)	Control (n=5)
Age[b]	13.6 [13.1-15.3]	16.6 [11.9-18.0]	15.5 [12.2-18.0]	15.0 [14.8 – 15.4]
Duration of disease (months)[b]	25 [4-109]	13 [0-24]	18 [7-43]	-
Female[c]	2 (66.6)	5 (100)	4 (80)	4 (80)
Total pBILAG score[d]	9 [7-10]	9 [8-13]	0	-
Ethnicity				
White British	-	3 (60)	4 (80)	4 (80)
Any other White background	-	1 (20)	-	-
Bangladeshi	1 (20)	-	-	-
Pakistani	1 (20)	-	1 (20)	-
Chinese	-	1 (20)	-	-
Latin American	-	-	-	1 (20)
Other	1 (20)	-	-	-
Family history				
Autoimmune diseases	-	3	3	-
Other	1	1	1	2
None	2	2	1	3
Medication[e]				
Steroids	2	5	2	-
DMARDs	3	5	5	-
Other	2	4	2	1
dsDNA (IU/L)	0	0	0 [0-7]	-
C3 (g/L)	1.13 [0.95-1.36]	1.07 [0.65-1.32]	1.11 [1.03-1.33]	-
ESR (mm/h)	1 [1-4]	2 [1-23]	8 [2-16]	-

Data are expressed as median and interquartile range [square brackets] or as total numbers with percentages (round brackets).

pBILAG. Paediatric British Isles Lupus Assessment Group; DMARDs, disease-modifying anti-rheumatic drugs; dsDNA, anti-double stranded DNA antibody; C3, complement component 3; ESR, erythrocyte sedimentation rate.

[a]Classification of patients into active disease in haematological domain (scored A/B), active disease in the renal domain (scored A/B) and inactive disease in all domains (D/E) was determined using the pBILAG.

[b]Age and disease duration at time of serum sample collection.

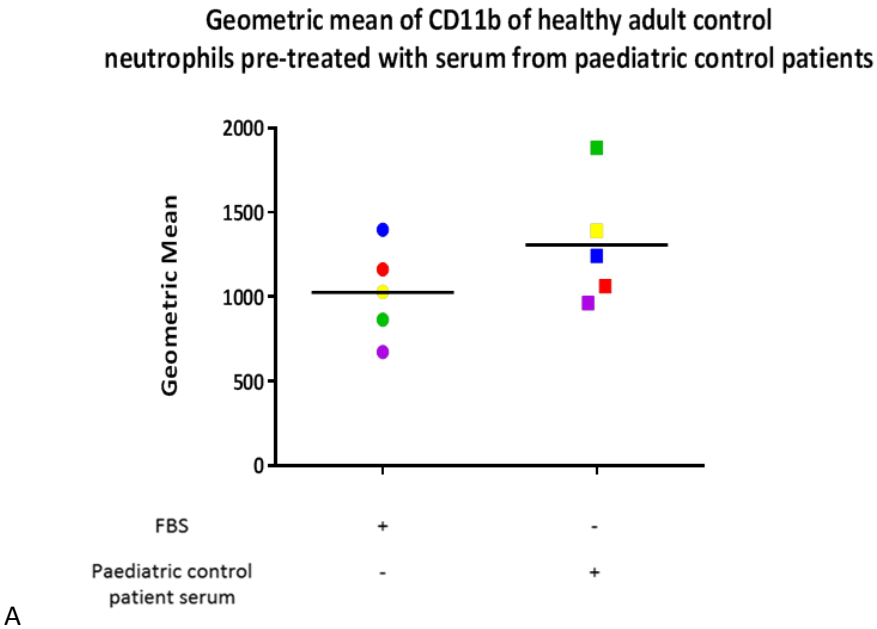
[c]Gender of patients.

[d]Total pBILAG score determined by the numeric scoring of disease classifications of the organ domains.

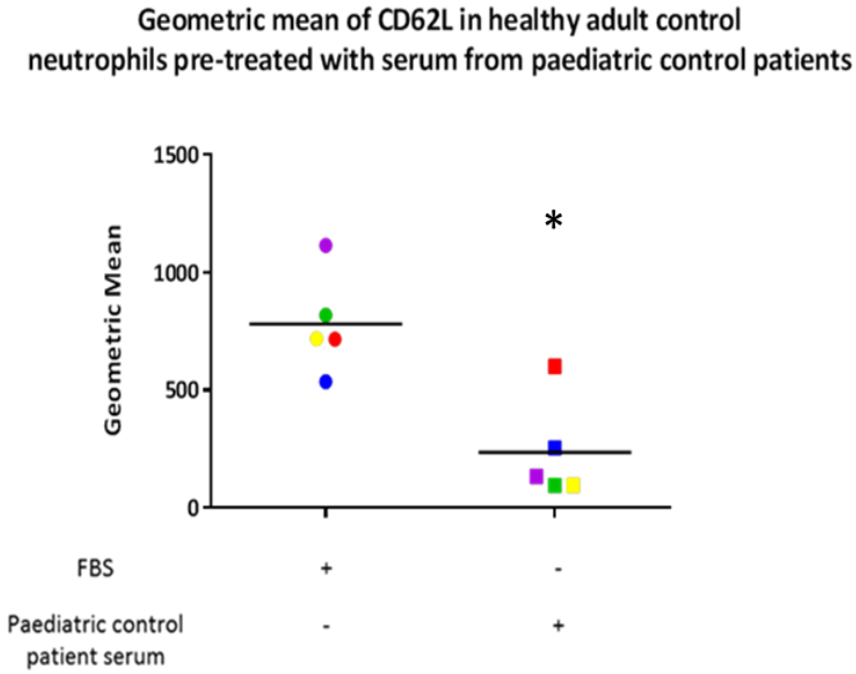
[e]Medication taken at time of serum sample collection.

4.5.7.2. The effect of JSLE patient and paediatric control sera on neutrophil activation

Neutrophils from healthy adult neutrophils were incubated in media/10% FBS or primed with 10% patient sera (Section 4.5.5.1) for 30mins and neutrophil activation was then analysed. Compared to FBS treated neutrophils, sera from paediatric control patients increased the expression of CD11b (1308 ± 161 vs 1025 ± 124, n=5) and significantly decreased the expression of CD62L (235 ± 96 vs 780 ± 95 n=5, p=0.031), indicating neutrophils were primed for activation (Figure 4.11). Compared to FBS treated neutrophils, sera from JSLE patients with inactive disease in all pBILAG domains significantly increased the expression of CD11b (1326 ± 120 vs 1025 ± 124, n=5, p=0.031) and significantly decreased the expression of CD62L (247 ± 65 vs 780 ± 95, n=5, p=0.031) indicating neutrophils were primed (Figure 4.12). Compared to FBS treated neutrophils, sera from JSLE patients with active disease in the renal pBILAG domain significantly increased the expression of CD11b (1414 ± 298 vs 1138 ± 214, n=5, p=0.031) significantly decreased the expression of CD62L (172 ± 48 vs 494 ± 56, n=5, p=0.031), indicating neutrophils were primed (Figure 4.13). Compared to FBS incubated neutrophils, sera from JSLE patients with active disease in the haematological pBILAG domain had no statistically significant effect on the expression of either activation markers (CD11b = 961 ± 171.2 vs 978 ± 217, CD62L = 576 ± 216.6 vs 822 ± 167, n=3), indicating that these neutrophils were not primed, although this may be due to low sample numbers of sera used (n=3 only available at time of assay being undertaken) (Figure 4.14).

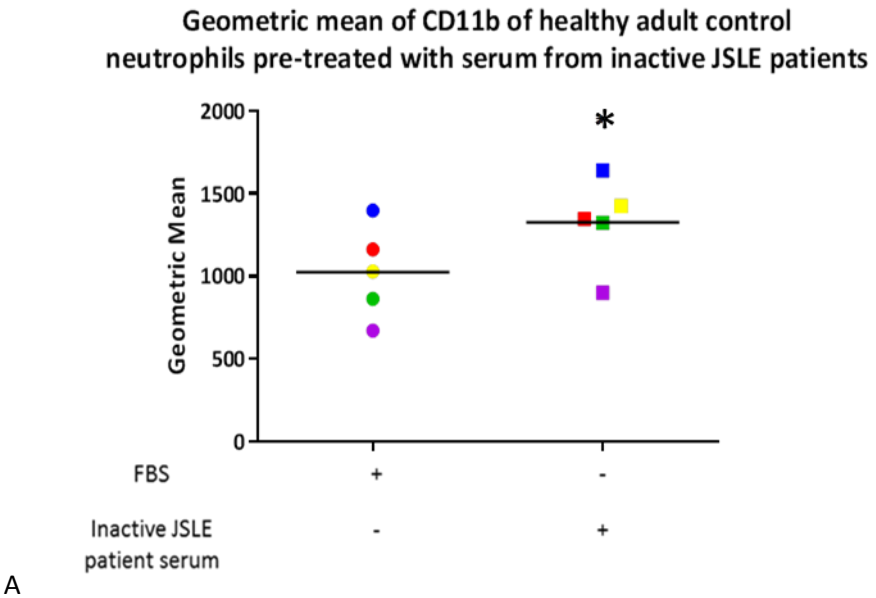


A



B

Figure 4.11. CD11b and CD62L expression analysis on neutrophils from healthy adult donors after 30mins priming with sera from paediatric control patients. Neutrophils were isolated from healthy adult donors and left naïve or were primed with sera from paediatric control patients. The neutrophils were stained with CD11b (A) and CD62L (B) and the relative expression via the geometric mean was measured using flow cytometry. An increase in CD11b (n=5; p>0.05) and a significant decrease in CD62L after serum pre-treatment (*, n=5, p=0.031), confirming neutrophil activation by sera from paediatric control patients



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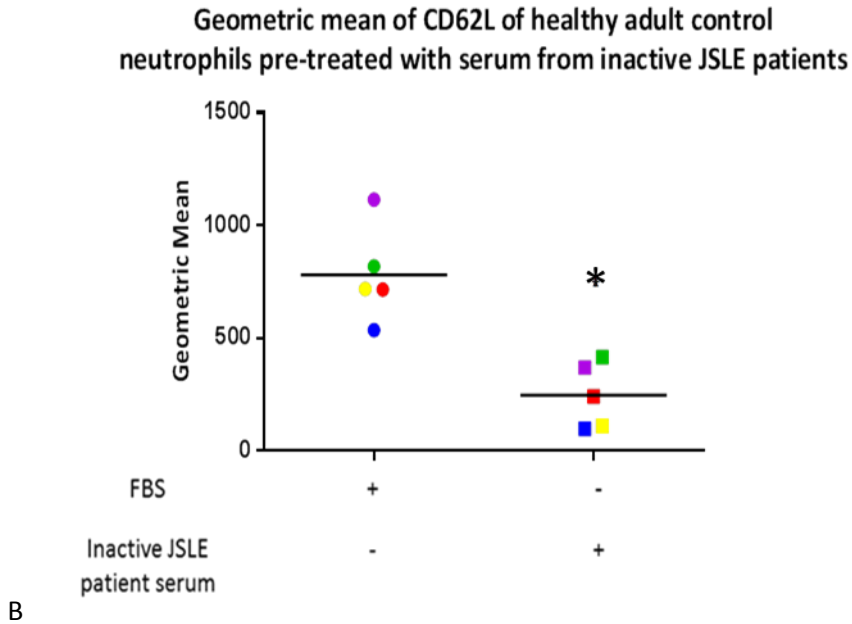
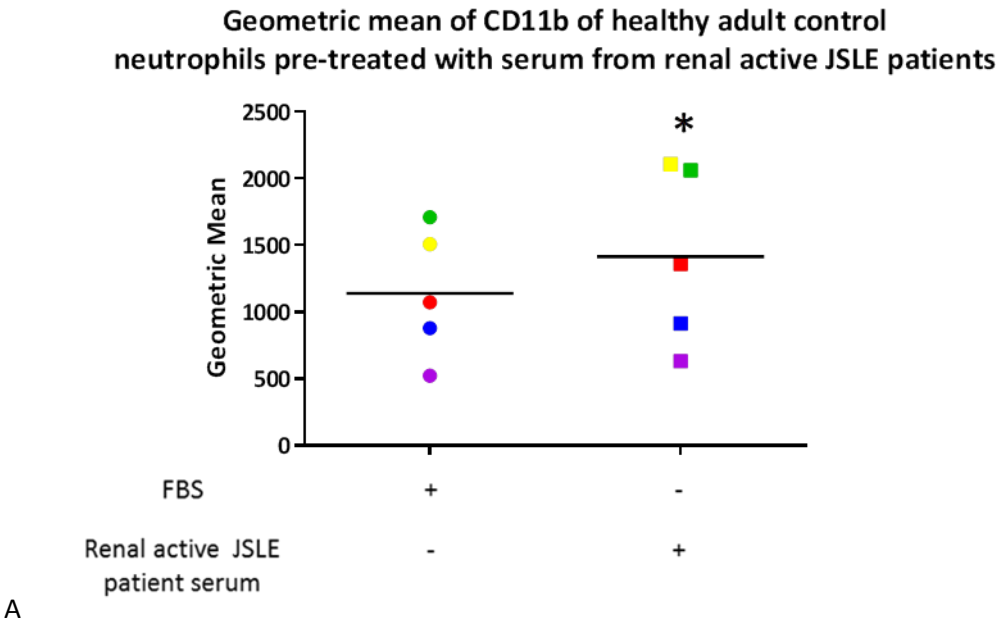


B

Figure 4.12. CD11b and CD62L expression analysis on neutrophils from healthy adults after 30mins priming with sera from JSLE patients with inactive disease in all pBILAG domains. Neutrophils were isolated from healthy adult donors and were left naïve or were primed with sera from JSLE patients with inactive disease in all pBILAG domains. The neutrophils were stained with CD11b (A) and CD62L (B) and the relative expression via the geometric mean was measured using flow cytometry. Increase in CD11b (*, n=5, p=0.031) and a significant decrease in CD62L after serum pre-treatment (*, n=5, p=0.031) confirming neutrophil activation by sera from patients with inactive JSLE.



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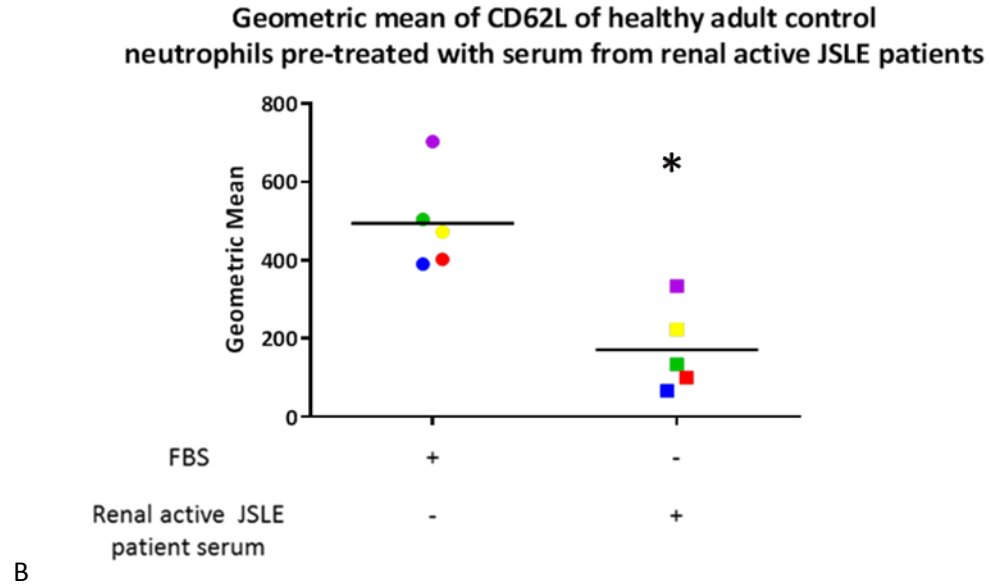
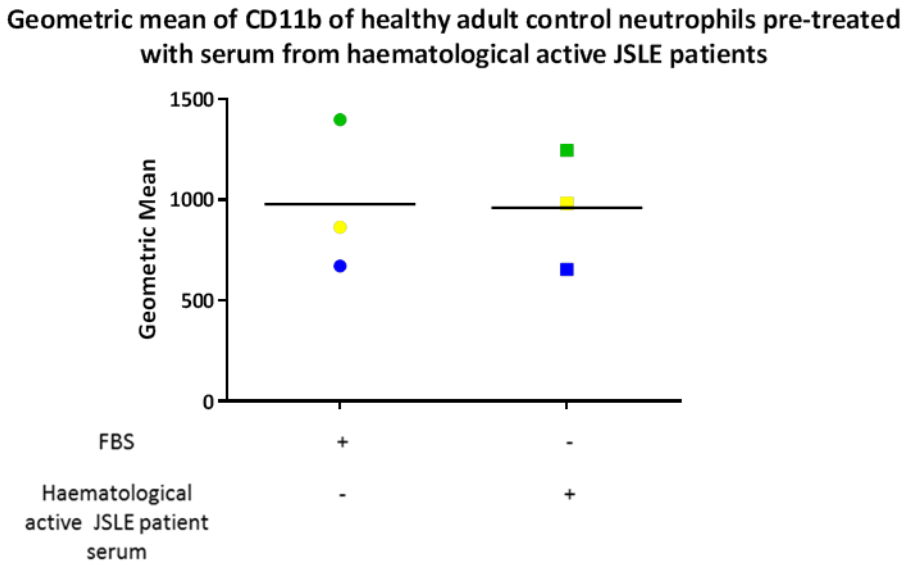


B

Figure 4.13. CD11b and CD62L expression analysis on neutrophils from healthy adults after 30mins priming with sera from JSLE patients with active disease activity in the renal pBILAG domain. Neutrophils were isolated from healthy adult donors and were left naïve or were primed with sera from JSLE patients with active disease in the renal pBILAG domain. The neutrophils were stained with CD11b (A) and CD62L (B) and the relative expression via the geometric mean was measured using flow cytometry. Increase in CD11b (*, n=5, p=0.031) and a significant decrease in CD62L after serum pre-treatment (*, n=5, p=0.031) confirming activation by sera from patients with renal active JSLE.



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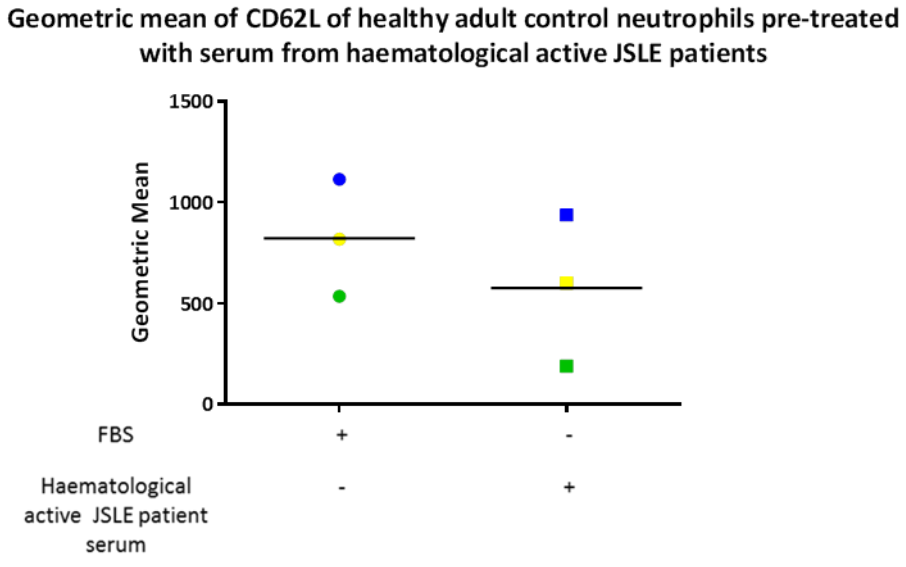


B

Figure 4.14. CD11b and CD62L expression analysis on neutrophils from healthy adults after 30mins priming with sera from JSLE patients with active disease in the haematological pBILAG domain. Neutrophils were isolated from healthy adult donors and were left naïve or were primed with sera from JSLE patients with active disease in the haematological pBILAG domain. The neutrophils were stained with CD11b and CD62L and the relative expression via the geometric mean was measured using flow cytometry (n=3).

4.5.7.3. Effect of JSLE patient and paediatric control patient sera on neutrophil apoptosis

JSLE neutrophils are more apoptotic than control neutrophils, and it has been shown that JSLE serum can increase neutrophil apoptosis (72, 110) (Section 4.1). In Section 4.5.5.2, both JSLE and paediatric control sera activated healthy neutrophils. In Section 4.5.4.3, it was demonstrated that the activation of neutrophils can abrogate the anti-apoptotic potential of IFNs and GMCSF, and a high IFN concentration can result in an increase in neutrophil apoptosis, as seen in Section 4.5.3.2. Therefore, the effect of each of the IFN subtypes on neutrophil apoptosis occurring as a result of treatment with patient sera was therefore investigated, with FBS treated neutrophils as a comparison.

Neutrophils were primed as above with control patient sera, each of the three patient sera as stated above (Section 4.4.5.2) or FBS and incubated for 6hrs with 10ng/ml of all three IFN subtypes or 5ng/ml of GMCSF. Apoptosis was measured and expressed by the fold change against unstimulated FBS-incubated neutrophils Within the renal active sera analysis, GMCSF significantly reduced apoptosis in FBS-incubated neutrophils compared to unstimulated, FBS-incubated neutrophils (0.3 ± 0.03 vs 1.0, n=5, p=0035) (Figure 4.17). Within the control sera analysis, the inactive sera analysis, and the haematological active sera analysis, this reduction of apoptosis by GMCSF, although observed, was not statistically significant. This may be due to the fact that one GMCSF experimental observation had to be omitted due to operator error, and therefore the complete data set would have reached significance. Additionally, there was no reduction in apoptosis in FBS-incubated neutrophils upon any IFN subtype, which seems to contradict data analysed previously (Figures 4.6, 4.10, 4.15, 4.16 and 4.18; Section 4.5.4 and Section 4.5.6). These contradictory results may be due to the small sample size, and the way the results were analysed (fold change versus percentage). This was particularly relevant for neutrophils that were stimulated with sera from JSLE patients who were active in the haematological pBILAG domain, as this was the smallest sample size, due to a loss of one of the GMCSF stimulated FBS control samples (which did not run through the flow cytometer successfully and thus had to be omitted). None of the IFN subtypes nor GMCSF had any marked effect on neutrophil apoptosis in neutrophils that had been pre-treated with either healthy control and JSLE patient sera. This suggests that the presence of heterologous serum primed neutrophils, regardless of origin of the serum (Figures 4.11-4.18). This priming lead to abrogation of GMCSF related reduction in apoptosis (Figures 4.15-4.18)These results compare to other experimental data seen previously (Section 4.5.4.3), in which priming of neutrophils with TNFα was able to abrogate the anti-apoptotic potential of

GMCSF (Figure 4.10). However, as none of the IFN subtypes had any effect on apoptosis in naïve/FBS incubated neutrophils in these experiments (contradictory to what was see in Section 4.5.4.3), the effect of serum on IFN apoptosis remains inconclusive (Figures 4.15-4.18).

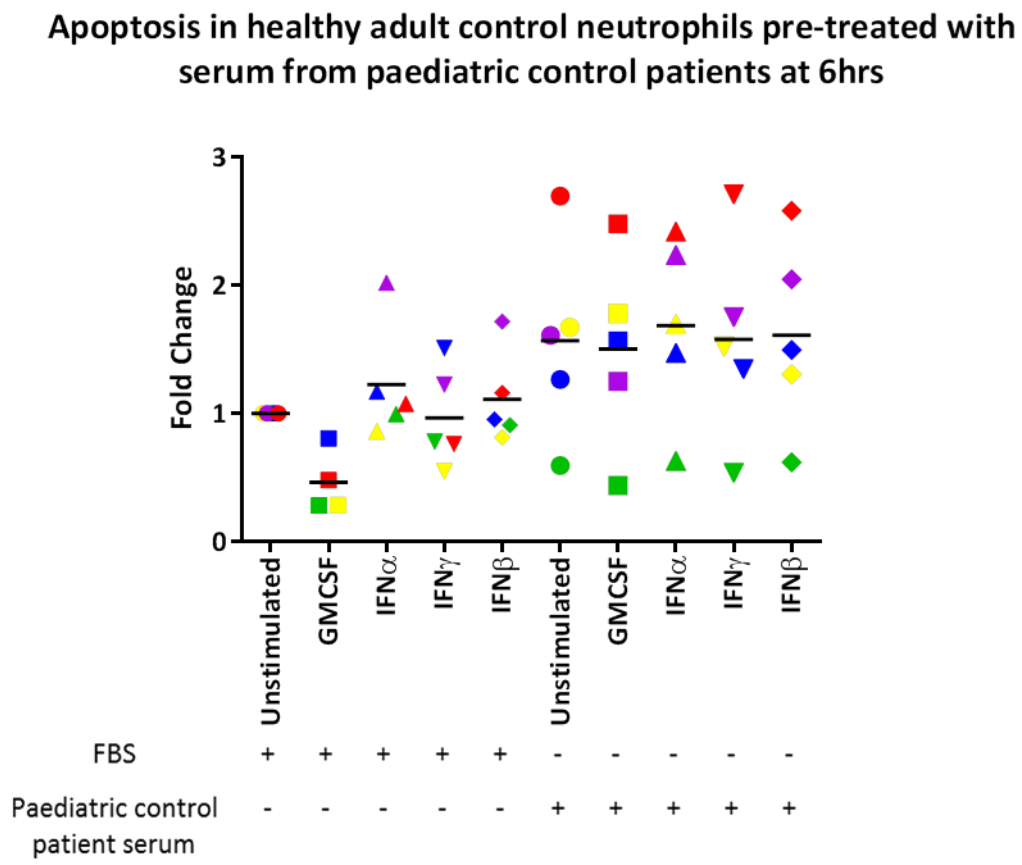


Figure 4.15. Apoptosis analysis of healthy adult neutrophils primed with sera from paediatric control patients, stimulated with 5ng/ml GMCSF, 10ng/ml IFNα, 10ng/ml IFNγ or 10ng/ml IFNβ for 6hrs. Neutrophils were isolated from healthy adult donors and were left naïve or were primed with 10% sera from paediatric control patients for 30mins, and subsequently stimulated with 5ng/ml GMCSF, 10ng/ml IFNα, 10ng/ml IFNγ or 10ng/ml IFNβ for 6hrs. Neutrophils were stained with annexin V FITC and PI, and the percentage of apoptosis was measured using flow cytometry (n=4-5).

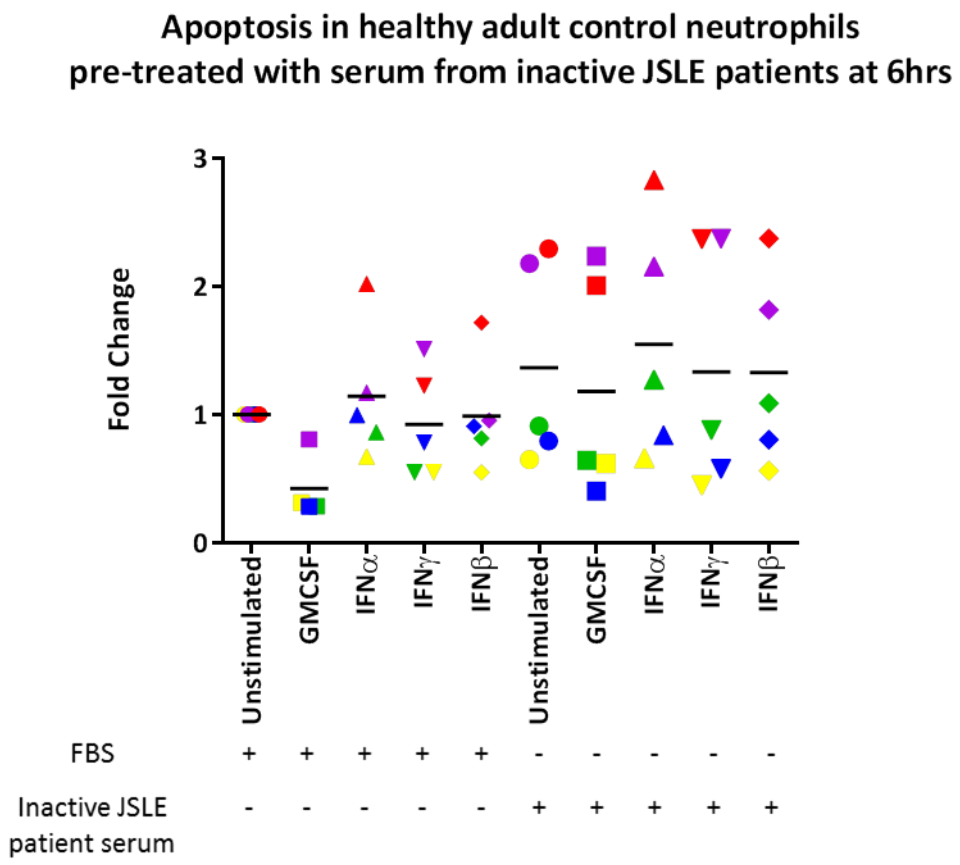


Figure 4.16. Apoptosis analysis of healthy adult neutrophils primed with sera from JSLE patients with inactive disease, stimulated with 5ng/ml GMCSF, 10ng/ml IFNα, 10ng/ml IFNγ or 10ng/ml IFNβ for 6hrs. Neutrophils were isolated from healthy adult donors and were left naïve or were primed with 10% sera from JSLE patients with inactive disease in all pBILAG domains for 30mins, and subsequently stimulated with 5ng/ml GMCSF, 10ng/ml IFNα, 10ng/ml IFNγ or 10ng/ml IFNβ for 6hrs. Neutrophils were stained with annexin V FITC and PI, and the percentage of apoptosis was measured using flow cytometry (n=4-5).

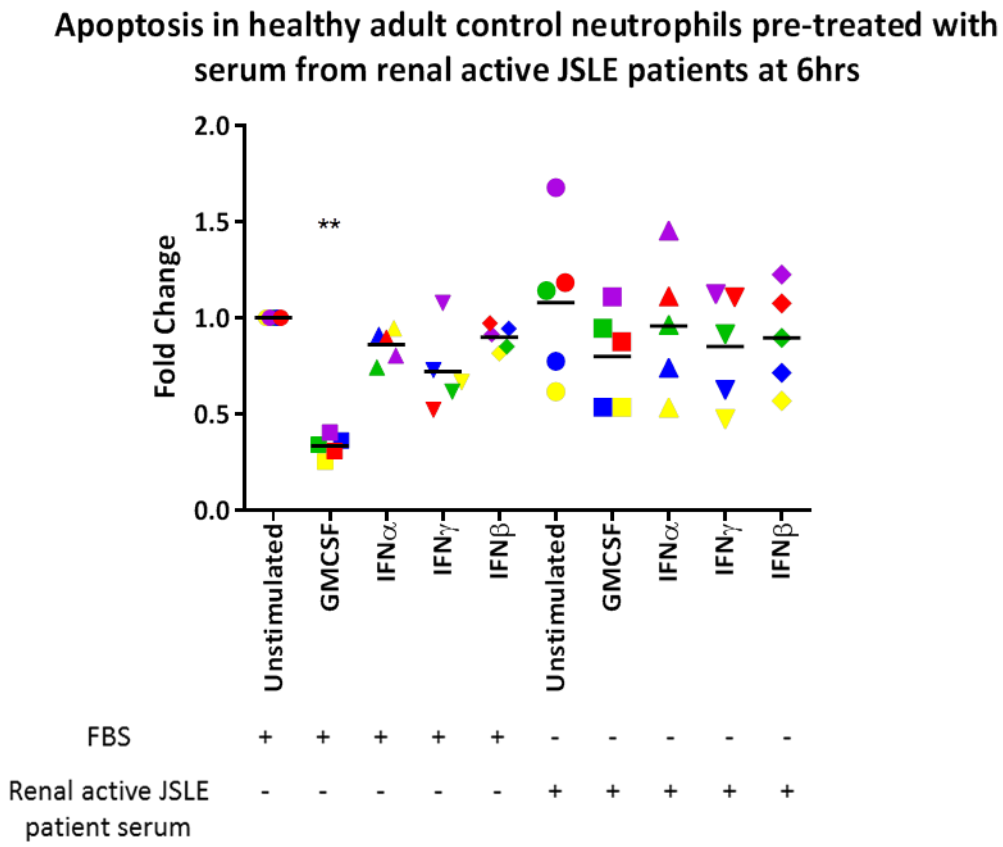


Figure 4.17. Apoptosis analysis of healthy adult neutrophils primed with sera from JSLE patients with active disease in the renal domain, stimulated with 5ng/ml GMCSF, 10ng/ml IFNα, 10ng/ml IFNγ or 10ng/ml IFNβ for 6hrs. Neutrophils were isolated from healthy adult donors and were left naïve or were primed with 10% sera from JSLE patients with active disease in the renal pBILAG domain for 30mins, and subsequently stimulated with 5ng/ml GMCSF, 10ng/ml IFNα, 10ng/ml IFNγ or 10ng/ml IFNβ for 6hrs. Neutrophils were stained with annexin V FITC and PI, and the percentage of apoptosis was measured using flow cytometry (n=5). GMCSF in FBS-incubated neutrophils significantly reduced apoptosis compared to unstimulated FBS-incubated neutrophils (n=5, p=0.0035).

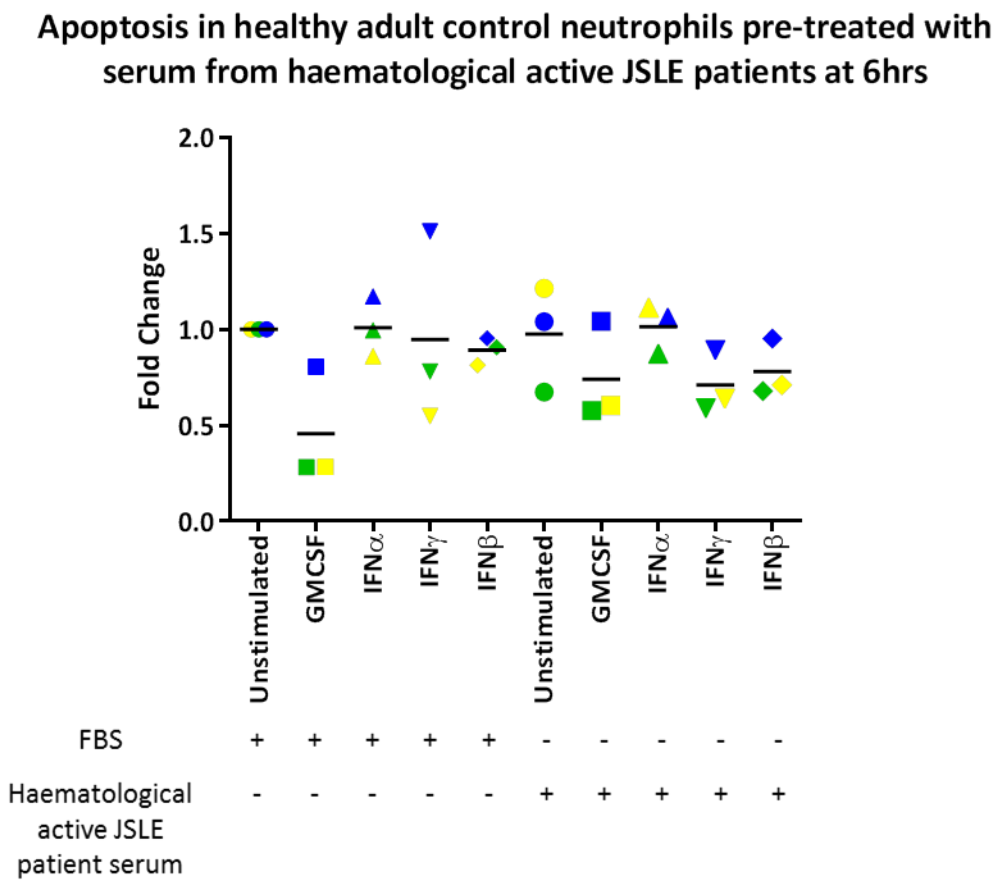


Figure 4.18. Apoptosis analysis of neutrophils primed with sera from JSLE patients with active disease in the haematological domain, stimulated with 5ng/ml GMCSF, 10ng/ml IFNα, 10ng/ml IFNγ or 10ng/ml IFNβ for 6hrs. Neutrophils were isolated from healthy adult donors and were left naïve or were primed with 10% sera from JSLE patients with active disease in the haematological pBILAG domain for 30mins and subsequently stimulated with 5ng/ml GMCSF, 10ng/ml IFNα, 10ng/ml IFNγ or 10ng/ml IFNβ for 6hrs. Neutrophils were stained with annexin V FITC and PI, and the percentage of apoptosis was measured using flow cytometry (n=3).

4.5.8. Investigating the role of IFNs on apoptosis in paediatric control and JSLE patient neutrophils

It has been shown that there is an increase in pro-apoptotic proteins within JSLE neutrophils (170) (Sections 1.5.3.1 and 4.1). As the cellular environment (serum effects) had already been investigated in part, it was important to determine whether the neutrophils from JSLE patients themselves responded differently to IFNs compared to neutrophils from control patients (83).

4.5.8.1. The activation state of paediatric control and JSLE patient neutrophils

Neutrophils from paediatric control and JSLE patient whole blood were isolated, stained with CD11b and CD62L, and the expression of these markers was analysed using flow cytometry. JSLE neutrophils had a higher expression of CD11b compared to paediatric control neutrophils (1260 ± 150, n=5, vs 814 ± 129, n=6, p=0.052). However, JSLE neutrophil expression of CD62L was comparable to that of paediatric control neutrophils (313 ± 37, n=5, vs 380 ± 66, n=6) (Figure 4.19). These data indicate that the JSLE neutrophils are activated compared to that of paediatric controls (as shown by the increase in CD11b).

Table 4.2. Clinical and demographic information of individuals providing blood for neutrophil activation marker experimental analysis.

Variables	JSLE (n=5)	Control (n=6)
Age (years)[a]	17.7 [14.5-18.9]	13.9 [8.2-15.4]
Duration of disease (months)[a]	40 [22-101]	-
Female[b]	5 (100)	2 (33.3)
Total pBILAG score[c]	3 [0-5]	-
Ethnicity		
White British	5 (100)	5 (100)
Family history		
Autoimmune diseases	2	1
Other	2	1
None	2	3
Medication[d]		
Steroids	2	-
DMARDs	5	-
Hydroxychloroquine	4	-
Mycophenolate	5	-
C3 (g/L)	1.1 [1.05-1.47]	-
C4 (g/L)	0.19 [0.07-0.24]	-
ESR (mm/h)	4 [3-12]	-

Data are expressed as median and range [square brackets] or as total numbers with percentages (round brackets)

pBILAG. Paediatric British Isles Lupus Assessment Group; C3, complement component 3; C4, complement component 4; DMARDs, disease-modifying anti-rheumatic drugs; ESR, erythrocyte sedimentation rate

[a]Age and disease duration time of neutrophil isolation and analysis

[b]Gender of patients

[c]Total pBILAG score determined by the numeric scoring of disease classifications of the organ domains

[d]Medication taken at time of serum sample collection

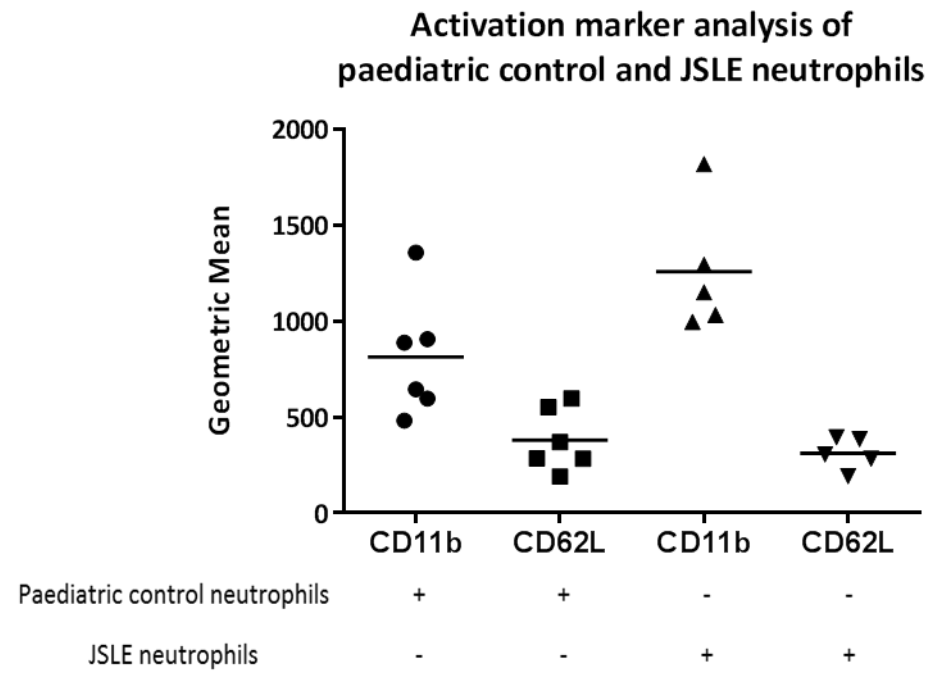


Figure 4.19. CD11b and CD62L expression analysis of neutrophils isolated from paediatric control patients and JSLE patients. Neutrophils were isolated from paediatric control patients and JSLE patients, were stained with CD11b and CD62L and relative expression via geometric mean was measured using flow cytometry. There was an increase in CD11b in JSLE neutrophils compared to paediatric control neutrophils, but this was not statistically significant (n=5-6).

4.5.8.2. The effect of IFNs on paediatric control patient neutrophil apoptosis

Healthy paediatric control patients used in this experimental analysis (n=5) had a median age of 10.4 years (7.7-14.9 years) and were 40% female (2 females and 3 males) (Table 4.3). Control patient neutrophils were incubated for 6hrs with 5ng/ml GMCSF and 10ng/ml IFNα, 10ng/ml IFNγ or 10ng/ml IFNβ. At 6hrs, GMCSF significantly reduced apoptosis in neutrophils from paediatric control patients compared to unstimulated neutrophils (5.5 ± 0.6% vs 18.1 ± 3.5%, n=5, p=0.021) (Figure 4.20). There was a suggestion that IFNγ also reduced apoptosis in neutrophils from paediatric control patients (13.6 ± 3.3%, n=5). IFNα (18.3 ± 3.2%, n=5), and IFNβ (16.5 ± 3.0%, n=5) had no overall effect on paediatric control patient neutrophil apoptosis (p>0.05) (Figure 4.20). These data are reflective of the results shown in healthy adult control neutrophils (Section 4.5.4.1), although IFNγ seems to be less effective in reducing apoptosis in children than adults.

4.5.8.3. The effect of IFNs on JSLE patient neutrophil apoptosis

JSLE patients recruited to this experimental analysis (n=5) had a median age of 14.9 years (14.4-18.9 years) and were 80% female (4 females and 1 male) (Table 4.3). JSLE patient neutrophils were incubated for 6hrs with 5ng/ml GMCSF and 10ng/ml IFNα, 10ng/ml IFNγ and 10ng/ml IFNβ. At 6hrs, GMCSF significantly reduced apoptosis in JSLE neutrophils compared to unstimulated neutrophils (6.1 ± 0.9% vs 19.1 ± 0.9%, n=5, p=0.037) (Figure 4.20). IFNα (21.5 ± 2.1%, n=5) and IFNβ (22.2 ± 1.6%, n=5) had no overall effect on control patient apoptosis (p>0.05) (Figure 4.20). IFNγ (15.0 ± 1.2%, n-=5) had a reducing effect on apoptosis, but this was not statistically significant. Additionally, there were no statistically significant differences in naïve and stimulated neutrophil apoptosis between control and JSLE patients. These data do not reflect what was expected from the literature (72). The fact that no statistically significant differences were noted in these JSLE patients may be that when their samples were collected, they may have their disease well managed or have medication masking the effects. Many demographic and clinical factors may be affecting the rate of neutrophil apoptosis measured here. Table 4.3 summarises some key demographic and clinical data for the patients included in this part of the study. The data indicates that the median pBILAG (disease activity score) was 1, which indicates that the disease was generally inactive in these patients. All were on a range of medication, including a couple on corticosteroids, which again may be affecting the rate of neutrophil apoptosis and how these neutrophils respond to IFNs. Small sample size precluded further investigation of the specific effects of medication on these findings. Overall, it is likely that the patients' neutrophils are

responding more similarly paediatric control neutrophils, in part due to low disease activity and/or the disease was well managed due to medication.

Table 4.3. Clinical and demographic information of individuals providing blood for neutrophil apoptosis experimental analysis.

Variables	JSLE (n=5)	Control (n=5)
Age (years)[a]	14.9 [14.4-18.5]	10.4 [7.7-14.8]
Duration of disease (months)[a]	41 [30-125]	-
Female[b]	4 (80)	2 (40)
Total pBILAG score[c]	1 [0-8]	-
Ethnicity		
White British	4 (80)	5 (100)
Bangladeshi	1 (20)	-
Family history		
Autoimmune diseases	2	1
Other	1	2
None	3	2
Medication[d]		
Steroids	4	-
DMARDs	5	-
Hydroxychloroquine	5	-
Mycophenolate	4	-
Other	1	-
OCP	1	-
dsDNA (IU/L)[e]	7 [0-202]	-
C3 (g/L)[f]	1.17 [1.06-1.27]	-
ESR (mm/h)	3 [1-25]	-

Data are expressed as median and range [square brackets] or as total numbers with percentages (round brackets)

pBILAG. Paediatric British Isles Lupus Assessment Group; DMARDs, disease-modifying anti-rheumatic drugs; dsDNA, anti-double stranded DNA antibody; C3, complement component 3; ESR, erythrocyte sedimentation rate

[a]Age and disease duration time of neutrophil isolation and analysis

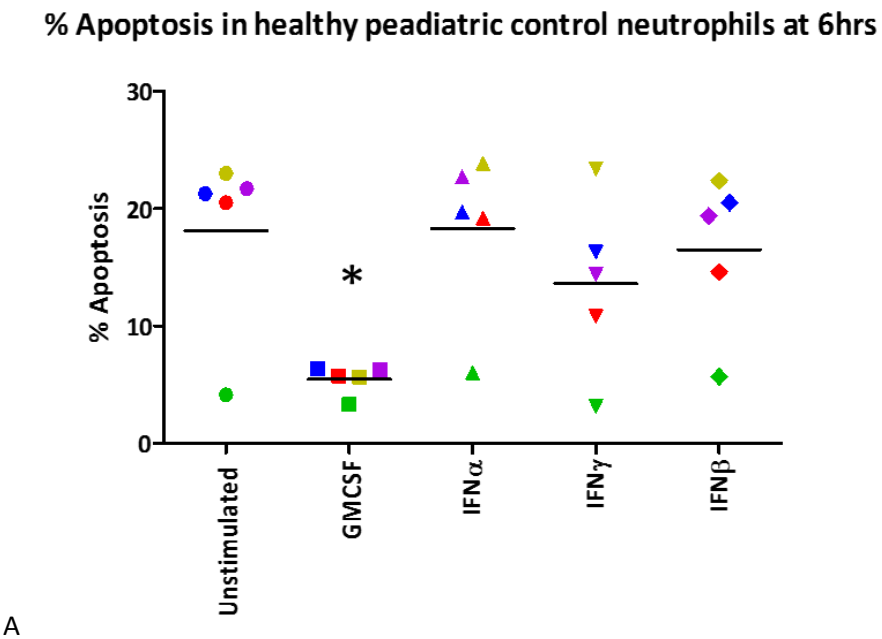
[b]Gender of patients

[c]Total pBILAG score determined by the numeric scoring of disease classifications of the organ domains

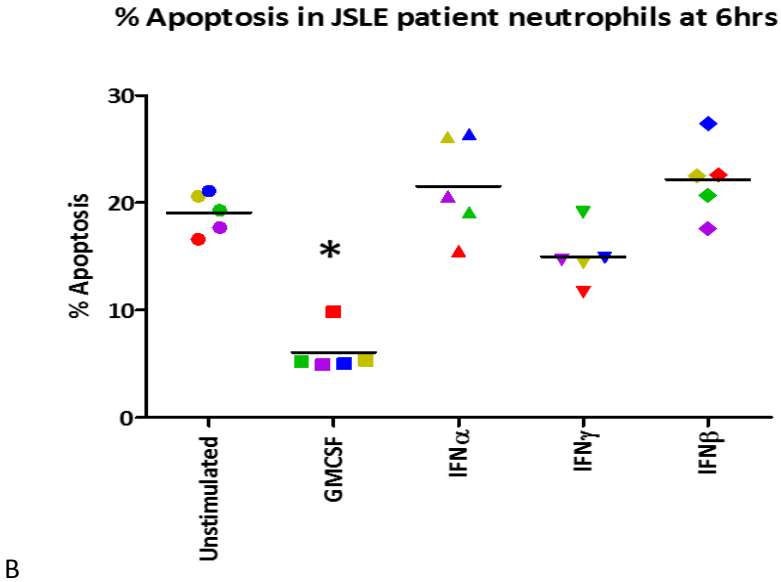
[d]Medication taken at time of serum sample collection

[e]Two patients had no dsDNA data at time of demographic analysis

[f]One patient had no C3 data at time of demographic analysis



A



B

Figure 4.20. Apoptosis analysis of neutrophils isolated from paediatric control patients and JSLE patients stimulated with 5ng/ml GMCSF, 10ng/ml IFNα, 10ng/ml IFNγ or 10ng/ml IFNβ for 6hrs. Neutrophils were isolated from paediatric control patients (A) or JSLE patients (B) and were left unstimulated or were stimulated with 5ng/ml GMCSF, 10ng/ml IFNα, 10ng/ml IFNγ or 10ng/ml IFNβ for 6hrs. Neutrophils were stained with annexin V FITC and PI, and the percentage of apoptosis was measured using flow cytometry and the percentage of apoptotic neutrophils was measured using flow cytometry. A significant reduction in apoptosis was found in GMCSF stimulated neutrophils in both control (*, n=5, p=0.021) and JSLE patients (*, n=5, p=0.037).

4.6. Discussion

Neutrophils have been shown to undergo increased apoptosis within both SLE and JSLE, and this may be due to dysregulation in both extrinsic environmental factors and intrinsic signalling pathways (71, 72, 110, 170). Neutrophils from SLE have been shown to be activated, and it confirmed here that neutrophils from JSLE are also activated; this may be an important factor in the increased neutrophil apoptosis seen (71, 72, 83). IFNs have been shown to be increased in the serum of SLE and JSLE patients, and IFN gene signatures have been shown to be increased in both SLE and JSLE patients (41, 68, 171, 172, 174) (Section 1.5.4.1). Importantly, high IFNα serum levels has been indicated to be a risk factor in developing SLE and IFN activity has be shown to be increased in patients with JSLE even before disease classification (190, 222) (Section 1.5.4.1). Along with the IFN gene signature as mentioned previously, there is also an increase in a granulocyte gene signature within PMBCs from JSLE patients (41). This therefore indicates an important interaction between neutrophils and IFNs in JSLE pathogenesis, particularly in regards to increased neutrophil apoptosis.

4.6.1. IFN subtypes and their effect on apoptosis in naïve and TNFα primed neutrophils

Within this study, it was it was important to initially investigate the role of IFNs on neutrophil apoptosis within naïve and primed neutrophils. Literature has shown that IFNs can be both pro- and anti-apoptotic on a variety of cells, including immune cells, although only an anti-apoptotic role by both type 1 and 2 IFNs has been demonstrated in naïve neutrophils previously (138, 141, 164, 165) (Section 1.5.3.1). However, no data had been published in regards to any differential apoptotic effects of IFNs on naïve and primed neutrophils or the role of IFNs on neutrophil apoptosis within SLE or JSLE.

It was confirmed that IFNs have a pro- and anti-apoptotic effect on neutrophils; this effect was dependent on concentration of IFNs and activation state of neutrophils (Section 4.5.3). At a high concentration, both IFNα and IFNγ reduced apoptosis in naïve neutrophils; however, within TNFα primed neutrophils, all three IFN subtypes increased the rate of apoptosis (Section 4.5.3). Following on from this, all three IFN subtypes could reduce the level of apoptosis in naïve using a lower concentration of IFNs, although to varying degrees, by which IFNγ reduced apoptosis the most. This effect was abrogated in neutrophils primed with TNFα (Sections 4.5.4, 4.5.6).

4.6.1.1. Use of TNFα-primed neutrophils to model JSLE neutrophils

It is important to note that TNFα can be also pro- and anti-apoptotic, and therefore careful consideration of the effect of TNFα on apoptosis was undertaken (221). Other priming agents were considered, but from review of the literate, the only other possibilities were IFNγ and GMCSF; these were not appropriate priming agents as both were important downstream conditions in this investigation (86, 90, 167) (Section 1.3.5). Here, there was no observed overall effect of TNFα on neutrophil apoptosis downstream, and therefore its use as a priming agent was suitable to model the JSLE neutrophils *in vitro*.

4.6.2. IFN subtypes and their effect on downstream apoptotic signalling proteins in naïve neutrophils

Apoptosis signalling is complex and is mediated by many pro and anti-apoptotic factors. GMCSF has been shown to reduce apoptosis through the stability of MCL1, a member of the anti-apoptotic BCL2 family (77, 223) (Section 1.3.4). Caspase 3, however, is known as a apoptosis effector protein, and is cleaved by both the extrinsic and intrinsic apoptosis pathways (73) (Section 1.3.4). Hence, these two contrasting apoptotic proteins were analysed in regards to IFN signalling pathway within naïve neutrophils to start elucidating how IFNs inflict their effect on apoptosis. Data here has shown that IFNs reduce the cleavage of caspase 3, which is likely to coincide with the reduced apoptosis seen, IFNs did not have any influence over the stability of MCL1; these observations have not been previously published (Section 4.5.5). However, IFNs are known to stimulate the JAK/STAT pathway, whereby STAT1 is pro-apoptotic and STAT3 is anti-apoptotic (138, 141) (Section 1.5.3.1). This IFN/JAK/STAT pathway will be further investigated in chapter 5 in regards to the differential effect of IFNs on apoptosis (138, 141) (Section 5.5.3). In brief, the STATs are likely to be fundamental to IFN signalling and apoptosis within JSLE. An increase in the activation of the STAT1 pathway has been indicated in PBMCs of SLE patients, and within a mouse model of lupus nephritis, and therefore it is highly likely that this pro-apoptotic pathway is heightened in JSLE neutrophils, and therefore contribute to the increased neutrophil apoptosis seen in JSLE (72, 194, 196).

4.6.3. IFN subtypes and their effect on apoptosis in patient serum primed neutrophils

This study confirmed that IFNs' differential roles on apoptosis was dependent on activation of neutrophils through priming with TNFα (Section 4.6.1). However, neutrophils within SLE and JSLE will be exposed to a variety of inflammatory factors and cytokines, and thus investigation of apoptosis in neutrophils exposed to paediatric control and JSLE patient sera

was an important next step (72, 224, 225). It was observed that patient serum induced neutrophil activation, as shown through increased CD11b and CD62L. However, there was no differences in the level of neutrophil activation upon priming with either paediatric control patient or JSLE patient sera, or between the different JSLE sera groups (Section 4.5.7.2). These observations may simply indicate that neutrophils will become activated when exposed to homogenous environments. As expected, the anti-apoptotic effect of IFNs was abrogated following neutrophil activation by patient sera, reflecting what was observed in the *in vitro* model (Sections 4.5.6 and 4.5.7.3). However, no increase in neutrophil apoptosis was observed in neutrophils primed with JSLE serum, contradictory to what other studies have shown (72, 110) (Section 4.5.7.3). Of note, contrary to the neutrophils used here, these studies used JSLE or paediatric control neutrophils; these neutrophils may be more susceptible to environmental apoptotic factors found in JSLE serum than adult neutrophils. Therefore, repeat of this study using paediatric control or JSLE neutrophils may be beneficial to fully investigate the role of IFNs in a JSLE environment.

4.6.4. IFN subtypes effect on apoptosis in patient neutrophils

Neutrophils have been shown to undergo increased apoptosis in both SLE and JSLE and may be partly due to their increased activation state as observed by the increase in CD11b in a published study and as shown here (71, 72, 83) (Sections 1.3.5 and 4.5.6.1). The increase of IFNs in serum, the IFN and granulocyte gene signature within JSLE PBMCs, and the pro-apoptotic effect IFNs can have on activated neutrophils indicate that IFNs may induce apoptosis in JSLE neutrophils (41, 68, 171, 172) (Section 1.5).

It has been shown here that JSLE patient neutrophil had comparable rates of apoptosis to paediatric control patient neutrophils, both unstimulated and stimulated with IFNs, which does not correlate with published data (71, 72) (Sections 1.4.3 and 4.5.8). The study by Midgley *et al*. had included patients with active disease, which may contribute to the increased apoptosis seen (72). Therefore, clinical data was analysed, and it was concluded that the JSLE patients, of whom the neutrophils were isolated from, had a low pBILAG score, and thus had inactive disease at sampling. Their health, therefore, may be comparable to that of paediatric control patients as their disease was well managed, and therefore may explain similar neutrophil responses and apoptosis. Thus, a larger study, containing JSLE patients with various levels of disease activity, would test whether disease activity and medication at time of sampling are important contributors to the dysregulated apoptosis seen (71, 72).

4.7. Summary

IFNs as a group can be both pro- and anti-apoptotic, and this depends on the activation state of the neutrophils and concentration of the IFNs. IFNs induce an anti-apoptotic effect through the reduced cleavage of caspase 3, but not through the stability of MCL1. Patient serum activated healthy adult neutrophils and abrogated the anti-apoptotic effect of IFNs. However, there were no observed differences between the different patient serum groups on neutrophil activation or apoptosis, which may be due to the complexity of human serum. Although result suggested JSLE neutrophils from inactive patients were slightly more activated than paediatric control neutrophils, there was not differences in the rate of apoptosis between patient groups. This may be due to the patients' disease being inactive and their medication influencing neutrophil function.

Chapter 5: The role of IFN signalling in neutrophils in JSLE

5.1. Introduction

Cytokines signal their effect through a series of proteins on the surface of and within the cell. The expression of receptors and proteins within cells differ between cell types and the environment they are in. Thus, the effect of cytokines differs depending on cell type and between different states of health and disease.

It is recognised that there is a difference in cell protein constitution within neutrophils from healthy individuals and individuals with JSLE (170). It has been shown that there is an increase in pro-apoptotic proteins and decrease in anti-apoptotic proteins within JSLE neutrophils compared to healthy paediatric controls (72, 170). This difference in protein constitution may be due to the likely activation of JSLE neutrophils (83) (Section 1.5.3.1, Section 4.5.8.1, Figure 4.19). It has been shown that TNFα activated neutrophils have an up-regulation of proteins and cellular markers, such as increased activation of ERK and p38 MAPK, and increased surface expression of CD11b and CD66b, compared to that of healthy, naïve neutrophils (84, 85, 95, 169) (Section 1.3.5).

It is well established that IFNs signal through the JAK/STAT pathway within a variety of cells, including neutrophils (138, 141, 166) (Section 1.5.1). Although the various IFN subtypes have similar and overlapping effects, there are key differences in their signalling pathways. Type 1 IFNs all signal through the IFNα receptor (IFNAR), albeit with varying potencies and effects (159, 160). Type 2 IFNs signal through the IFNγ receptor (IFNGR) (161, 162).

Each IFN receptor is made up of two chains, and the expression and dimerization of these chains contribute to which downstream signalling pathways are activated (146, 147) (Section 1.5.2). In summary, particular the IFNGR chains have differential expression on three immune cell lines, and this observed difference can contribute to either cell proliferation or apoptosis. (161). IFNGR1 has been shown to be consistently expressed on different immune cell lines (such as myeloid, B cell and T cell lines), but IFNGR2 has differential expression levels depending on cell type and environment (161). In particular IFNGR2 had limited expression on T cell line and NK cell line; however the myeloid cell line (the closest cell line to neutrophils) have high IFNGR2 membrane expressions (161). The environment has an important role to play in the expression of these receptor chains. IFNGR2 can have increased expression in serum-depleted T cells, and within cells that have a high IFNGR2 expression,

IFNγ induced apoptosis (161). Additionally, the expression of IFNGR2 on T cells extracted from healthy volunteers can influence whether IFNγ induces proliferation (when IFNGR2 is low) or apoptosis (when IFNGR2 is high) (162). This suggests that the IFN receptor chain expression is interchangeable, and the IFN effect on apoptosis is dependent on the relative expression of its receptor chains.

As noted previously, IFNAR is understood to be important in the increased IFN signature found in JSLE patients (41, 187) (Section 1.5.4.1). Previous studies have shown that murine lupus could be induced by 2,6,10,14-Tetramethylpentadecane (TMPD), also known as pristine, a compound that can induce autoimmune disease, in particular SLE (187, 226). This drug-induced lupus model was associated with high levels of type 1 IFNs and caused an increase in ISGs such as IP10, Mx1 and IRF-7 in mouse PBMCs (187, 227). However, within the IFNAR deficient mice, these genes did not increase in expression after TMPD treatment, suggesting that the IFNα receptor is important in lupus-associated IFN gene signature expression.

Additionally, in TMPD treated mice, there was a high level of ANAs in the sera (187). IFNAR knockout mice also had an increase in ANAs upon TMPD treatment, but is reduced compared to control mice treated with TMPD (187). A significant reduction of anti- nuclear (n)RNP/Sm, anti-Su, anti-chromatin, anti-dsDNA and anti-single stranded (ss)DNA is seen in the knockout IFNAR mice treated with TMPD compared with TMPD treated control mice (187). This shows that IFNAR and thus type 1 IFNs contribute to the SLE autoantibody production, that in turn contribute to disease pathogenesis.

In the kidney, glomerular cellularity is increased in TMPD-control mice, however this is unchanged in TMPD-treated, IFNAR knockout mice (187). Proteinuria was decreased and immune deposits were unchanged in TMPD treated IFNAR knock out mice compared to TMPD treated control mice (187). This suggests that within lupus nephritis, IFNAR is required for increased glomerular cellularity and may be involved in the increased proteinuria seen in lupus patients; however, IFNAR is not involved in immune deposits in the kidney.

IFNs signal through the phosphorylation of the different STATs; the activation of STATs can result in very different endpoints. In particular, it has been shown that STAT1 is considered to be pro-apoptotic, and STAT3 is considered to be anti-apoptotic (138, 141). The IFN receptor chain expressions may be important in determining which STAT is phosphorylated. It has been shown via EMSA that IFNγ induces phosphorylation of STAT1 in the myeloid cell line, and that this may be associated with the high expression of IFNGR2 in these cells (161).

It was shown that IFNγ was able to only slightly induce phosphorylation of STAT1 within the T cell lines, and this may relate to the low expression of IFNGR2 on the membrane (161). It was also shown that depleting T cell line of serum lead to an increase IFNGR2 expression, which lead to an increase in IFNγ-induced STAT1 phosphorylation, and an increase in apoptosis (161). Thus, the increase in apoptosis may be due to the increase in STAT1 phosphorylation and therefore activation, which may be reliant on the increased IFNGR2 expression on the membrane. This part of the IFN/JAK/STAT pathway may therefore be important in JSLE neutrophil apoptosis.

Within this thesis, it was shown that both type 1 and type 2 IFNs phosphorylate STAT1 and STAT3, and activation of neutrophils alters the level of phosphorylation (Section 5.5.3). It has been shown that there is proportionally more STAT1 and phosphorylated STAT1 (pSTAT1) in PBMCs in adult-onset SLE compared to healthy controls, and IFNγ was able to effectively phosphorylate more STAT1 in SLE PBMCs than in healthy controls (194). This up-regulation of STAT1 in SLE PBMCs positively correlated with dsDNA levels, indicating a link between STAT1 and a marker of disease activity (194). Additionally, STAT1 has also been implicated in the mouse model of lupus nephritis, where by the expression of both STAT1 and pSTAT1 were higher in lupus nephritis mice compared to healthy controls (196). There is a high level of STAT1 in lupus nephritis mesangial cells, and both IFNγ and IFNα are able to phosphorylate STAT1 in these cells, albeit IFNα less so than IFNγ (196). This difference in IFN-induced pSTAT1 may be due to receptor and potency differences mentioned previously. This indicates an important role of STAT1 in SLE pathogenesis.

5.1.1. Summary

IFNs can be both pro- and anti-apoptotic, and this function depends on the phosphorylation of pro-apoptotic STAT1 or phosphorylation of anti-apoptotic STAT3. This differentiation may depend on the activation state of the neutrophils; stressful influences can modulate the IFNγ receptor chains in particular, and switch IFNγ signalling from proliferative to apoptotic. The IFNα receptor also is paramount to contributing to the increase in the IFN signature seen in JSLE. Additionally, the STAT1 pathway is increased in SLE PBMCs and a mouse modal in lupus nephritis, and therefore is likely to be important in SLE pathogenesis. Thus, investigation into the IFN signalling pathway is required to determine whether IFN-induced STAT1 phosphorylation contributes to the increase in neutrophil apoptosis seen in JSLE, and thus may become a potential therapeutic target.

5.2. Chapter hypothesis

IFNs exert their effect through the JAK/STAT pathway. Their influence on neutrophil functions is dependent on the expression of IFN receptor chains and which STAT is phosphorylated, whereby STAT1 is considered pro-apoptotic, and STAT3 is considered anti-apoptotic. Therefore, it is hypothesized that within activated neutrophils, as found in JSLE, the IFN receptor expressions differs from that of healthy naïve neutrophils, and this drives the IFN subtypes to activate the STAT1, pro-apoptotic signalling pathway.

5.3. Chapter aim and objectives

Aim 3: To investigate the IFN signalling pathway in naïve and primed neutrophils and its relation to apoptosis.

- Objective 1: Using flow cytometry, measure the IFN receptor expression on naïve and TNFα-primed healthy adult neutrophils.
- Objective 2: Using flow cytometry, measure the IFN receptor expression on paediatric control and JSLE patient neutrophils.
- Objective 3: Using Western blotting, measure the relative expression of pSTAT1 and pSTAT3 in naïve and TNFα-primed healthy adult neutrophils.
- Objective 4: Using both flow cytometry and Western blotting, measure the ability of fludarabine phosphate and S1459 to reduce pSTAT1/β actin expression ratio (and therefore possibly inhibiting pSTAT1) within healthy adult neutrophils and their effect on the rate of neutrophil apoptosis. .

5.4. Methods

5.4.1. IFN receptor and activation marker analysis

Neutrophils were isolated from healthy adults (Section 2.5.2). Neutrophils were treated with 1µg/ml TNFα, 1µg/ml IFNα, 1µg/ml IFNβ or 1µg/ml of IFNγ for 30mins at 37°C, 5% CO_2 in a 96 well plate, using $1x10^5$ neutrophils per condition. The plate was centrifuged at 1800 RPM for 5mins, and the supernatant was decanted. Neutrophils were washed in 100µl of PBS/0.5% BSA, and the plate was centrifuged at 1500 RPM for 5mins. The supernatant was decanted and 100µl of PBS/0.5% BSA was added. PE (eBioscience, UK) and APC isotype controls

(eBioscience, UK [for CD62L], Miltenyi Biotec, UK [for IFNAR2], R&D Systems [for IFNGR2]), and IFNAR1 (Sino Biological, China/Stratech, UK), IFNAR2 (Miltenyi Biotec, UK), IFNGR1 (eBioscience, UK) and IFNGR2 (R&D Systems, UK) were added to neutrophils at a final concentration of 1µg/ml (or ~ 1µg/ml for IFNAR2 and its APC isotype). Single and dual stain CD11b and CD62L were added to unstimulated and TNFα/IFN stimulated neutrophils at a final concentration of 1µg/ml. Neutrophils were incubated at 4°C for 30mins, and the plate was centrifuged at 1500 RPM for 5mins. The supernatant was decanted and 200µl PBS/0.5% BSA was added. The neutrophils were transferred to 1.5ml/2ml microcentrifuge tubes and run through Guava EasyCyte, using a pre-set programme.

5.4.2. IFN receptor and activation marker analysis on paediatric control and JSLE neutrophils

Neutrophils were isolated from healthy adults (Section 2.5.2). Neutrophils were plated at $1x10^5$ neutrophils per condition and stained (Section 5.4.1).

5.4.3. Analysis of phosphorylation of STAT1 and STAT3 within naïve and primed neutrophils

Neutrophils were isolated from healthy adults (Section 2.5.2). Neutrophils were seeded at $5x10^5$ –$1x10^6$ and left untreated or treated with 1ng/ml TNFα for 30mins 37°C, 5% CO_2. Neutrophils were stimulated 10ng/ml IFNα, 10ng/ml IFNγ or 10ng/ml IFNβ for 15mins at 37°C, 5% CO_2. Proteins were extracted and analysed for pSTAT1/β actin and pSTAT3/STAT3 expression ratios using Western blotting (Section 2.7).

5.4.4. STAT1 inhibition analysis

5.4.4.1. Fludarabine phosphate dose response

Fludarabine phosphate (Sigma-Aldrich, UK) was diluted at 1µg/µl in dimethyl sulfoxide (DMSO). Neutrophils were isolated from healthy adults as described in the technical methods chapter (section 2.5.2) and were seeded at $5x10^5$ in a 24 well plate. Neutrophils were either unstimulated or treated with 25µM fludarabine phosphate, 50µM fludarabine phosphate or 100µM fludarabine phosphate for 2hrs. Additional neutrophils were treated with an equivalent volume of DMSO (for 25µM fludarabine phosphate only) to check for any DMSO-induced STAT1 phosphorylation. Neutrophils were primed with 1µg/ml TNFα for 30mins at 37°C, 5% CO_2, and stimulated with 10µg/ml IFNα for 15mins at 37°C, 5% CO_2. Proteins were extracted and analysed for pSTAT1/β actin expression ratios using Western blotting (Section 2.7).

5.4.4.2. DMSO dose response

Since fludarabine phosphate (Section 5.4.4.1) was initially diluted at 1µg/µl DMSO, and thus as a control, the effect of DMSO on apoptosis was investigated alongside fludarabine phosphate dose response. Neutrophils were isolated from healthy adults (Section 2.5.2) and were seeded at $5x10^5$ in 24 well plates. Neutrophils were either unstimulated or treated with 4.5µl DMSO (equates to 25µM fludarabine phosphate), 9µl DMSO (equates to 50µM fludarabine phosphate) or 18µl DMSO (equates to 100µM fludarabine phosphate) for 2hrs at 37°C, 5% CO2. Apoptosis was analysed (Section 2.6.2.2), and results analysed using FlowJo.

5.4.4.3. Fludarabine phosphate time course

Fludarabine phosphate was diluted at 10µg/µl in DMSO then diluted further to 1µg/µl with media. Neutrophils were isolated from healthy adults (Section 2.5.2) and were seeded at $1x10^6$ in a 24 well plate. Neutrophils were untreated or treated with 50µM of fludarabine phosphate for 30, 60 and 120mins at 37°C, 5% CO_2. Neutrophils were stimulated with 10ng/ml IFNα and 10ng/ml IFNγ for 15mins at 37°C, 5% CO_2. Proteins were extracted and analysed for pSTAT1/β actin and pSTAT3/STAT3 expression ratios using Western blotting (Section 2.7).

5.4.4.4. The effect of S1495 on STAT1 phosphorylation in A549 cells (positive control)

S1459 (Abcam, UK) was reconstituted at 10µg/µl in DMSO, and then diluted further to 1µg/µl with media A549 cells were grown to confluency in T75 flask in high glucose Dulbecco's modified eagle's medium (DMEM) (Sigma) with 10% heat-inactivated FBS (Life Technologies, UK), and 0.1% of gentamicin (Sigma) (known so forth as 'DMEM'). DMEM was removed and A549 cells washed with 5ml PBS and were lifted using 1ml trypsin (Sigma). 4ml DMEM was added to neutralise the trypsin. The cells were centrifuged at 2000 RPM for 5mins. The supernatant was removed and cells were re-suspended in 5ml of DMEM. 1ml of A549 cells was added to two wells in a 24 well plate, and 500µl PBS was added to the surrounding wells. Cells were left to settle for up to 6hrs. DMEM was removed from wells and cells were washed with 1ml PBS. Add 1ml of serum-free DMEM with 0.1% gentamicin and 10µl of S1495 (final concentration 10µg/ml). The cells were incubated for 18hrs and then stimulated with 10ng/ml IFNα and 10ng/ml IFNγ for 15mins. The cells were lysed by removing the DMEM, washing with 1ml PBS and adding 100µl lysis buffer. Wells were scraped with a tip, and supernatants containing proteins were transferred to wells, and heated/vortexed as per protocol (Section 2.7.1). Proteins were analysed for pSTAT1/β actin and pSTAT3/STAT3 expression ratios using Western blotting (Section 2.7).

5.4.4.5. The effect of S1495 on STAT1 and STAT3 phosphorylation in neutrophils

Neutrophils were isolated from healthy adults (Section 2.5.2). Neutrophils were seeded at $5x10^5$ - $1x10^6$ in a 24 well plate and treated with either 5µg/ml or 10µg/ml S14-95 for 1hr at 37°C, 5% CO_2. Neutrophils were naïve (10µg/ml S1495 investigation) or primed with 1µg/ml TNFα for 30mins at 37°C, 5% CO_2 (5µg/ml S1495 investigation) and subsequently stimulated with 10ng/ml IFNα, 10ng/ml IFNγ or 10ng/ml of IFNβ individually (10µg/ml S1495 investigation), or 10ng/ml IFNα and 10ng/ml IFNγ combined (10µg/ml S1495 investigation) for 15mins at 37°C, 5% CO_2. Proteins were extracted and analysed for pSTAT1/β actin and pSTAT3/STAT3 expression ratios using Western blotting (Section 2.7).

5.4.4.6. S14-95 dose response on naïve and primed neutrophil, and priming trial

Neutrophils were isolated from healthy adults (Section 2.5.2). Neutrophils were seeded at $1x10^5$ in a 96 well plate. Neutrophils were either left naïve or primed with 1µg/ml TNFα for 30mins, and subsequently treated with a dose response 1, 5 or 10µg/ml S1495, or one dose of 10µg/ml S1495 alone, and incubated for 6hrs at 37°C, 5% CO_2. Apoptosis was analysed (Section 2.6.2.2) and results analysed on FlowJo.

5.4.4.7. S1495 effect on IFN apoptosis on TNFα-primed neutrophils

Neutrophils were isolated from healthy adults (Section 2.5.2). Neutrophils were seeded at $1x10^5$ in a 96 well plate. Neutrophils were either unstimulated or treated with 5µg/ml or 10µg/ml S1495 for 1hr at 37°C, 5% CO_2. Neutrophils were also primed with 1µg/ml TNFα 30mins at 37°C, 5% CO2, and subsequently stimulated with 10ng/ml IFNα, 10ng/ml IFNγ and 10ng/ml IFNβ for 6hrs at 37°C, 5% CO_2. Apoptosis was analysed (Section 2.6.2.2) and results analysed on FlowJo.

5.4.5. Statistical analysis

P values were analysed in GraphPad Prism 6. Friedman's test with Dunn's *post hoc* multiple comparison tests was conducted for analysis of the majority of data sets. One-tailed Wilcoxon matched-pairs signed rank test was used for analysis of activation marker expression. Kruskal-Wallis test with a *post hoc* Dunn's multiple comparison test was conducted for analysis of comparing multiple comparisons between JSLE and paediatric control patient data sets. For detailed statistical methodology, Section 2.10.

5.5. Results

5.5.1. IFN receptor expression on naïve and TNFα-primed adult neutrophils

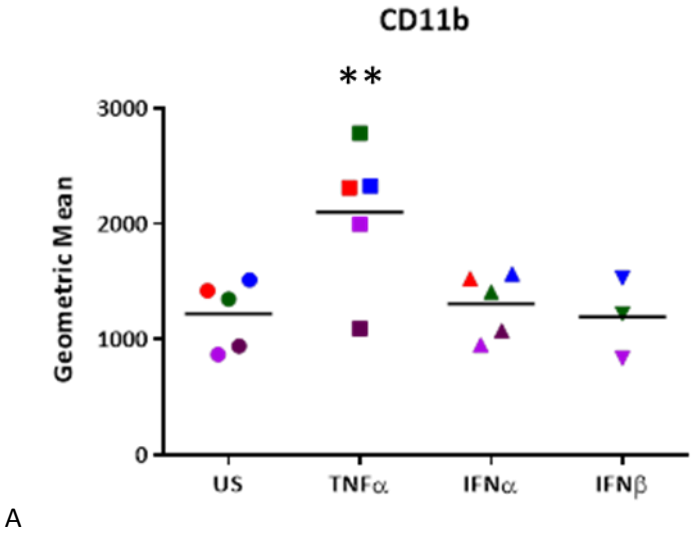
Each IFN subtype binds to its own receptor; IFNAR (type 1) and IFNGR (type 2). Each receptor is made up of a chain 1 (α chain) and chain 2 (β chain). Published data suggest that differential expression of these chains is commonly found between cell types, and the effect of the IFN signalling is dependent on the expression of the chains (159, 161, 162) (Sections 1 5.2 and 5.1). It has also been established that signalling pathways differ not only between naïve and primed neutrophils, but between neutrophils from JSLE patients and paediatric control patients (84, 85, 170) (Sections 1.3.5 and 5.1). Therefore, receptor chains may also differ between naïve and primed neutrophils, and paediatric control and JSLE neutrophils. Thus, the first aim was to investigate how naïve and primed adult healthy neutrophils differ in their IFN receptor expressions.

5.5.1.1. TNFα, but not IFNα or IFNβ, primes healthy adult control neutrophils for activation as measured by CD11b and CD62L expression

Neutrophils were stimulated with 1μg/ml TNFα, 1μg/ml IFNα or 1μg/ml IFNβ for 30mins and measured for CD11b and CD62L. TNFα activated the neutrophils, as shown through the increase in CD11b (2102 ± 282 vs 1218±131, n=5, p=0.0031) and decrease in CD62L (88 ± 16 vs 387 ± 55, n=5, p=0.023) compared to unstimulated neutrophils (Figure 5.1). However, IFNα and IFNβ had no overall effect on the expression of either CD11b (IFNα = 1305 ± 124, n=5; IFNβ = 1195 ± 201, n=3) or CD62L (IFNα = 389 ± 52, n=5; IFNβ = 382 ± 89, n=3) and thus did not demonstrate activation these neutrophils (Figure 5.1).

5.5.1.2. TNFα, but not IFNγ, primes healthy adult control neutrophils for activation as measured by CD11b and CD62L expression

Neutrophils were stimulated with 1μg/ml TNFα or 1μg/ml IFNγ for 30mins and measured for CD11b and CD62L. Again, TNFα-primed the neutrophils, as shown through the increase in CD11b (2227 ± 310 vs 1242 ± 107, n=5, p=0.0031) and decrease in CD62L (68 ± 6 vs 375 ± 51, n=5, p=0.0089) compared to unstimulated neutrophils (Figure 5.2). However, IFNγ did not have an overall effect on expression of either CD11b (1325 ± 120, n=5) or CD62L (368 ± 50, n=5) compared to unstimulated neutrophils, and thus did not prime these neutrophils for activation (Figure 5.2).



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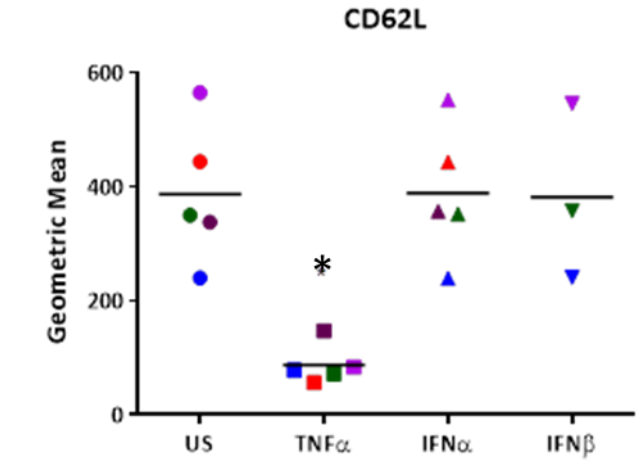
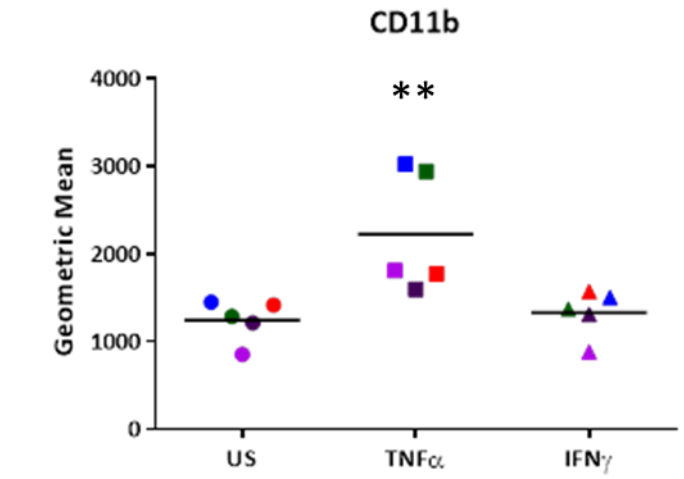
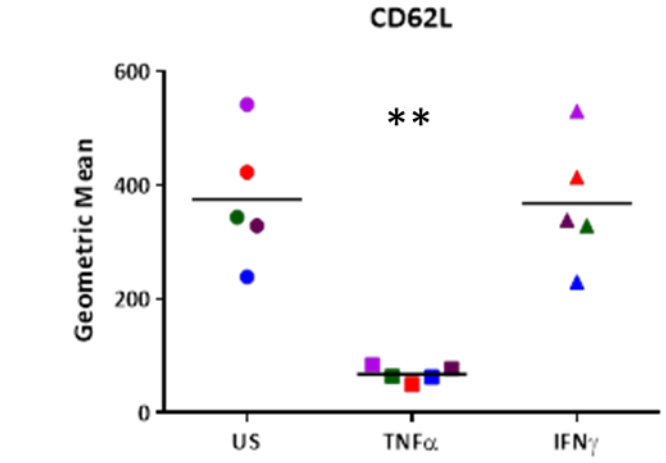


B

Figure 5.1. CD11b and CD62L expression analysis on naïve and 1µg/ml cytokine-primed neutrophils at 30mins. Neutrophils were isolated from healthy adult donors and were left naïve or were primed with 1µg/ml TNFα, 1µg/ml IFNα or 1µg/ml IFNβ for 30mins. Neutrophils were stained with antibodies for CD11b (A) and CD62L (B), and the relative expression via geometric mean was measured using flow cytometry. TNFα significantly primed neutrophils for activation as shown by the increase in CD11b (A) (**, n=5, p=0.0031) and decrease in CD62L (B) (*, n=5, p=0.023). IFNα and IFNβ had no overall effect on the expression of CD11b or CD62L (n=3-5).



A



B

Figure 5.2. CD11b and CD62L expression analysis on naïve and 1µg/ml cytokine-primed neutrophils at 30mins. Neutrophils were isolated from healthy adult donors and were left naïve or were primed with 1µg/ml TNFα or 1µg/ml IFNγ for 30mins. Neutrophils were stained with CD11b and CD62L antibodies, and the relative expression via geometric mean was measured using flow cytometry. TNFα significantly primed the neutrophils for activation as shown by the increase in CD11b (A) (**, n=5, p=0.0031) and decrease in CD62L (**, n=5, p=0.0089). IFNγ had no overall effect on the expression of CD11b or CD62L (n=5).

5.5.1.3. Expression of IFNAR chains 1 and 2 on naïve and primed neutrophils.

Receptor chains IFNAR1 and IFNAR2 were measured using flow cytometry after 30mins stimulation with 1µg/ml TNFα, 1µg/ml IFNα or1µg/ml IFNβ. TNFα decreased the expression of IFNAR1 (54 ± 11 vs 133 ± 37, n=5, p=0.014) compared to unstimulated neutrophils, but had no overall effect on IFNAR2 (59 ± 8 vs 58±10, n=5) (Figure 5.3). Neither IFNα (IFNAR1 = 125 ± 35; IFNAR2 60 ± 10; n=4-5) nor IFNβ (IFNAR1 = 117 ± 52; IFNAR2 54 ± 6; n=4-5) have an effect on either chain (Figure 5.3).

5.5.1.4. Expression of IFNGR chains 1 and 2 on naïve and primed neutrophils

Receptor chains IFNGR1 and IFNGR2 were measured after 30 min stimulation with 1µg/ml TNFα or 1µg/ml IFNγ. TNFα increased the expression of IFNGR2 (58 ± 7 vs 47 ± 3, n=5) compared to unstimulated neutrophils, but this was not significant. However, TNFα also had no overall effect on IFNGR1 (40 ± 4 vs 41 ± 2, n=5) (Figure 5.4). There was an increase in IFNGR2 upon IFNγ stimulation (59 ± 10 vs 47 ± 3, n=5) compared to unstimulated neutrophils, but this was not significant, and IFNγ had no overall effect on IFNGR1 (39 ± 4, n=5) (Figure 5.4).

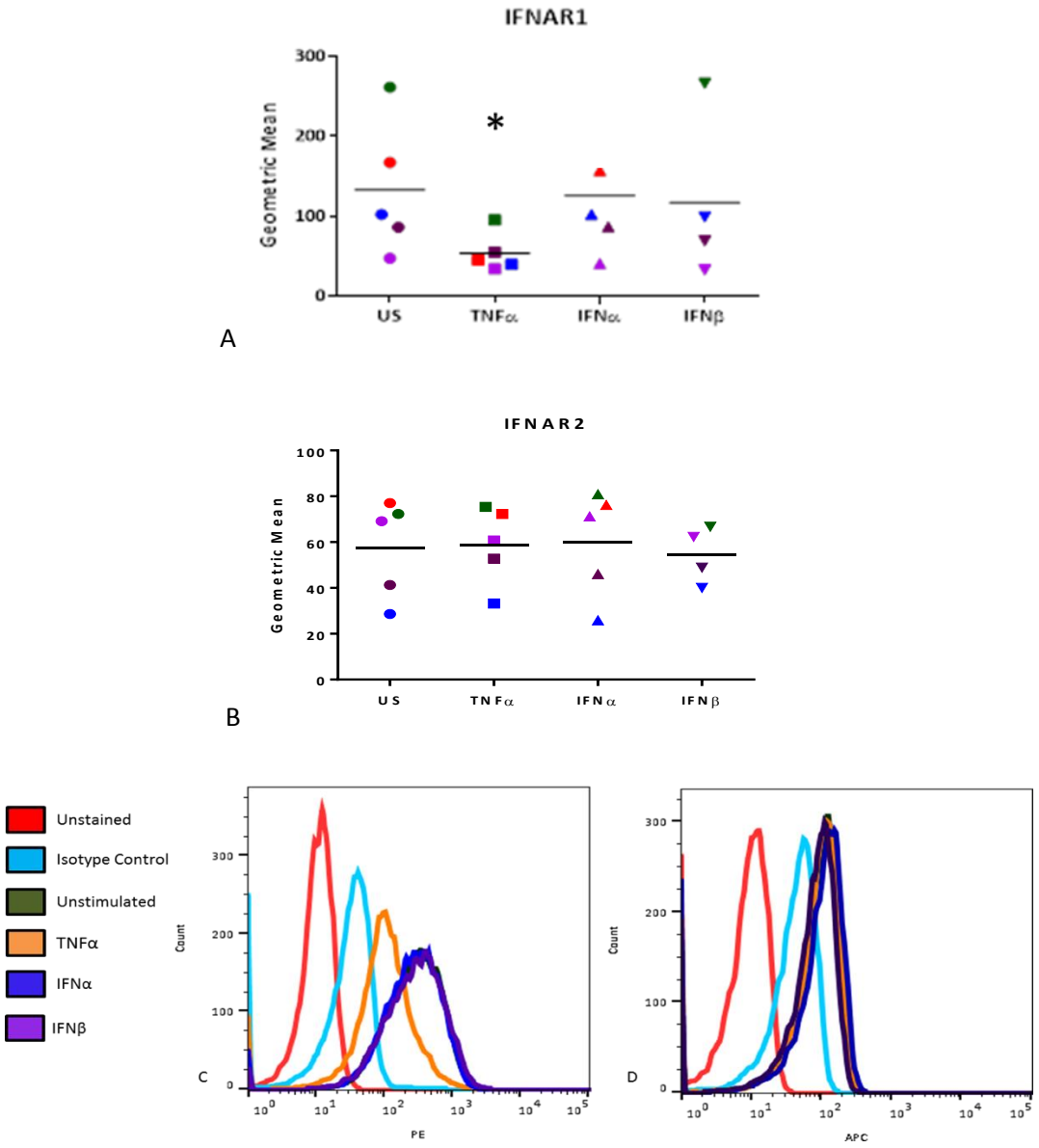


Figure 5.3. IFNα receptor chain analysis on naïve and 1µg/ml cytokine-primed neutrophils at 30mins. Neutrophils were isolated from healthy adult donors and were left naïve or were primed with 1µg/ml TNFα, 1µg/ml IFNα or 1µg/ml IFNβ for 30mins. Neutrophils were stained with IFNAR1 and IFNAR2 antibodies, and the relative expression via geometric mean was measured using flow cytometry for IFNAR1 (A+C) and 2 (B+D). TNFα reduced the expression of IFNAR1 (*, n=5, p=0.014) (A+C), but there was little effect of any of the cytokines on IFNAR2 (n=4-5) (B+D). Representative peaks are shown, where unstained is red, isotype controls are light blue, unstimulated neutrophils are dark green, TNFα stimulated neutrophils are orange, IFNα stimulated neutrophils are dark blue and IFNβ stimulated neutrophils are purple (C+D).

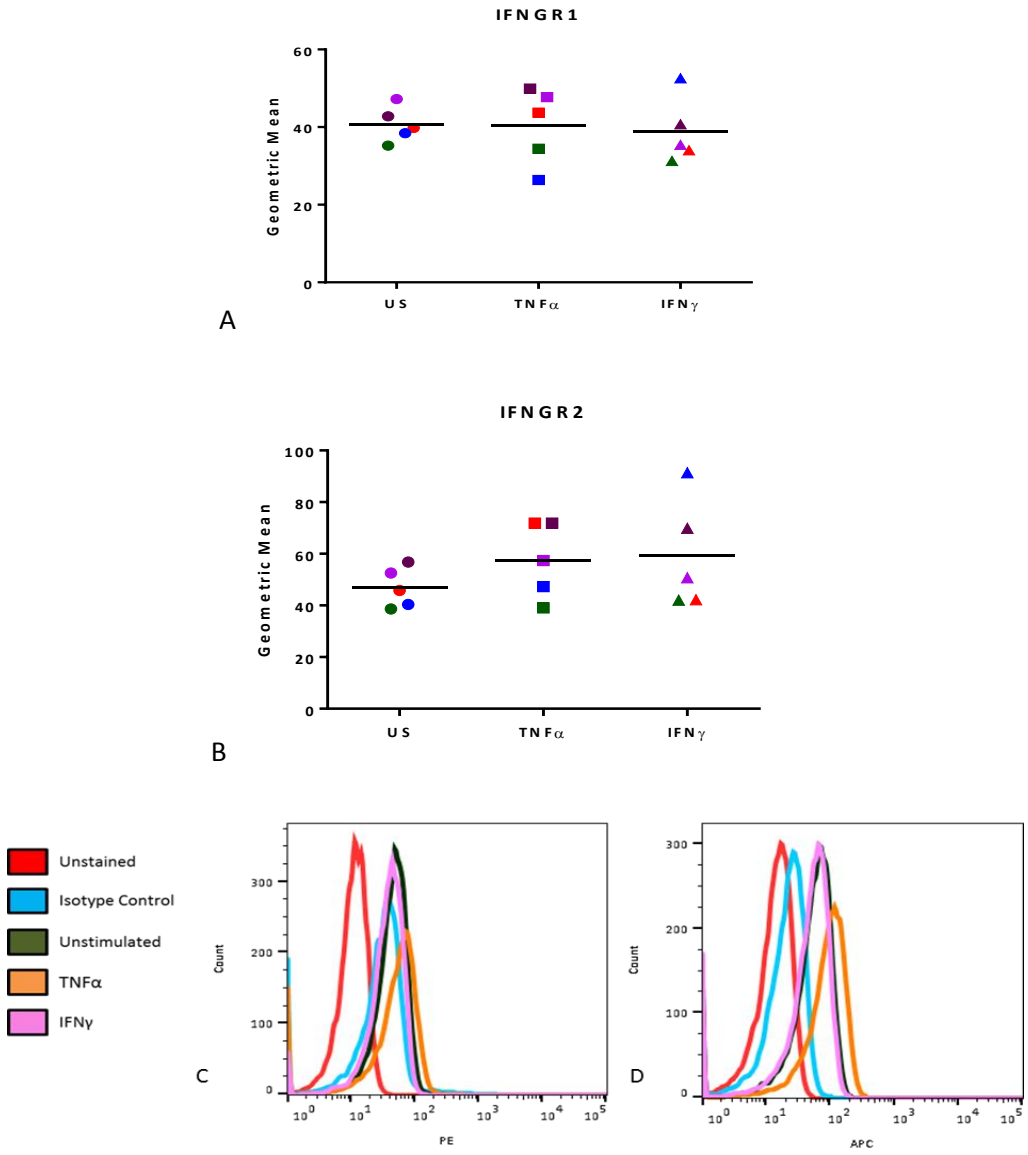


Figure 5.4. IFNγ receptor chain analysis on naïve and 1µg/ml cytokine-primed neutrophils at 30mins. Neutrophils were isolated from healthy adult donors and were left naïve or were primed with 1µg/ml TNFα or 1µg/ml IFNγ for 30 mins. Neutrophils were stained with IFNGR1 and IFNGR2 antibodies and the relative expression via geometric mean was measured using flow cytometry for IFNGR1 (A+C) and 2 (B+D). There is suggestion that TNFα and IFNγ increased the expression of IFNGR2 (n=5) (B+D), but there was little effect of any of the cytokines on IFNGR1 (n=5) (A+C). Representative peaks are shown, where unstained is red, isotype controls are light blue, unstimulated neutrophils are dark green, TNFα stimulated neutrophils are orange, IFNγ stimulated neutrophils are pink (C+D).

5.5.2. IFN receptor expression and activation states in neutrophils from JSLE patients and paediatric control patients

Based on data presented in Section 5.5.1, the next step was to investigate whether there was a difference in the activation state between neutrophils from JSLE patients and paediatric control patients. Additionally, to determine whether differences in activation states influences the expression of the receptor chains. This hypothesis stems from literature that suggests SLE neutrophils are activated compared to control neutrophils, which may influence IFN receptor expression; the activation of neutrophils and any IFN receptor changes may link to the increase in apoptosis reported in published studies (72, 83, 161). (Sections 1.4.3, and 1.5.2).

JSLE neutrophils had a higher expression of CD11b compared to paediatric control neutrophils (1260 ± 150, n=5, vs 814 ± 129, n=6, p=0.052), although JSLE CD62L was comparable to that of paediatric control neutrophils (312 ± 37, n=5, vs 380 ± 66, n=6) (Figure 5.5). This indicates that the JSLE neutrophils are activated compared to that of paediatric controls (as shown by the increase in CD11b), but not quite as activated as TNFα-primed healthy adult neutrophils (as shown by the expression of CD62L) (Figures 5.1, 5.2, and 5.5 respectively). CD11b is an integrin that forms part of differentiation antigen Mac-1, which is involved in various functions such as endothelium attachment, chemotaxis and phagocytosis whereas CD62L, or L-Selectin is an adhesion molecule and mediates leukocyte rolling (97, 101). Thus, TNFα-primed healthy adult neutrophils may be increasing their membrane antigens to respond to the surrounding environment, whilst losing their adhesive and rolling ability. In contrast, JSLE neutrophils may enhance their responsiveness to the environment through up-regulation of CD11b but retain their adhesive and rolling ability through CD62L.

Generally, there were no significant difference between expression of the different IFN receptors in neutrophils from JSLE patients and paediatric control patients. IFNAR1 and IFNAR2 expressions were slightly higher in JSLE neutrophils compared to paediatric control neutrophils (IFNAR1 = 140 ± 41, n=5, vs 123 ± 23, n=6; IFNAR2 = 89 ± 16, n=5, vs 69 ± 4, n=6) (Figure 5.6). However, these differences were not statistically significant. IFNGR1 expression was similar in JSLE and paediatric control neutrophils (43 ± 2, n=5, vs 44 ± 3, n=6) (Figure 5.7). Again, there was a slight increase of IFNGR2 in JSLE neutrophils compared to paediatric control neutrophils, although this was not statistically significant (60 ± 4, n=5, vs 52 ± 5, n=6) (Figure 5.7). These observations, namely that there was no significant difference between the JSLE and paediatric control neutrophils, can be considered in light of the clinical characteristics of the patients at the time of sampling (see Table 5.1). The JSLE patients'

disease activity status was in a state of relatively low current disease activity at the time of sampling, as evidenced by the low medium pBILAG score. This would be most likely due to the effective clinical management of their condition. The results may be affected by the medication that all the JSLE patients were on (Table 5.1).

Table 5.1. Clinical and demographic information of individuals providing blood for neutrophil activation marker and IFN receptor chain experimental analysis.

Variables	JSLE (n=5)	Control (n=6)
Age (years)[a]	17.7 [14.5-18.9]	13.9 [8.2-15.4]
Duration of disease (months)[a]	40 [22-101]	-
Female[b]	5 (100)	2 (33.3)
Total pBILAG score[c]	3 [0-5]	-
Ethnicity		
White British	5 (100)	5 (100)
Family history		
Autoimmune diseases	2	1
Other	2	1
None	2	3
Medication[d]		
Steroids	2	-
DMARDs	5	-
Hydroxychloroquine	4	-
Mycophenolate	5	-
C3 (g/L)	1.1 [1.05-1.47]	-
C4 (g/L)	0.19 [0.07-0.24]	-
ESR (mm/h)	4 [3-12]	-

Data are expressed as median and range [square brackets] or as total numbers with percentages (round brackets)

pBILAG. Paediatric British Isles Lupus Assessment Group; C3, complement component 3; C4, complement component 4; DMARDs, disease-modifying anti-rheumatic drugs; ESR, erythrocyte sedimentation rate

[a]Age and disease duration time of neutrophil isolation and analysis

[b]Gender of patients

[c]Total pBILAG score determined by the numeric scoring of disease classifications of the organ domains

[d]Medication taken at time of serum sample collection

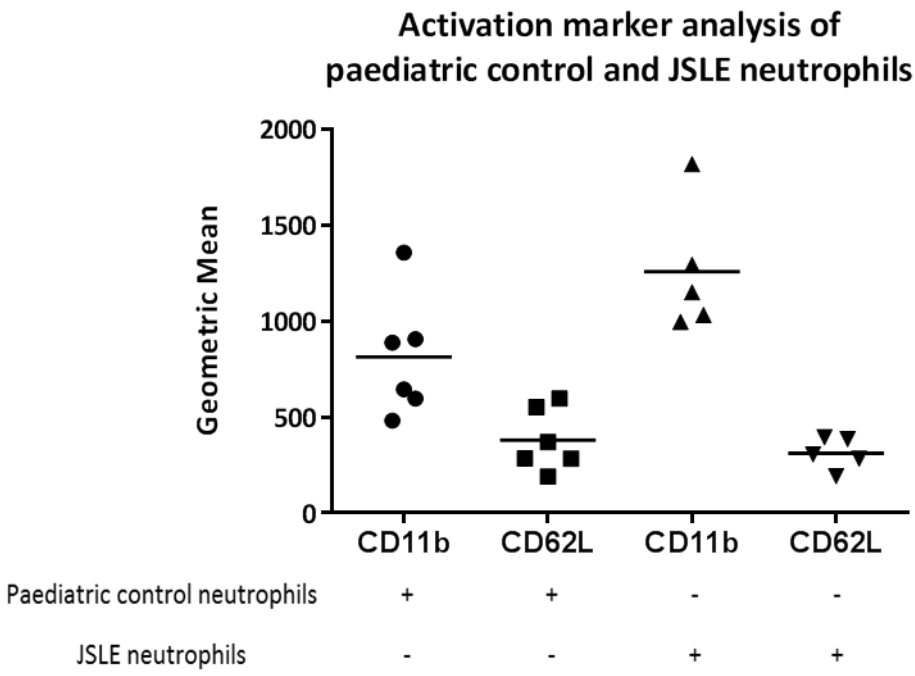


Figure 5.5. CD11b and CD62L expression analysis of neutrophils isolated from paediatric control patients and JSLE patients. Neutrophils were isolated from paediatric control patients and JSLE patients, were stained with CD11b and CD62L and the relative expression via geometric mean was measured using flow cytometry. There was an increase in CD11b in JSLE neutrophils compared to paediatric control neutrophils (n=5-6, p=0.052).

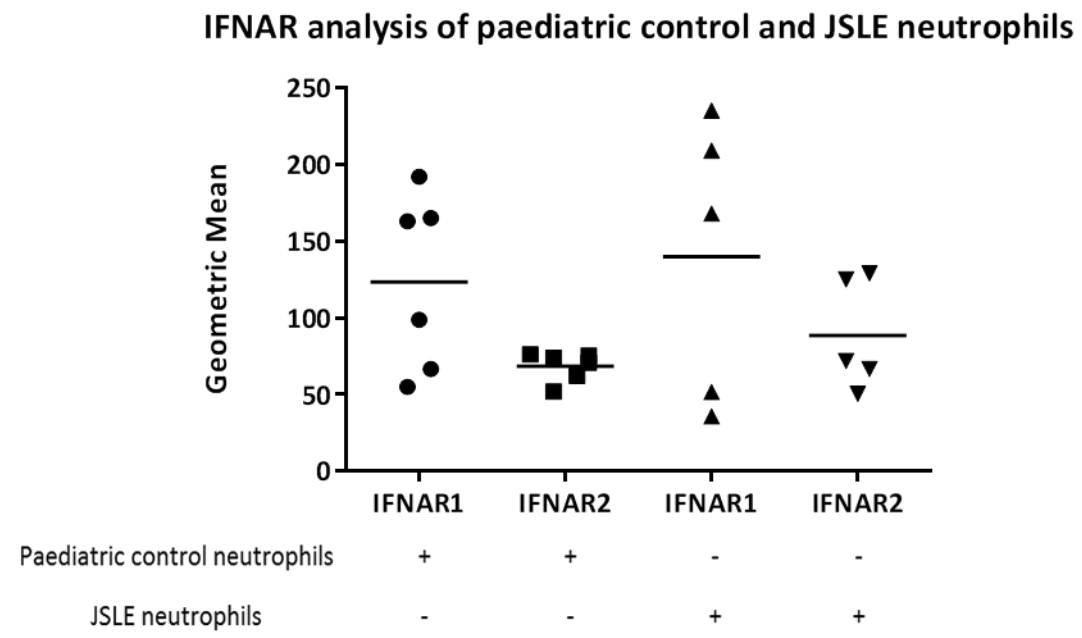


Figure 5.6. IFNα receptor chain expression analysis on neutrophils from paediatric control patients and JSLE patients. Neutrophils were isolated form paediatric control patients and JSLE patients, were stained with IFNAR1 and IFNAR2 and the relative expression via geometric mean was measured using flow cytometry (n=5-6).

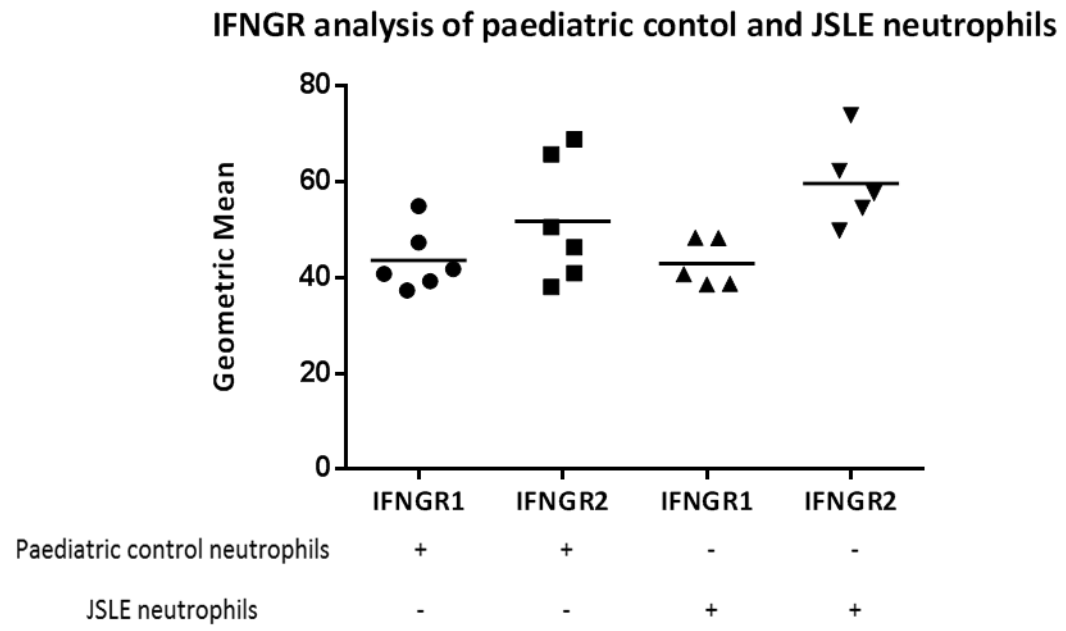


Figure 5.7. IFNγ receptor chain expression analysis on neutrophils from paediatric control patients and JSLE patients. Neutrophils were isolated form paediatric control samples and JSLE samples and were stained with IFNAR1 and IFNAR2 the relative expression via geometric mean was measured using flow cytometry (n=5-6).

5.5.3. Phosphorylation of STAT1 and STAT3 in naïve and TNFα-primed neutrophils

The next step was to investigate the activation of the proteins immediately downstream from IFN receptors. This is important to investigate in the context of data observed and previously summarised regarding the differential effect of IFN stimulation on apoptosis. STAT1 has been shown to be pro-apoptotic and STAT3 has been shown to be anti-apoptotic (138, 141) (Section 1.5.3.1). Thus, the phosphorylation of STAT1 and STAT3 was measured in naïve and primed neutrophils.

Neutrophils were primed with 1ng/ml TNFα for 30mins, and then stimulated with 10ng/ml IFNα, 10ng/ml IFNγ, or 10ng/ml IFNβ for 15mins. Protein was extracted, and pSTAT1/β actin and pSTAT3/STAT3 expression ratios were measured using Western blotting.

It was shown that both pSTAT1 and pSTAT3 were expressed upon all IFN subtypes (Figure 5.8, 5.9), although this effect was not always statistically significant. IFNβ significantly increased the pSTAT3/STAT3 ratio in naïve neutrophils compared to unstimulated naïve neutrophils (15.6 ± 10.2 vs 0, n=5, p=0.0018) (Figure 5.8). IFNγ significantly increased pSTAT1/β actin ratio in TNFα-primed neutrophils compared to unstimulated naïve and primed neutrophils (4.4 ± 2.5, vs 0, n=5, p=0.0014) (Figure 5.9). Importantly, TNFα alone did not cause any phosphorylated STAT expression, and thus any differences in phosphorylation state between naïve and primed neutrophils were due to activation state and not a compounding cytokine effect (Figure 5.8, Figure 5.9).

There was a reduction in pSTAT3/STAT3 expression ratios in primed neutrophils compared to naïve neutrophils (IFNα = 1.1 ± 0.3 vs 3.3 ± 1.2; IFNγ 0.4 ± 0.1 vs 3.6 ± 2.7; IFNβ 3.1 ± 1.2 vs 15.6 ± 10.2; n=5) (Figure 5.8). This indicates a reduction in the ability of IFNs to phosphorylate STAT3, and thus may reduce the IFN anti-apoptotic effect in primed neutrophils. There was an increase in pSTAT1/β actin expression ratio in primed neutrophils compared to naïve neutrophils in the presence of IFNγ (4.4 ± 2.5 vs 1.4 ± 0.5, n=5) (Figure 5.9). This effect was not seen with IFNα (1.1 ± 0.4 vs 1.2 ± 0.4, n=5) or IFNβ (1.6 ± 0.7 vs 1.5 ± 0.6, n=5) (Figure 5.9). This indicates that, although there seems to switch to increasing pSTAT1/β actin expression ratio (which may be due to either increased phosphorylation of STAT1 or increased expression of STAT1) from pSTAT3/STAT3 expression ratio, suggesting a switch from the anti-apoptotic to pro-apoptotic signalling pathway, it is dependent on the presence of IFNγ.

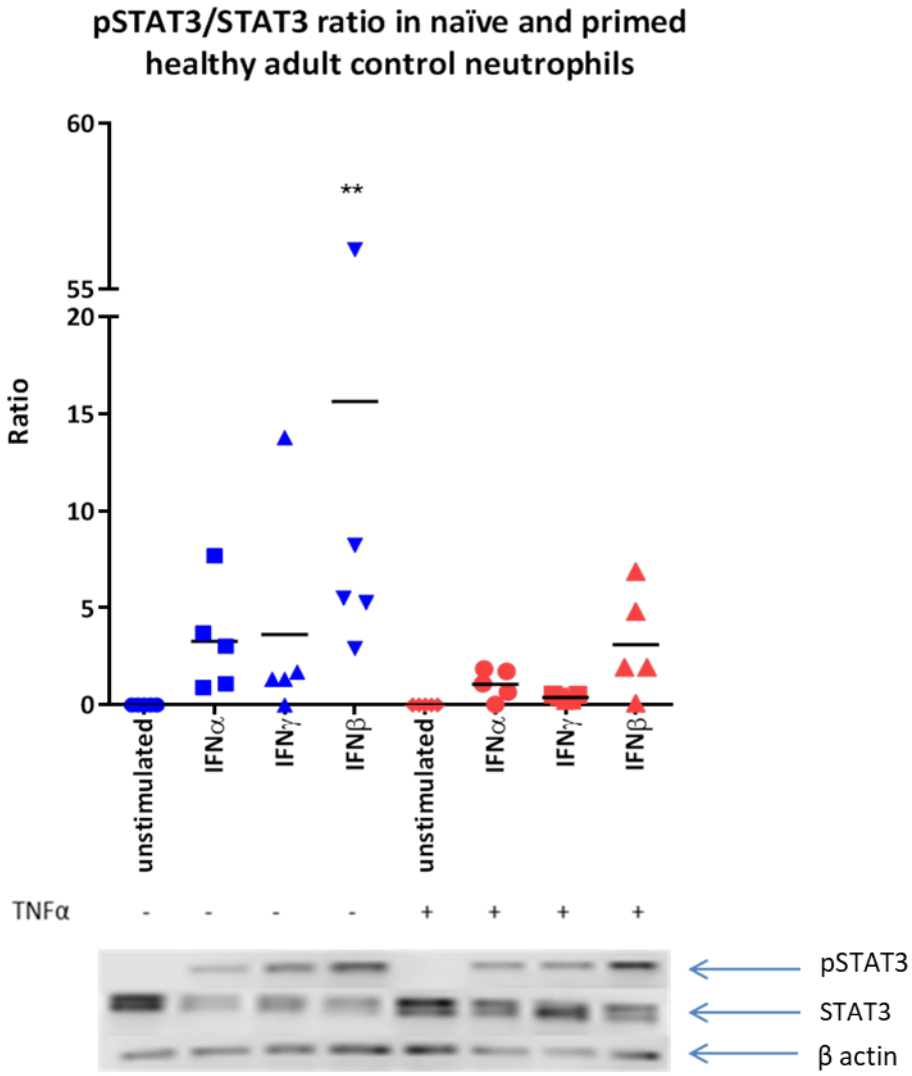


Figure 5.8. The phosphorylation of STAT3 in naïve and TNFα-primed healthy adult neutrophils, stimulated with 10ng/ml IFNα, 10ng/ml IFNγ or 10ng/ml IFNβ for 15mins. Neutrophils were isolated from healthy adult donors and were left naïve (blue) or were primed 1ng/ml TNFα (red) for 30mins. Neutrophils were subsequently stimulated with 10ng/ml IFNα, 10ng/ml IFNγ or 10ng/ml IFNβ. Proteins were extracted, and pSTAT3/STAT3 expression ratios were measured using Western blotting. All IFN subtypes phosphorylated STAT3. However, increased pSTAT3/STAT3 expression ratios correlated more with IFNα and IFNβ (whereby IFNβ significantly increased the pSTAT3/STAT3 expression ratio in naïve neutrophils compared to unstimulated naïve neutrophils; **, n=5, p=0.0018). Priming of the neutrophils with TNFα reduced the pSTAT3/STAT3 in all IFNα and IFNβ-stimulated neutrophils, compared to naïve neutrophils stimulated with IFNα and IFNβ (n=5).

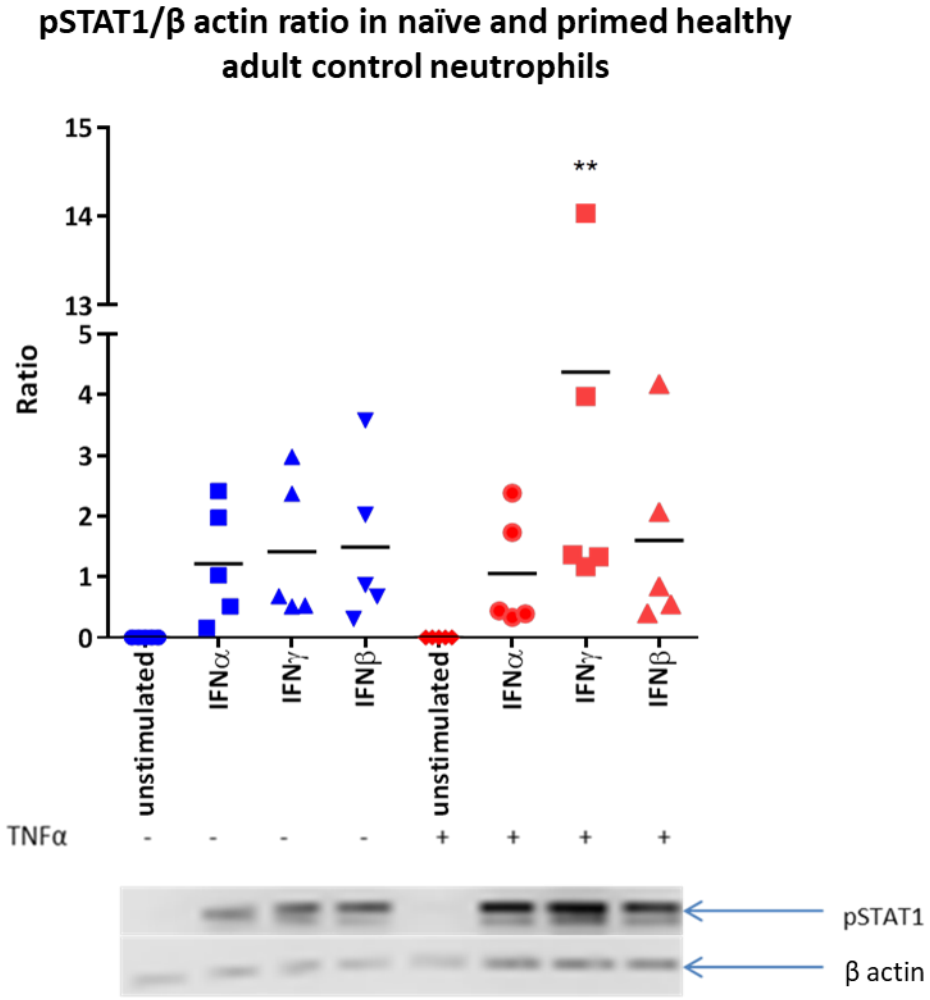


Figure 5.9. The phosphorylation of STAT1 in naïve and TNFα-primed healthy adult neutrophils, stimulated with 10ng/ml IFNα, 10ng/ml IFNγ or 10ng/ml IFNβ for 15mins. Neutrophils were isolated from healthy adult donors and left naïve (blue) or were primed 1ng/ml TNFα (red) for 30mins. Neutrophils were subsequently stimulated with 10ng/ml IFNα, 10ng/ml IFNγ or 10ng/ml IFNβ. Proteins were extracted, and pSTAT1/β actin expression ratios were measured using Western blotting. All IFN subtypes increased the pSTAT1/β actin expression ratio (n=5). However, the pSTAT1/β actin expression ratio increase correlated more with IFNγ. IFNγ significantly increased the pSTAT1/β actin expression ratio in TNFα-primed neutrophils, compared to naïve and TNFα-primed neutrophils without IFN stimulation (**, n=5, p=0.0014). This may be due to either increased phosphorylation of STAT1, or increased expression of STAT1. It is of note that the STAT1 antibody was not able to be optimised in this thesis, and therefore the STAT1 expression was not analysed.

5.5.4. Investigating the ability of fludarabine phosphate to inhibit STAT1

STAT1 has a pro-apoptotic role, and its increase in primed neutrophils, (as shown in Figure 5.9) may be important in IFN-related neutrophil apoptosis (Section 1.5.3.1). It may therefore be a potential therapeutic target in JSLE neutrophil apoptosis (138, 194). Therefore, the next specific objective was to investigate if STAT1 could be inhibited by specific inhibitors in neutrophils.

5.5.4.1 Fludarabine phosphate dose and time course

The first STAT1 inhibitor used was fludarabine phosphate. The initial experiments were to determine an appropriate concentration of fludarabine phosphate to use to inhibit the phosphorylation of STAT1. It is of note the fludarabine phosphate was dissolved at 1µg/µl in DMSO.

At 2hrs, it was shown that neither TNFα, DMSO, nor fludarabine phosphate at any concentration tested induced any phosphorylation of STAT1. Only IFNα resulted in an increase in the pSTAT1/β actin expression ratio. This indicates that IFNα is needed to activate this pathway (Figure 5.10). It was shown that TNFα-priming increased pSTAT1/β actin expression ratio in neutrophils stimulated with IFNα compared to naïve neutrophils stimulated with IFNα (1.27 vs 0.65, n=1) (Figure 5.10). This confirms that in this assay, as shown previously in the data arising from experiments presented above (see Figure 5.9), IFNα is required to induce the JAK/STAT pathway. At 50µM, fludarabine phosphate reduced the amount of pSTAT1 in IFNα-stimulated, TNFα-primed neutrophils the most compared to IFNα-stimulated, TNFα-primed neutrophils only. This indicates that this was the most appropriate concentration to use for subsequent experiments (no inhibitor = 1.27, 25µM = 0.99, 50µM = 0.57, 100µM = 0.63, n=1) (Figure 5.10).

As fludarabine phosphate was initially diluted in DMSO at 1µg/µl, it was important to test if there was any potential effect of DMSO on neutrophil apoptosis directly. Thus, neutrophils were incubated with equivalent volumes of DMSO to those used in the dose response of fludarabine phosphate for 2hrs (4.5µl DMSO in 500µl of neutrophils equates to 25µM of fludarabine phosphate, 9µl DMSO in 500µl of neutrophils equates to 50µM of fludarabine phosphate, and 18µl DMSO in 500µl of neutrophils equates to 100µM of fludarabine phosphate). The level of apoptosis was then measured. It was shown that all volumes of DMSO induced an increase in apoptosis compared to unstimulated neutrophils (4.5µl DMSO = 8.4 ± 1.1%, 9µl DMSO = 13.7 ± 2.8%, 18µl DMSO = 21.5 ± 4.6%, unstimulated = 5.3 ± 0.9%, n=5) with 18µg/ml becoming significant (p=0.0007) (Figure 5.11). Thus, suspending

fludarabine phosphate in less DMSO was required to inhibit STAT1 phosphorylation without DMSO-affected apoptosis.

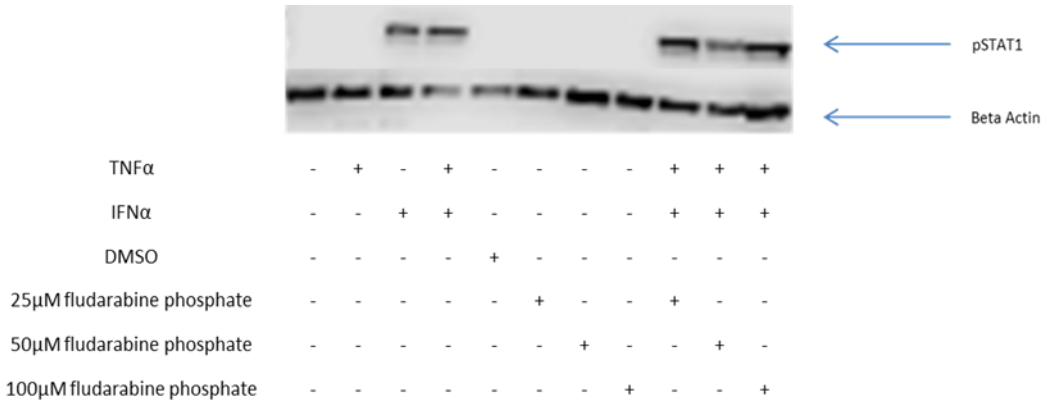


Figure 5.10. Dose response of fludarabine phosphate at 2hrs on TNFα-primed healthy adult neutrophils, stimulated with 10μg/ml of IFNα for 15mins. Neutrophils were isolated from healthy adult donors and were left unstimulated or treated with 25μM, 50μM or 100μM of fludarabine phosphate, or 25μM equivalent (v/v) of DMSO for 2hrs. Neutrophils were subsequently primed with 1μg/ml TNFα for 30mins and stimulated with 10μg/ml IFNα for 15mins. Protein was extracted and pSTAT1/β actin expression ratios was measured using western blotting. TNFα, DMSO, and fludarabine phosphate alone treatments did not induce any pSTAT1 expression. 50μM of fludarabine phosphate reduced the pSTAT1/β actin expression ratio in TNFα primed, IFNα treated neutrophils the most compared to the TNFα primed, IFNα treated neutrophil control, and subsequent experiments used this concentration (n=1).

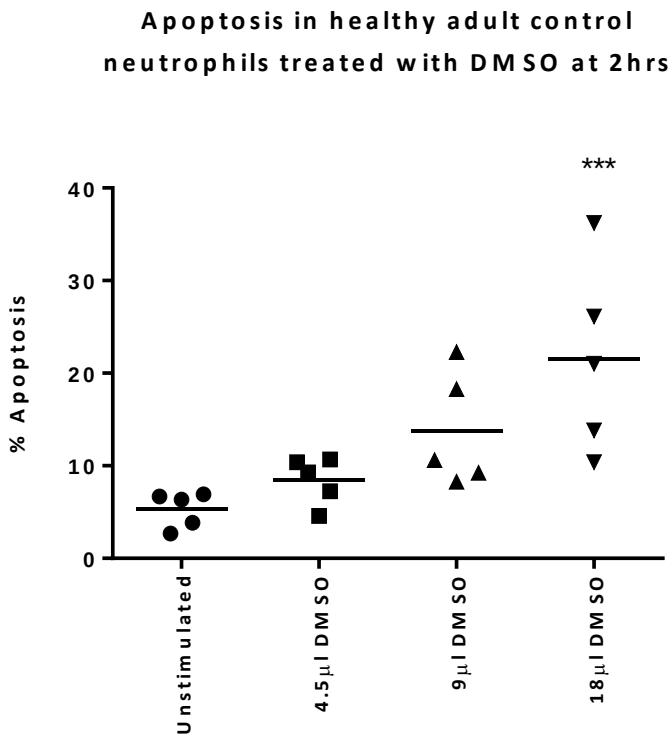


Figure 5.11. Apoptosis analysis of healthy adult neutrophils following a 2hr dose response of DMSO. Neutrophils were isolated from healthy adult donors and were left unstimulated or treated with equivalent v/v of DMSO to fludarabine phosphate dose response used in Figure 5.10. Specifically, 4.5µl corresponds to 25µM of fludarabine phosphate, 9µl corresponds to 50µM of fludarabine phosphate, and 18µl corresponds to 100µM of fludarabine phosphate originally constituted at 1µg/µl. Neutrophils were incubated for 2hrs, and apoptosis was measured using annexin V FITC and PI. All volumes of DMSO induced apoptosis with 18µl DMSO being significant (***, n=5, p=0.0007) and thus fludarabine phosphate needs to be re-constituted in smaller amounts of DMSO.

The next stage in optimisation was to do a time course using 50μM of fludarabine phosphate. Fludarabine phosphate was re-suspended initially at 10μg/μl in DMSO, then diluted more so to 1μg/μl using media. This enabled dilution of the fludarabine phosphate at lower concentrations of DMSO which would not induce neutrophil apoptosis.

Neutrophils were treated with 50μM of fludarabine phosphate for 30, 60 and 120mins, and subsequently stimulated with 10ng/ml IFNα and 10ng/ml IFNγ for 15mins. There was some indication of possible pSTAT1 inhibition through reduction of pSTAT1/β actin expression ratio at all time points by fludarabine phosphate when compared to neutrophils without fludarabine phosphate as fold change baseline (30mins = 0.9 ± 2 of baseline; 60mins = 0.9 ± 0.1 of baseline; 120mins = 0.8 ± 0.2 of baseline; n=5) (Figure 5.12). There was also an indication of inhibition of pSTAT3 through the reduction of pSTAT3/STAT3 expression ratio, which was seen in particular at 120mins (30mins = 0.9 ± 0.2 of baseline; 60mins = 1.1 ± 0.3 of baseline, 120mins = 0.7 ± 0.1; n=5) (Figure 5.13).

Importantly, this indication of a dual inhibition of both STAT1 and STAT3 indicated that fludarabine phosphate is inappropriate to use as a STAT1 inhibitor.

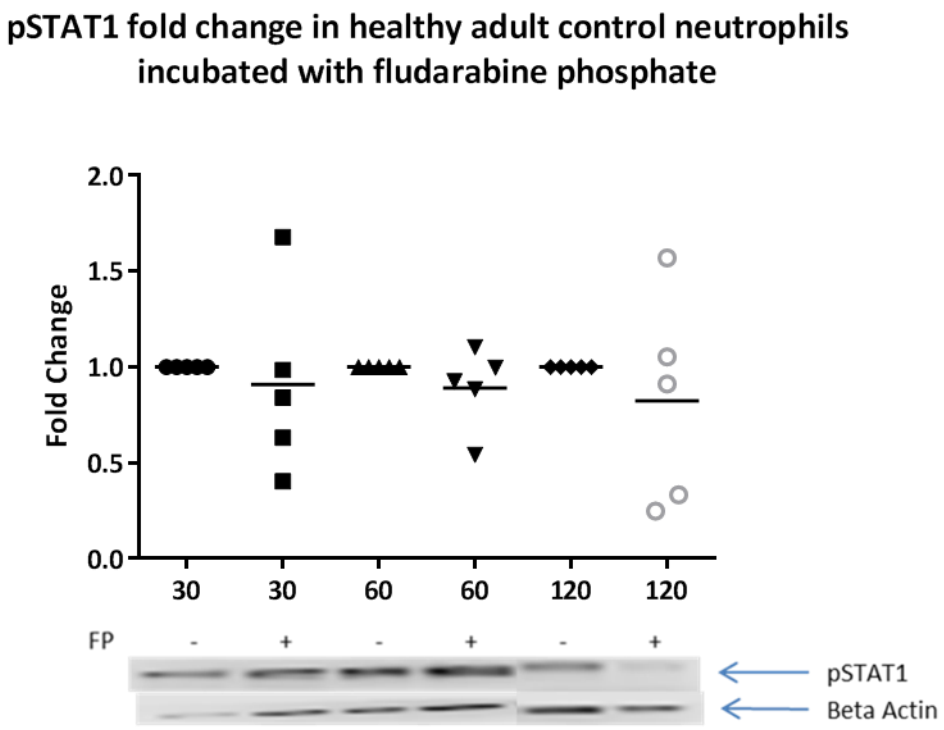


Figure 5.12. Fludarabine phosphate time response in healthy adult neutrophils stimulated with 10ng/ml IFNα and 10ng/ml IFNγ. Neutrophils were isolated from healthy adult donors and were left unstimulated or treated with or without 50μM of fludarabine phosphate for 30, 60 and 120mins, and subsequently stimulated with 10ng/ml IFNα and 10ng/ml of IFNγ for 15mins. Protein was extracted and pSTAT1/β actin expression ratios was measured by Western blotting (n=5).

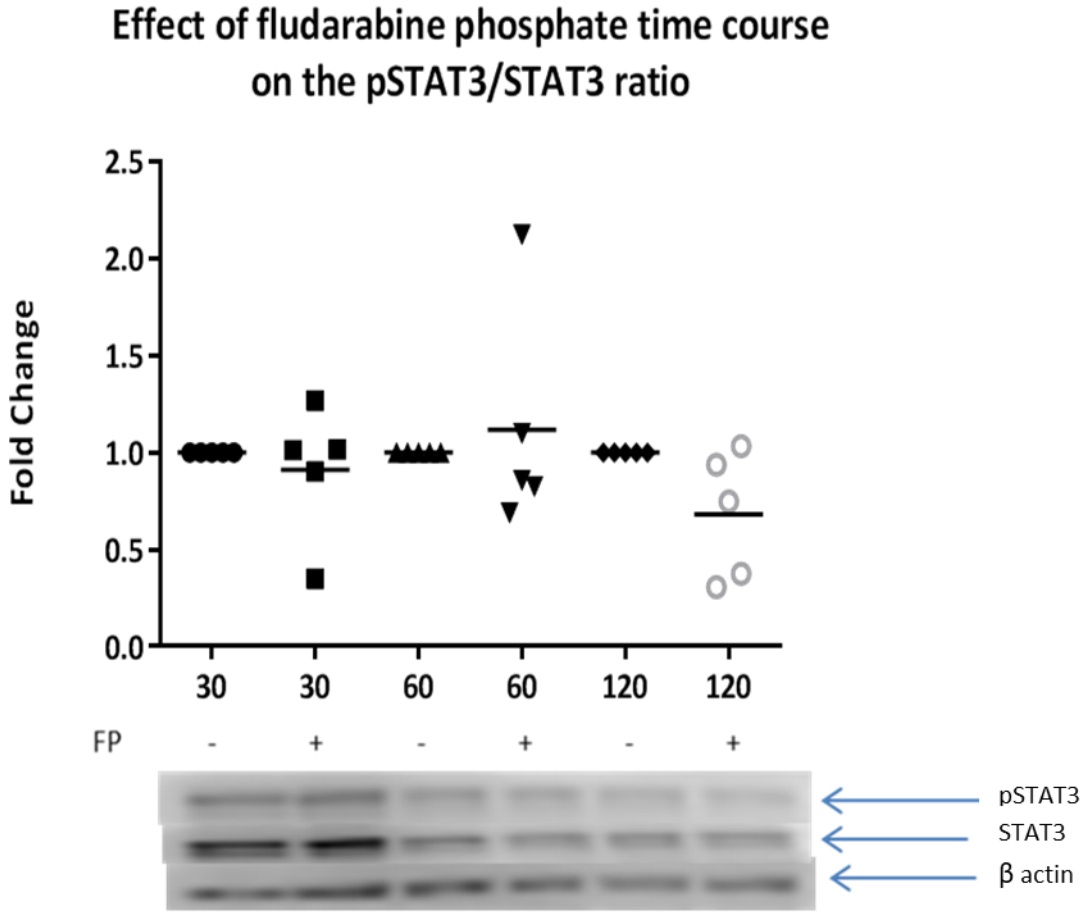


Figure 5.13. Fludarabine phosphate time response in healthy adult neutrophils stimulated with 10ng/ml IFNα and 10ng/ml IFNγ for 15mins. Neutrophils were isolated from healthy adult donors and were left unstimulated or treated with 50µM of fludarabine phosphate for 30, 60 and 120mins, and subsequently stimulated with 10ng/ml IFNα and 10ng/ml of IFNγ for 15mins. Protein was extracted and pSTAT3/STAT3 expression ratios was measured by Western blotting (n=5).

5.5.5. Inhibition of STAT1 phosphorylation by S1495

The above experiments demonstrated that fludarabine phosphate was not an appropriate pSTAT1 specific inhibitor to use in these experiments, an alternative STAT1 inhibitor was selected. S1495 is a compound isolated from the fungus Penicillium species and was shown to inhibit STAT1 phosphorylation in a variety of cells (201, 202). The aim of the next set of experiments was to investigate the ability of S1495 to inhibit STAT1 phosphoryation.

5.5.5.1. pSTAT1/β actin expression ratio reduction in A549 cells (positive control)

Similar to fludarabine phosphate, S1495 requires a solvent to initially dissolve it. Following on from the preliminary data from fludarabine phosphate experiments outlined above, and DMSO apoptosis analysis (Figure 5.12), S1495 was diluted at 10µg/µl in DMSO, and subsequently diluted to 1µg/µl with media.

Based on the study by Yao *et al.* (201), A549 cells were used as a positive control for S1495-related STAT1 phosphorylation A549 cells were left un-treated or treated with 10µg/ml of S1495 for 18hrs serum-free DMEM, and stimulated with 10ng/ml IFNα and 10ng/ml IFNγ for 15mins. Cells were lysed and the pSTAT1/β actin expression ratio was analysed. This was repeated for another passage of A549 cells. There was a reduction in the ratio of pSTAT1 to β actin upon S1495 treatment compared to untreated cells (0.3 vs 1.2 for 1st passage; 1.4 vs 2.0 for 2nd passage) (Figure 5.14). Additionally, S1495 had little effect on pSTAT3/STAT3 expression ratios compared to untreated cells (0.007 vs 0.06 for 1st passage; 0.07 vs 0.1 for 2nd passage) (Figure 5.15). This indicates that the S1495 reduces the pSTAT1/β actin expression ratio without effecting STAT3 phosphorylation and therefore may be a suitable STAT1 phosphorylation specific inhibitor in neutrophils.

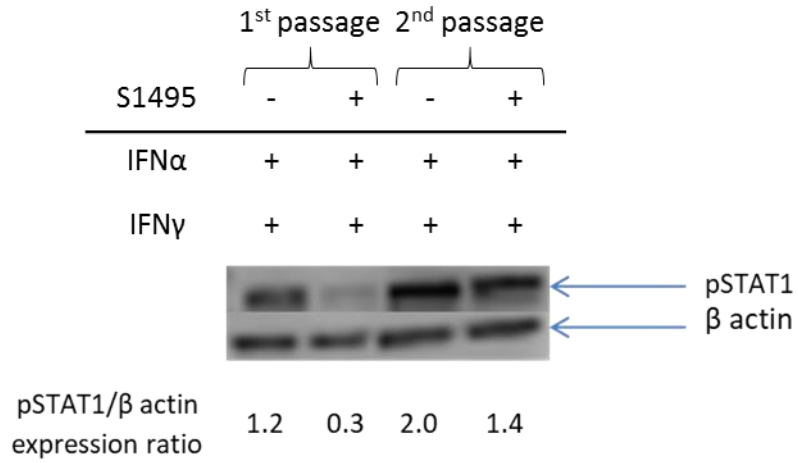


Figure 5.14. S1495 pre-treatment on STAT1 phosphorylation in IFN stimulated A549 cells. Based on study by Yao *et al.* (201), A549 cells were used as a positive control in investigation into the use of S1495 as a STAT1 phosphorylation inhibitor. After 18hrs pre-treatment, S1495 was able to reduce pSTAT1/β actin expression ratio in A549 cells stimulated with 10ng/ml IFNα and 10ng/ml IFNγ for 15mins. This was consistent across two passages and therefore indicates that S1495 may be an effective STAT1 phosphorylation inhibitor.

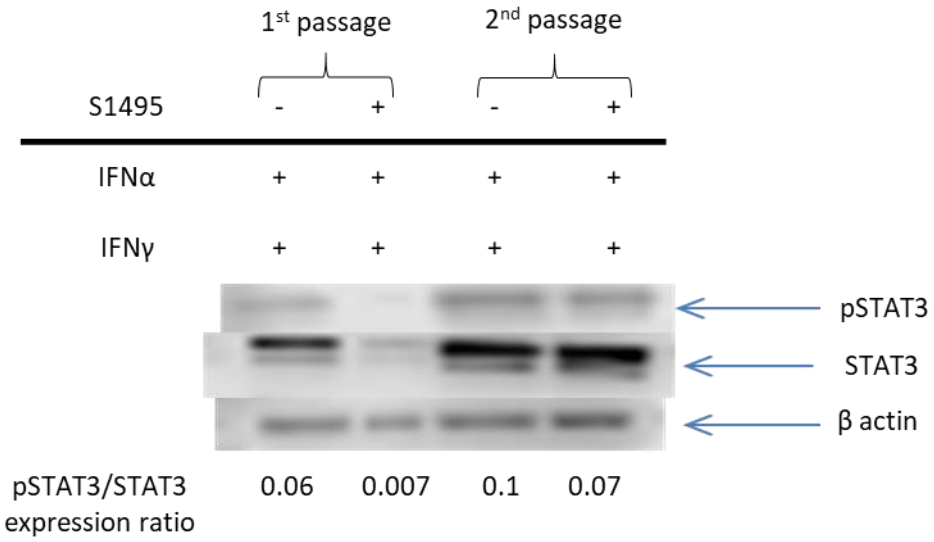


Figure 5.15. S1495 pre-treatment on STAT3 phosphorylation in IFN stimulated A549 cells. Based on study by Yao *et al.* (201), A549 cells were used as a positive control in investigation into the use of S1495 as a STAT1 phosphorylation specific inhibitor. After 18hrs pre-treatment, S1495 had no significant effect on pSTAT3/STAT3 expression ratio in A549 cells stimulated with 10ng/ml IFNα and 10ng/ml IFNγ for 15mins. This was consistent across two passages and therefore indicates that S1495 may be an effective STAT1 phosphorylation specific inhibitor.

5.5.5.2. Dose response of S1495 on apoptosis

Neutrophils were treated with a dose response of S1495 for 6hrs (1μg/ml, 5μg/ml and 10μg/ml), and apoptosis was measured. Little difference was seen between doses of S1495, indicating that it does not appear to affect naïve neutrophil apoptosis significantly at any of the doses tested (unstimulated = 12.7 ± 5.4%; 1μg/ml = 12.4 ± 5.2%; 5μg/ml = 12.6 ± 5.5%; 10μg/ml = 15.5 ± 6.7%; n=3) (Figure 5.16). This was reassuring as it is hypothesised in this study that STAT1 is more likely to be activated in IFN-stimulated, TNFα-primed neutrophils. Thus, its effect on apoptosis may be more pronounced, and so STAT1 inhibition by S1495 may only affect apoptosis in these conditions. From this experiment, as there was little difference in effect between different concentrations used, it was initially decided pragmatically that 10μg/ml of S1495 was an appropriate concentration for subsequent experiments, modelling previous published studies that used this concentration (202).

5.5.5.3. The effect of S1495 on pSTAT1 and pSTAT3 expression

Following on from the dose response above, the effect of S1495 on STAT1 and STAT3 phosphorylation was investigated. Here, it was shown that the S1495 reduced the pSTAT1/β actin expression ratio in neutrophils from healthy adult donors, following 1hr S1495 treatment at 10μg/ml and subsequent stimulation with 10ng/ml IFNα and 10ng/ml IFNγ, for 15mins (0.46 ± 0.23 vs 0.78 ± 0.37; n=5) (Figure 5.17). This suggests that S1495 may be inhibiting STAT1 phosphorylation or reducing overall STAT1 expression. However, donor dependency was observed, whereby the S1495 had little inhibitory effect on IFN-related pSTAT1 in 2 of 5 donors (Figure 5.16). Reassuringly, there was no overall effect on the IFN-related pSTAT3 expression (0.20 ± 0.05 vs 0.16 ± 0.05; n=5) (Figure 5.18), suggesting that, unlike fludarabine phosphate, S1495 is a specific STAT1 inhibitor.

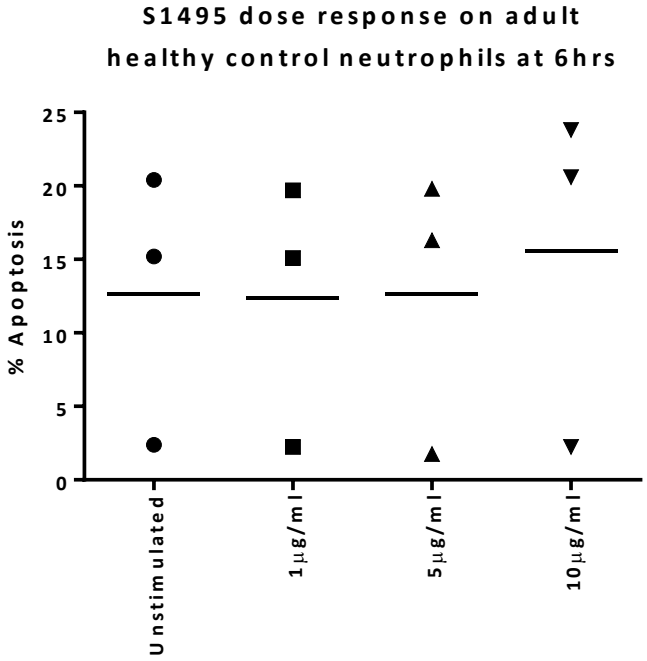


Figure 5.16. Apoptosis analysis in neutrophils from healthy adult donors following a S1495 dose response at 6hrs. Neutrophils were isolated from healthy adult donors and were left unstimulated or treated with a dose response of 1, 5 or 10µg/ml S1495 for 6hrs. Neutrophils were stained with annexin V FITC and PI and apoptosis was calculated as a percentage. No overall differences were seen between concentrations; however, 10µg/ml was used in other studies (n=5).

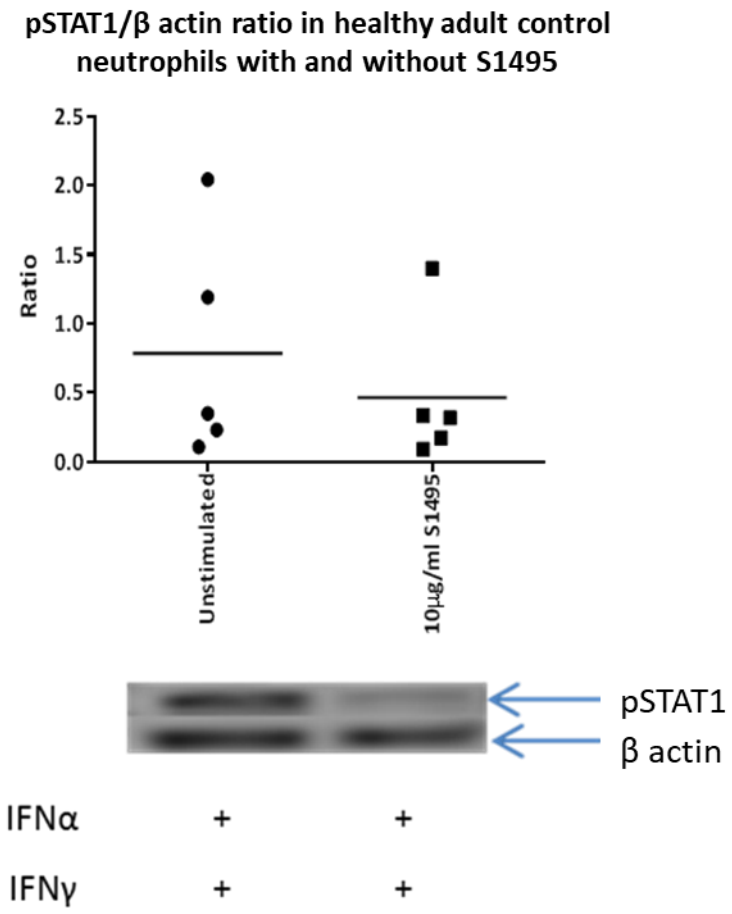


Figure 5.17. pSTAT1/B actin expression ratios after 1hr S1495 treatment in healthy adult neutrophils stimulated with 10ng/ml of IFNα and 10ng/ml IFNγ for 15mins. Neutrophils were isolated from healthy adults and were left unstimulated or treated with S1495 for 1hr and both conditions subsequently stimulated with 10ng/ml IFNα and 10ng/ml IFNγ for 15mins. Protein was extracted, and pSTAT1/β actin expression ratios was measured using Western blotting (n=5).

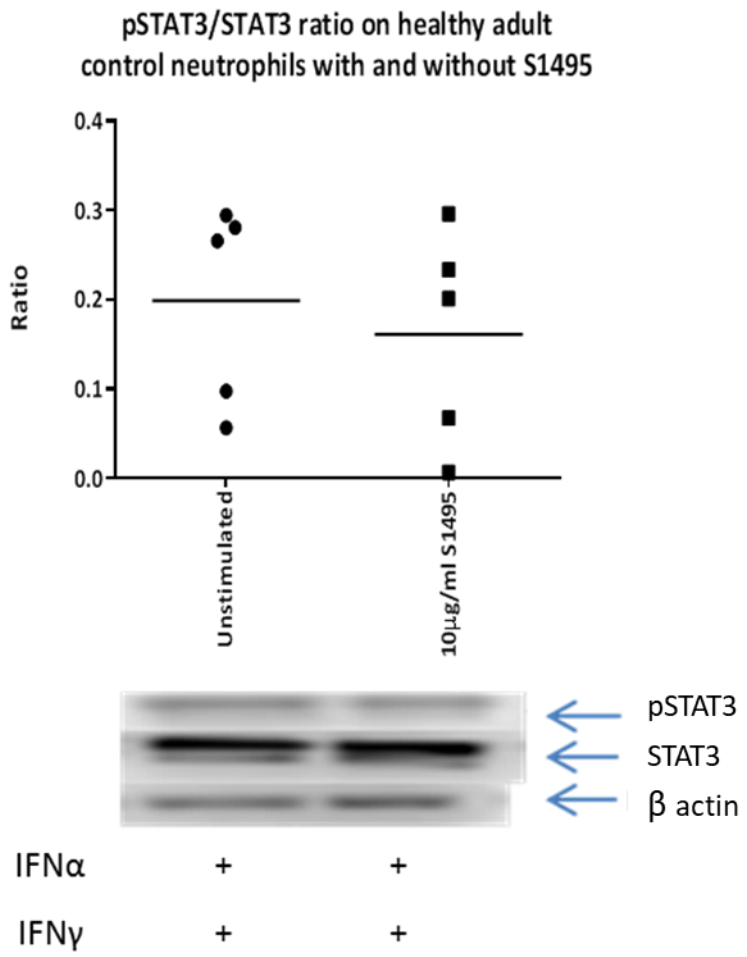


Figure 5.18. pSTAT3/STAT3 expression ratios after 1hr S1495 treatment in healthy adult neutrophils treated stimulated with 10ng/ml of IFNα and 10ng/ml IFNγ for 15mins. Neutrophils were isolated from healthy adult donors and were left unstimulated or were treated with S1595 for 1hr and both conditions were subsequently stimulated with 10ng/ml IFNα and 10ng/ml IFNγ for 15mins. Protein was extracted and pSTAT3/STAT3 expression ratios was measured using Western blotting (n=5).

5.5.5.4. The effect of S1495 on neutrophil apoptosis

Following on from the investigations above (Section 5.5.5.3), the effect of S1495 on apoptosis in TNFα-primed, IFN stimulated neutrophils was investigated. As has been shown in this study (see above) such neutrophils have increased STAT1 phosphorylation, which may lead to the increase in apoptosis seen with the high concentration of IFNs. It is hypothesised that in the presence of STAT1 inhibition with S1495, that IFNs are forced to signal through the anti-apoptotic STAT3 in primed neutrophils, where they may typically induce STAT3 phosphorylation, and thus become anti-apoptotic.

Neutrophils were pre-treated with 10μg/ml S1495, primed with 1μg/ml TNFα for 30mins and stimulated with 10ng/ml IFNα, 10ng/ml IFNγ or 10ng/ml IFNβ for 6hrs. Initially, data suggested that in primed neutrophils, S1495 had an anti-apoptotic effect on neutrophils, and IFNs had no additional effect on this reduction in apoptosis (unstimulated = 12.3 ± 1.2% vs 25.1 ± 2.6%; IFNα = 12.4 ± 3.3% vs 26.6 ± 3.0%; IFNγ = 15.0 ± 1.8% vs 27.3 ± 3.8%; IFNβ =12.9 ± 2.0% vs 26.6 ± 3.0% n=3) (Figure 5.19).

Thus, an experiment was conducted to investigate whether priming neutrophils initially with TNFα would affect how S1495 modulates apoptosis through STAT1 inhibition. Neutrophils were primed with 1μg/ml TNFα for 30mins, then treated with S1495 for 6hrs. This did demonstrate again that the S1495 still had an overall anti-apoptotic effect on primed neutrophils, but not on naïve neutrophils (unstimulated = 7.7 ± 1.7% vs 10.4 ± 2.5%; TNFα =19.2 ± 3.7% vs 27.1 ± 9.0%; n=3) (Figure 5.20). This indicated that, contrary to what was hypothesised, S1495 actually reduced apoptosis when neutrophils were primed with TNFα, but without any IFN stimulation. Therefore, any reduction in apoptosis by S1495 is not via the phosphorylation of anti-apoptotic STAT3. This was regardless of whether the neutrophils were primed with TNFα first or stimulated with S1495 first.

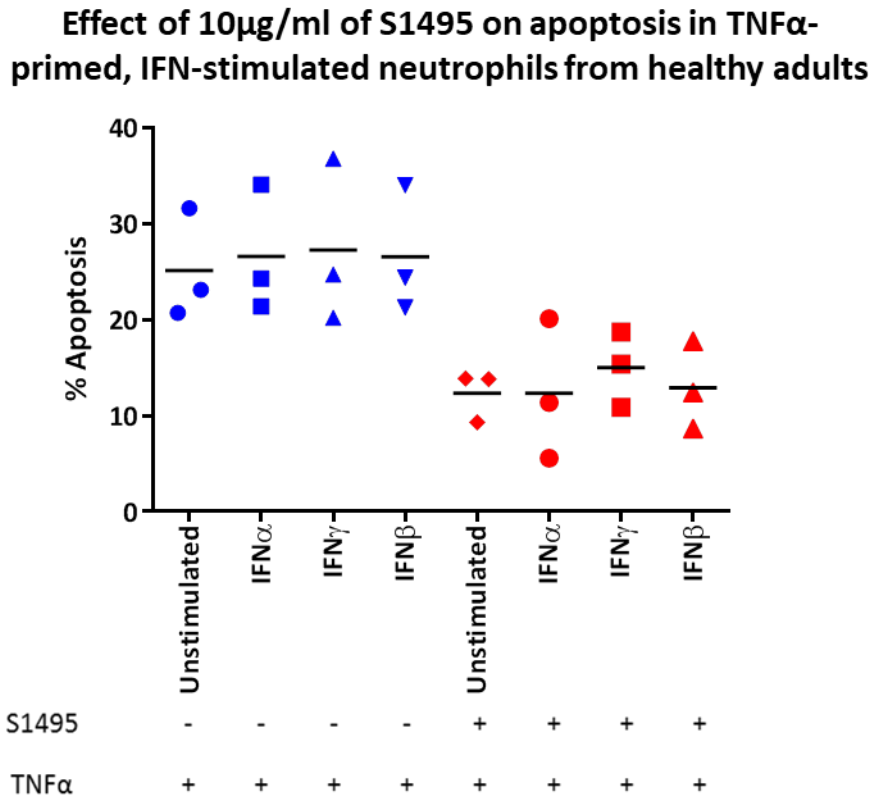


Figure 5.19. The effect of 10µg/ml of S1495 on 10ng/ml IFN-related apoptosis in TNFα-primed healthy adult neutrophils at 6hrs. Neutrophils were isolated from healthy adult donors and left unstimulated (blue) or treated with 10µg/ml S1495 (red) for 1hr. Neutrophils were primed with 1µg/ml TNFα and stimulated with 10ng/ml IFNα, 10ng/ml IFNγ or 10ng/ml IFNβ for 6hrs. Neutrophils were stained for annexin V FITC and PI, and the apoptosis was calculated as a percentage. As expected, IFNs lost their ability to reduce apoptosis primed neutrophils without S1495. However, S1495 had reduced apoptosis effect in primed neutrophils with no additional effect from the IFNs (n=3).

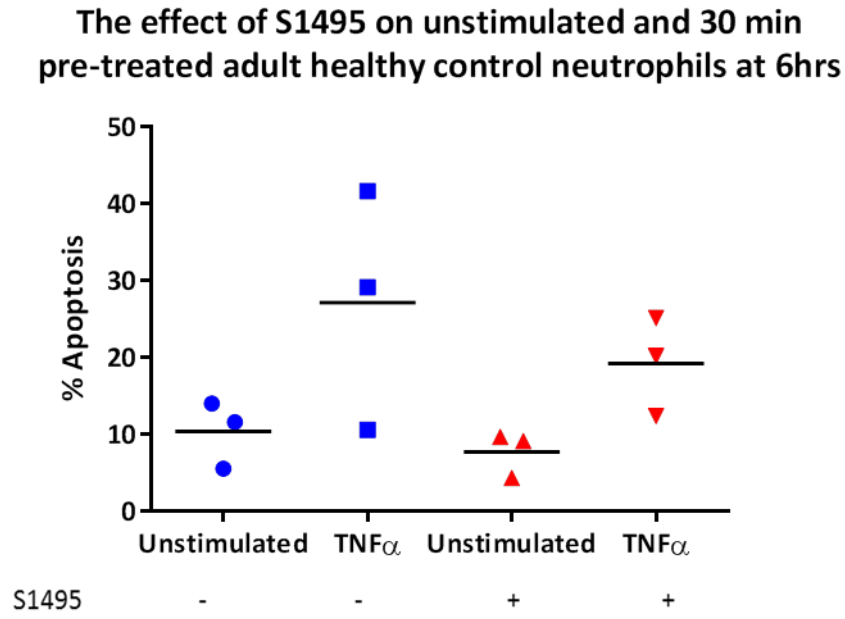


Figure 5.20. The effect of S1495 on apoptosis in naïve and primed neutrophils from healthy adults. Neutrophils were isolated from healthy adult donors and either left naïve or primed with 1µg/ml TNFα. Neutrophils were either left unstimulated (blue) or treated with 10µg/ml S1495 (red) for 6hrs, and neutrophils were stained with annexin V FITC and PI. Apoptosis was measured and presented as a percentage. Here, TNFα had an apoptotic effect at 1µg/ml, an effect not seen in other experiments. S1495 had no effect on unstimulated neutrophil nor TNFα-primed neutrophil apoptosis (n=3).

Thus, another dose response experiment was conducted, to see if in TNFα-primed neutrophils, a lower concentration of S1495 may be more appropriate in inhibiting STAT1 without having an additional anti-apoptotic effect when IFNs are not present. Neutrophils were primed with 1μg/ml TNFα for 30mins, and subsequently treated with S1495 for 6hrs. It was shown that again, 10μg/ml of S1495 was able to reduce apoptosis in primed neutrophils compared to unstimulated primed neutrophils, but this was not seen at lower concentrations (unstimulated = 29 ± 4.6%; 1μg/ml = 29 ± 4.7%; 5μg/ml = 32 ± 5.4%; 10μg/ml = 23 ± 2.0%; n=3) (Figure 5.21).

It has to be clarified that, although at 5μg/ml the inappropriate S1495 anti-apoptotic effect was lost, that S1495 was still able to reduce pSTAT1/β actin expression ratios at this concentration indicating that S1495 is possibly inhibiting STAT1 phosphorylation. Neutrophils were treated with 5μg/ml S1495, primed with 1μg/ml TNFα for 30mins and stimulated with 10ng/ml IFNα, 10ng/ml IFNγ, or 10ng/ml IFNβ for 15 mins. Using Western blotting, it was confirmed that at 5μg/ml, S1495 was able to reduce pSTAT1/β actin expression radios in primed neutrophils stimulated with IFNα, IFNγ or IFNβ but again this was donor dependent (donor 1: unstimulated = 0 vs 0.05; IFNα = 0.24 vs 0.08; IFNγ = 0.56 vs 0.42; IFNβ = 0.57 vs 0.47; donor 2: unstimulated = 0 vs 0.04; IFNα= 0.30 vs 0.18; IFNγ = 1.02 vs 1.23; IFNβ = 0.62 vs 1.34) (Figure 5.22). Interestingly there was some level expression of pSTAT1 in primed neutrophils without S1495 or IFN stimulation, which was inhibited in the presence of S1495 at 5μg/ml (Figure 5.22). Thus, TNFα may phosphorylate STAT1 in neutrophils but only at a high concentration (1μg/ml) as opposed to 1ng/ml initially used in STAT1 experiments (Figure 5.9, Figure 5.22).

It was now important to analyse the effect of 5μg/ml S1495 on TNFα-primed neutrophil apoptosis in the presence of IFNs. Neutrophils were pre-treated with 5μg/ml S1495, primed with 1μg/ml TNFα for 30mins and stimulated with 10ng/ml IFNα, 10ng/ml IFNγ, or 10ng/ml IFNβ for 6hrs. Unlike at 10μg/ml, at 5μg/ml S1495 had no effect apoptosis of primed neutrophils, and IFNs had no effect in the presence of S1495 at this concentration (unstimulated = 19.5 ± 2.2% vs 22.2 ± 2.7%; IFNα = 24.0 ± 2.8% vs 23.9 ± 3.1%; IFNγ = 23.3 ± 3.2% vs 24.4 ± 2.1%; IFNβ =23.8 ± 3.1% vs 23.0 ± 2.2% n=3) (Figure 5.23). This was regardless of the concentration of neutrophils seeded. Neutrophils from one donor were seeded at $5x10^4$ neutrophils per 100μl per condition compared to $1x10^5$ neutrophils per 100μl per condition for other donors, with no difference noted.

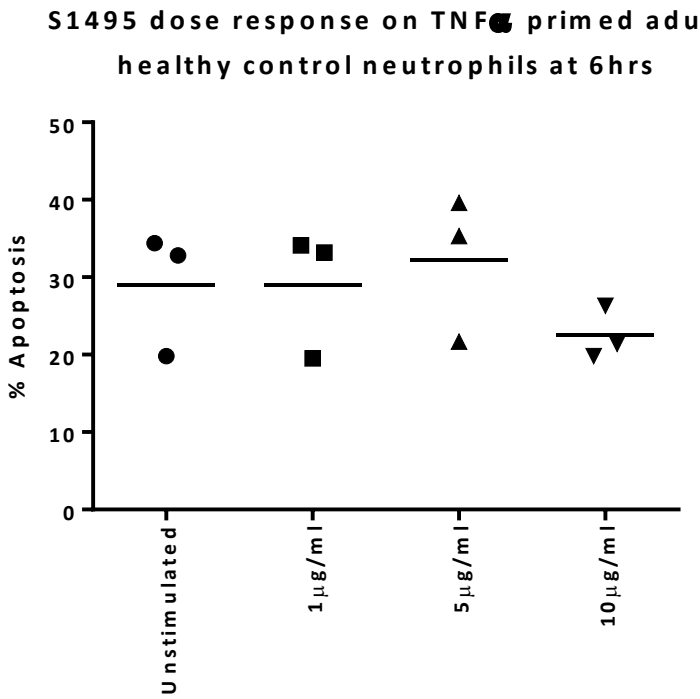


Figure 5.21. The effect of S1495 dose response on apoptosis in TNFα-primed neutrophils from healthy adults. Neutrophils were isolated from healthy adult donors and were primed with 1μg/ml TNFα for 30mins and treated with a dose response of 1, 5 or 10μg/ml S1495 for 6hrs. Neutrophils were stained with annexin V FITC and PI and apoptosis was calculated as a percentage. On average, only 10μg/ml S1495 reduced apoptosis (n=3).

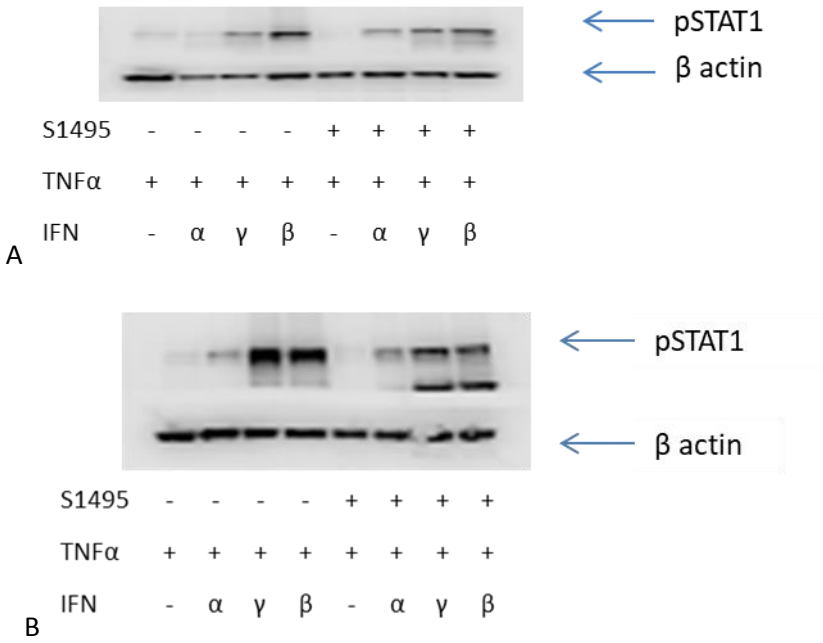


Figure 5.22. The effect of 5µg/ml of S1495 on STAT1 phosphorylation in primed neutrophils from healthy adults. Neutrophils were isolated from healthy adult donors and left unstimulated or treated with 5µg/ml of S1495 for 1hr. Neutrophils were primed with 1µg/ml TNFα and stimulated with 10ng/ml IFNα, 10ng/ml IFNγ or 10ng/ml IFNβ for 15mins. Proteins were extracted and pSTAT1/β actin expression ratios were measured using western blotting. S1495 at this concentration can inhibit pSTAT1 in IFNγ and IFNβ stimulated neutrophils (B) but this seems to be donor dependent (n=2).

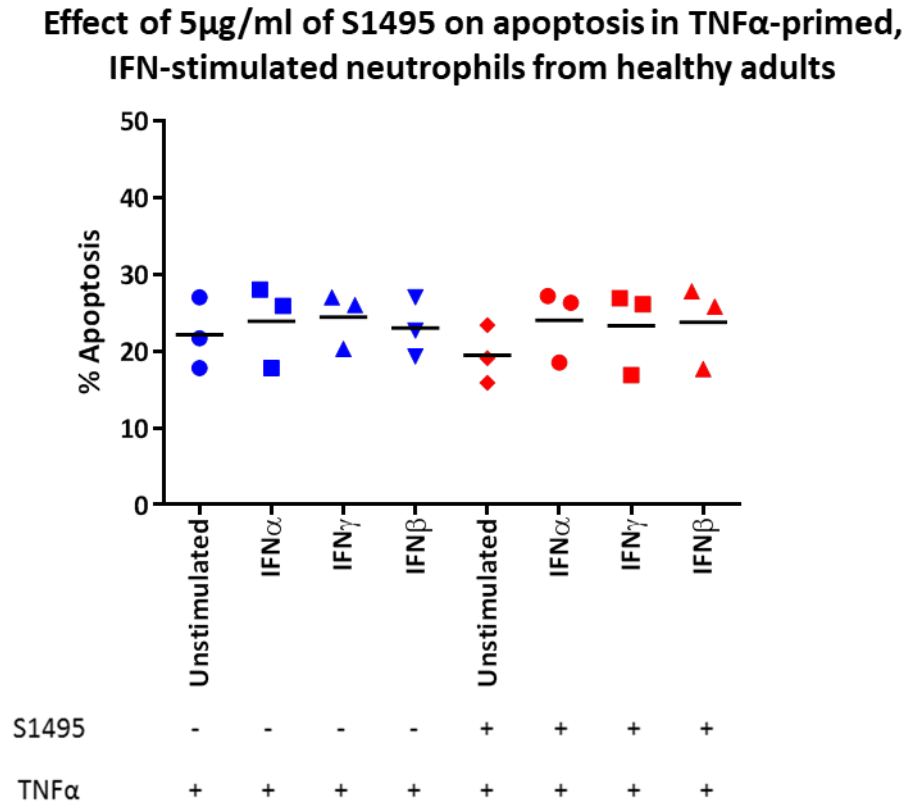


Figure 5.23. The effect of 5µg/ml of S1495 on IFN-related apoptosis in primed neutrophils from healthy adults. Neutrophils were isolated from healthy adult donors and left unstimulated (blue) or treated with 5µg/ml S1495 (red) for 1hr. Neutrophils were primed with 1µg/ml TNFα and stimulated with 10ng/ml IFNα, 10ng/ml IFNγ or 10ng/ml IFNβ for 6hrs. Neutrophils were stained for annexin V FITC and PI, and the apoptosis was calculated as a percentage. S1495 had no effect on apoptosis in TNFα-primed neutrophils (n=3).

5.6. Discussion

In this chapter, it has been shown that as the IFN subtypes exert their effect through the JAK/STAT pathway, this may be important pathway in JSLE neutrophil apoptosis. It was shown that there was an up-regulation of IFNGR2 and subsequent increased phosphorylation of pro-apoptotic STAT1 in TNFα-primed neutrophils, and down-regulation of IFNAR1 and decreased phosphorylation of anti-apoptotic STAT3. This suggests that the JAK/STAT signalling pathway may have a role in JSLE neutrophil apoptosis and that IFNγ in may be an important effector of this pathway. Although efforts were made to see if STAT1 inhibition promotes IFN-induced anti-apoptosis via signalling through pSTAT3, this was not always possible using the two inhibitors investigated. However, the pathway has been shown to be up-regulated in SLE (194), and as it is important in neutrophil apoptosis, it still highlights a potential therapeutic in JSLE.

5.6.1. IFN receptor expression and the JAK/STAT pathway

Published data has shown that IFN receptor chain expression can influence different parts of the JAK/STAT pathway, and an increase in IFNGR2 is linked to increased IFNγ related pSTAT1 and apoptosis (161, 162) (Sections 1.5.2, and 5.1).

The data in this chapter showed that in TNFα-primed neutrophils, as confirmed by changes in CD11b and CD62L expression, there was a significant decrease in IFNAR1, and a trending increase in IFNGR2 (Section 5.5.1). IFNs had no overall effect on activation or IFN receptor chain expression although there was suggestion that IFNγ could increase IFNGR2 (Section 5.5.1). Downstream, there was a reduction in pSTAT3/STAT3 expression ratio and an increase in pSTAT1/β actin expression ratio in these activated neutrophils (Section 5.5.3). The link suggests that the reduction in type 1 IFN-induced pSTAT3 is linked to the reduction in IFNAR1; that in activated neutrophils, the ability of IFNα and IFNβ to phosphorylate STAT3 was reduced (Section 5.5.3). Additionally, as published data indicated, there was an increase in type 2 IFN-induced pSTAT/β actin ratio (Section 5.5.3). It was shown in activated neutrophils, IFNγ-related pSTAT1/β actin expression ratio is increased (although whether this increase was due to increase pSTAT1 expression or an increase in overall STAT1 expression was not concluded due to issues with the pan STAT1 antibody), and this increase may be linked to increased apoptosis seen in these cells. This supports the evidence that indicates this pathway is important in SLE and lupus nephritis pathogenesis (161, 162, 194, 196) (Section 1.5).

These findings are important and supportive of the previous findings in Sections 4.5.3.2 and 4.5.4.3 with regards to neutrophil apoptosis. It seems that there is a reduction in the anti-apoptotic signalling pathway and an increase in the pro-apoptotic signalling pathway via decrease in pSTAT3/STAT3 expression ratio and increase in pSTAT1/β actin expression ratio (which may be due to an increase in pSTAT1 expression or overall STAT1 expression) which may be contributing the differential apoptotic effects of IFNs on neutrophils (138, 141) (Sections 1.5, and 4.5.3-4.5.7). Although apoptosis was only seen at high concentrations of IFNs in activated neutrophils, it still may be possible that IFNγ in particular can induce apoptosis in JSLE through an increase in IFNGR2 expression suggested by published studies on T cells and increase pSTAT1/β actin expression ratio seen here (161, 162) (Sections 1.5 and 5.1).. Alternatively, activated neutrophils, which represent neutrophils found in SLE and JSLE patients have reduced IFNAR1 (83, 90). This may reduce the activation of STAT3; this reduction of anti-apoptotic STAT3 activation could contribute to the increase in neutrophil apoptosis seen in SLE and JSLE patients (71, 72, 141) (Section 1.4.3).

5.6.2. IFN receptors in JSLE neutrophils

This study showed that there was an increase in CD11b in freshly isolated JSLE neutrophils compared with paediatric control neutrophils. This indicates that the JSLE neutrophils are more active, most likely due to the pro-inflammatory environment in which they came from. However, there was no comparable reduction in CD62L in JSLE neutrophils. This reflects well with a published study which has already shown that neutrophils are activated in adult-onset SLE patients (83) (Section 1.3.5). However, although the increase in CD11b correlates well with activation of healthy adult neutrophils though TNFα-priming, the lack of change in CD62L between JSLE and paediatric control neutrophils did not reflect that of the *in vitro* model. This inconsistency may be due high concentration of TNFα used in the model; the concentration is higher than the range of cytokine concentrations typically found in JSLE serum and plasma (68, 72, 172, 225). However, the environment in which SLE and JSLE neutrophils are in is complex, and there is evidence to suggest both an increase and decrease in neutrophil priming agents with SLE and JSLE serum (72, 86, 90, 168, 171) (Sections 1.5.4 and 4.1). A published study has shown that GMCSF, a known neutrophil priming agent that can induce the expression of CD11b, has been shown to be decreased in JSLE serum, indicating that the JSLE environment may be less likely to activate neutrophils (72, 86, 168, 228) (Section 1.3.5). However, although not shown here, it has been suggested that IFNγ, can also prime neutrophils (90) (Sections 1.3.5 and 5.5.1.2). As IFNγ is increased in SLE and therefore likely to be increased in JSLE, it may be involved in activation of neutrophils (171).

However, these conflicting studies may reflect the differences in SLE and JSLE patients and their diseases (7) (Section 1.1). Following from this, the patients who contributed neutrophils to be analysed were noted to have inactive disease (Section 5.5.2); therefore, CD62L expression on neutrophils from these patients may not significantly differ from that of paediatric control neutrophils. Therefore, although these neutrophils may be activated, activation of neutrophils via the pro-inflammatory environment within JSLE patients with inactive disease may not be as extreme in comparison to TNFα-primed neutrophils *in vitro* or indeed compared to neutrophils from JSLE patients with active disease. It would therefore be interesting to investigate these markers on neutrophils from patients with more active disease to fully elucidate how disease activity affects neutrophil activation.

Following on from this activation analysis, IFN receptor chain expressions were analysed (Section 5.5.2). There were no significant differences in the expression of IFNAR chains and IFNGR chains between JSLE and paediatric control neutrophils. The observed similarities of IFNGR chain expression between JSLE and paediatric control neutrophils was as expected; these results reflect the *in vitro* model mentioned above. Therefore, unlike published observations on T cells, IFNGR chain expression is likely to be always stable on neutrophils regardless of environmental factors, activation or JSLE disease, and thus any differential effect of IFNγ on neutrophil apoptosis (Section 4.5.3.2) is not due to the expression of IFNGR chains (161, 162) (Sections 1.5.2 and 5.1). The similarities in IFNAR expression between JSLE and paediatric control neutrophils, do not reflect the adult *in vitro* model. However, again the neutrophils analysed for IFN receptor chains were from patients who had inactive disease; therefore, similarities in IFN receptor chains on neutrophils from inactive JSLE patients and paediatric control patients may be due to the JSLE patients' good health at time of sampling (Section 5.5.2). As with neutrophil activation, it would be beneficial to extend this study to neutrophils from JSLE patients with active disease; these neutrophils may be more active than those from inactive JSLE patients, and this increased activation may result in decreased IFNAR1.

This observed similarity in IFN receptor chain expression in JSLE patients and healthy controls (and thus, allowing IFNs to phosphorylate the anti-apoptotic STAT3) may explain why there was not an increase in apoptosis in JSLE neutrophils seen in the previous chapter (Section 4.5.8); the receptor chain expression is not primed to induce STAT1 related apoptosis (Section 5.5.2). However, sample size makes it difficult to see a difference between cases and controls. Additionally, JSLE patients, of whom the neutrophils were used in these experiments, and were found to have relatively inactive disease in general. Therefore, these

patients used may not fully represent the JSLE patient population as a whole. JSLE patients are heterogeneous through disease characteristics and medication exposure, may be other sub-groups of JSLE patients in whom the JAK/STAT pathway is more of a drug therapy target than in others. Additionally, the JSLE patients used in this study had a low medium pBILAG score indicating overall as a group, their disease was inactive or relatively inactive, and well controlled by the medication they are on. This may also contribute to the comparable IFN receptor chain expression between JSLE patients and healthy controls. Thus, more sampling, preferably from those patients that have very active disease, would be needed to fully investigate this signalling pathway.

5.6.3. STAT1 inhibition

As these data have indicated, IFNs can have a pro-apoptotic effect in neutrophils, and this is via the phosphorylation of STAT1 (Section 5.5.3). If STAT1 phosphorylation is inhibited, IFNs may not be able to have a pro-apoptotic effect in neutrophils. If they are unable to phosphorylate STAT1, IFNs may have to phosphorylate STAT3, and thus induce an anti-apoptotic signalling pathway in activated neutrophils. This may then be beneficial in reducing the number of apoptotic neutrophils in JSLE.

5.6.3.1. Fludarabine phosphate as a STAT1 inhibitor

The first inhibitor of STAT1 that was investigated was fludarabine phosphate. It is a known chemotherapeutic medication; but was also suggested to inhibit STAT1 made it an interesting inhibitor as it may have had therapeutic potential in SLE (200, 229) (Section 1.5.4.3).

This assessment of this inhibitor was associated with several experimental issues (Section 5.5.4). The first was that it needed DMSO to dissolve it. Initial experiments showed that although fludarabine phosphate was effective at reducing the pSTAT1/β actin expression ratio at 50μM, the equivalent amount of DMSO significantly induced apoptosis. This was a fundamental flaw in the experimental set up; the carrier solution had an independent effect on neutrophils additional (and in theory opposite) to that of fludarabine phosphate.

Fludarabine phosphate was therefore diluted in less DMSO, and a time course was investigated using 50μM fludarabine phosphate as the optimal concentration for reducing pSTAT1/β actin expression ratio and therefore probably inhibiting STAT1, either through reducing pan STAT1 expression, or inhibiting specifically the phosphorylation of STAT1. At 2hrs, fludarabine phosphate had STAT1 inhibition ability shown through the reduction of the pSTAT1/β actin expression ratio. However, the pSTAT3/STAT3 expression ratio was also reduced, indicating that STAT3 was also inhibited under these conditions. Again, this

demonstrated another problem with using this as an inhibitor in this model. Any inhibitory effect fludarabine phosphate had was not STAT1 specific, and that it also influenced STAT3 meant that it was not able to subsequently be used as a specific 'STAT1 inhibitor'. Thus, further investigation into fludarabine phosphate was halted.

5.6.3.2. S1495 as a STAT1 inhibitor

S1495 was the second STAT1 inhibitor to be investigated (Section 5.5.5). More recent studies have used S1495 to inhibit IFNγ-induced pSTAT1 in J774 mouse macrophages, suggesting it may inhibit pSTAT1 in human neutrophils (201, 202). S1495 needed a solvent to dissolve in, and in this study, it was dissolved in DMSO similar to that of fludarabine phosphate (10µg/µl in DMSO, then diluted further to 1µg/µl in media)). However, it did not dissolve quite as well as fludarabine phosphate. At time of order and reconstitution, there was no suggestion from the company of which solvent was most suitable, and communication with technical support and data shown in this study on the effect of DMSO on apoptosis suggested DMSO would be suitable. However, recent searches have suggested methanol as solvent of choice for S1495 reconstitution (http://www.abcam.com/s14-95-ab144515.html). Thus, this may explain why S1495 didn't dissolve in DMSO quite as well as fludarabine phosphate, and methanol may be considered in any future S1495 studies. Additionally, the use of DMSO as a solvent for S1495, although diluted, may have some impact on the results shown in Section 5.5.5.

Unlike fludarabine phosphate, S1495 at 10µg/ml was shown to inhibit IFN-induced increase in pSTAT1/β actin expression ratio in neutrophils after 1hr incubation without any obvious effect on the pSTAT3/STAT3 expression ratio. This S1495 concentration and time course was selected based on previous published data observed in the J774 mouse macrophage cell line (therefore a useful starting protocol for work in neutrophils) (202). Additionally, another study showed that S1495 had an inhibitory effect on STAT1 phosphorylation in immortalised human cell lines (albeit at a higher concentration) indicating that inhibition of STAT1 by s1495 may be also be viable in human cells (201). These studies, however, did not show any comparative data on the effect of S1495 on other STATs, particularly STAT3 (201, 202). Therefore, here it was shown the selectivity of S1495 on STAT1 inhibition, without affecting STAT3, although its effect on other STATs remain to be elucidated. However, this possible pSTAT1 inhibition was donor dependent, and only reduced pSTAT1/β actin expression ratios in three out of five donors in initial experiments. This variability was deemed acceptable; however careful analysis on the effect S1495 on apoptosis would be needed due to the high probability donor-dependent variability.

Next, it was of interest to investigate how the STAT1 inhibition by S1495 affected apoptosis, a function that which was not investigated by other published studies using S1495 as a STAT1 inhibitor (201, 202). It was shown here that S1495 had no effect on apoptosis in naïve neutrophils; this may be due to the fact that type 1 IFNs in particular signal through the anti-apoptotic STAT3 more in these naïve neutrophils, exerting an anti-apoptotic effect (Section 4.5.3 and Section 4.5.4). Therefore, as it was shown that S1495 has no effect on STAT3 phosphorylation, it was unlikely to affect IFN signalling in naïve neutrophils in regards to apoptosis. However, the S1495 reduced apoptosis in TNFα-primed neutrophils, which was unaffected by IFN stimulation. It is likely that, within primed neutrophils treated with S1495, any reduction in apoptosis is not through the anti-apoptotic IFN/STAT3 pathway. This also correlates with the observation that S1495 had no effect on IFN-induced STAT3 phosphorylation; there was no increased IFN-induced pSTAT3/STAT3 expression ratios when pSTAT1/β actin expression ratios were reduced by S1495 (Figure 5.16), and therefore no downstream increase in an IFN induced anti-apoptotic effect. As with IFNs, TNFα can be both pro- and anti-apoptotic on neutrophils (221). Here, it was shown that TNFα does not activate either STAT1 or STAT3. It may be possible that S1495 has another target that is part of the TNFα pathway; this may lead to TNFα reducing apoptosis in neutrophils in the presence of S1495. Thus, S1495 is not ideal to investigate how STAT1 inhibition affects the IFN pathway and its effect on apoptosis in TNFα-primed neutrophils *in vitro*. More investigation is required to fully investigate the IFN/STAT1 pathway in regards to neutrophil apoptosis, and whether inhibition of this pathway would be therapeutically beneficial in JSLE.

5.7. Summary

In this chapter, it has been shown that TNFα-primed neutrophils have dysregulated IFN receptor chain expressions, and this may lead to IFN-induced increase in pSTAT1/β actin expression ratio, decrease in pSTAT3/STAT3 expression ratios, and the dysregulation of apoptosis, as demonstrated also in chapter 4. However, JSLE neutrophils do not completely reflect the activated adult neutrophils, which may be due to a variety of reasons, including the fact that TNFα was used at a high concentration and JSLE neutrophils were from patients with inactive disease. Efforts were made to inhibit STAT1, known to be pro-apoptotic and increased in SLE. Important preliminary data explored potential inhibitors of STAT1 in this model, with progress was made in understanding a potential role of S1495 as a specific inhibitor. However, the S1495-mediated reduction in apoptosis in TNFα-primed neutrophils led us to question whether S1495 modulates the TNFα pathway, leading to a TNFα anti-

apoptotic effect. Thus, the role of STAT1 in JSLE needs further investigation, particularly in regard to IFN-related neutrophil apoptosis, and whether STAT1 inhibition is potentially therapeutic in JSLE.

Chapter 6: Discussion

6.1. Concept of study

This study aimed to show how interactions between the different IFN subtypes and neutrophils might contribute to the pathogenesis of JSLE. There were many aspects to investigate, as the roles and functions of both neutrophils and the IFN subtypes are diverse and extensive, and many immune functions have been shown to be dysregulated within both adult-onset and JSLE (72, 110, 112, 113, 115-118, 123, 163, 170, 189, 208, 230) (Section 1.5). Neutrophil function is particularly impaired in SLE and with increased, dysregulated neutrophil apoptosis noted in both SLE and JSLE, neutrophils are thought to be a source of autoantigens in these conditions (19, 72, 114, 230) (Section 1.4.3).

A detailed literature review concluded that both the IFN gene expression and serum protein level were increased within SLE and JSLE, and indeed that increased IFN activity may actually take place before the onset of clinical symptoms of SLE (41, 68, 171, 172, 174, 190) (Section 1.5). This highlights that both type 1 and type 2 IFNs may have an important and key role in JSLE pathogenesis. In fact, review of the literature indicates that IFNs may be important in predicting SLE onset and also have a probable role in the induction of SLE pathogenesis (190). Therefore, the mechanism of action of type 1 and type 2 IFNs on neutrophils was investigated in regard to neutrophils within JSLE.

Published data has shown that not only is there an increased type 1 IFN gene signature within PBMCs of JSLE patients, there is also an increased expression of granulocyte-associated genes (41) (Section 1.5). These data indicate that IFNα, which is increased in sera from both adult-onset SLE and JSLE patients, could potentially, either directly or indirectly, interact with neutrophils to contribute to pathogenesis of JSLE (163, 231). Within adult-onset SLE, there is indication that IFNγ genes are also up-regulated, and that this increase correlates with both disease activity and with expression of type 1 IFN genes (174). IFNγ signalling pathways, via STAT1, have also been shown to be up-regulated within PBMCs of SLE patients (194). Additionally, the STAT1 pathway is up-regulated in a lupus nephritis model in MRL/lpr mice (196). These data suggest that not only are different IFN subtypes up-regulated in adult-onset SLE and JSLE, but also their signalling pathways are up-regulated and thus may be important in disease pathogenesis.

Although a careful review of the literature underlined a number of important studies investigating the role of the different IFN subtypes and their interaction with neutrophils and

their impact on neutrophil function in both adult-onset SLE and JSLE, detailed investigation into how the specific IFN subtypes interact with neutrophils in JSLE was limited to date.

The aim therefore of this thesis was to investigate the interaction of IFN sub-types with neutrophils, and how IFNs can influence neutrophil function and cell death in the context of JSLE. This was achieved by: a) Investigating the effect of the IFN subtypes on neutrophil chemotaxis, phagocytosis and NETosis; b) Investigating the role of IFN subtypes on neutrophil apoptosis; and c) Investigating the underlying IFN signalling pathways in naïve and TNFα-primed neutrophils with respect to neutrophil apoptosis.

6.2. IFNs and neutrophil function

6.2.1. Chemotaxis

The first function to be investigated was chemotaxis (Sections 1.3.1, 1.4.3, 3.1, 3.5.1 and 3.5.2). There is contradicting published data in how neutrophil chemotaxis is altered within SLE and JSLE. One study suggests an increase in chemotactic activity through an increase of plasma chemokines (118) (Section 3.1). However, other studies have suggested a decrease in chemotactic activity (116, 117) (Section 3.1). Many factors can influence and/or regulate neutrophil chemotaxis, in both health and disease, specifically in JSLE. It has been shown that a younger age of disease onset, renal involvement, and increased infection incidence may be associated with reduced neutrophil chemotactic function (117). Ethnicity of patients may also affect whether, and how, the neutrophil chemotaxis is dysregulated. In a population of Taiwanese JSLE patients, infection, disease activity and/or medication has no influence on chemotaxis in JSLE, although there was some (non-statistically significant) suggestion of reduced chemotaxis in JSLE neutrophils (112). However, within two populations of SLE patients from the USA neutrophils were demonstrated to have a reduced chemotactic ability and a reduced chemotactic activity within SLE serum (116, 117). Additionally, in contrast to the USA SLE patient populations, within a Chinese population of SLE patients, there was an observed increase in plasma chemokine concentration, although it was not clear if this was associated with increased neutrophil chemotaxis (118). Consideration of patient ethnicity and other clinical variables such as age of onset and organ involvement may be important when investigating chemotaxis within JSLE patients. In addition, considering basic demographic and medical information, such as any recent infections of the healthy volunteers contributing neutrophils to the *in vitro* models used, may greatly influence the results obtained from chemotaxis assays.

As neutrophils within SLE and JSLE are exposed to high levels of IFNs, it may be that the different IFN subtypes are priming neutrophils to respond to chemokines differently, as opposed to acting as chemokines themselves (68, 171, 172). IFNγ in particular has been suggested to prime neutrophils, whilst both IFNγ and IFNβ have been indicated to have roles in chemotaxis (90, 177-179). The next step into investigating neutrophil chemotaxis therefore was to initially prime neutrophils before testing their random and directed migration. IFN priming of the neutrophils did not have any overall effect on either random migration or directed migration to fMLP and IL-8. These experiments were carried out on healthy control neutrophils and, as literature has suggested, IFNγ and IFNβ may have a prominent role in chemotaxis in other scenarios. This included influencing immune cells than other than neutrophils and towards chemoattractants other than IL-8 and fMLP (112, 116-118). It would be interesting to conduct similar experiments on JSLE neutrophils (and other immune cells) to see if IFNs may contribute to the dysregulated chemotaxis already proposed in SLE and JSLE. The lack of any significant changes of the IFN priming on chemotaxis on healthy adult control neutrophils demonstrated in this present study may be due to many factors. As noted previously, within SLE patients, age of onset, renal involvement and also infection can enhance the reduced chemotaxis, and ethnicity is important in whether neutrophils have increased or reduced chemotactic activity (112, 116-118). The chemotaxis studies presented in this thesis were in a healthy adult cohort, and therefore these specific factors noted in SLE patients that may influence chemotaxis were not relevant. So, for example, whilst infection may be a factor that impacts on chemotaxis, it is unlikely that this is causing any effect here as the volunteers were all healthy at time of sampling. This means that although these results have not shown any significant IFN involvement in neutrophils in a healthy, non-pathological state, the IFNs may still be involved in a disease setting such as lupus where they too may be influenced by cytokines increased during inflammation or infections.

Additionally, other studies have investigated chemotaxis using other chemokines/chemoattractants in addition to those studied above (118, 177-179). It has been shown that chemokines are up-regulated in SLE, in which GROα, another neutrophil chemokine, is one; however, importantly for this published study, it was also shown that there was no difference in IL-8 between SLE patients and healthy controls (118). Thus, although this study has shown that IFNs don't influence chemotaxis towards IL-8 and fMLP, IFNs may influence neutrophil migration towards other chemokines. This has been reinforced by another study which showed that IFNγ can up-regulate CCR1 and CCR3 on neutrophils which have been shown to bind chemokines such as MCP3 and RANTES (179-182). To

understand further the role of IFNs on chemotaxis in a disease setting, an understanding of chemokine expression within JSLE disease is important. IFNs and other cytokines may prime neutrophils to respond differently to chemokines, however, a reduction/increase in neutrophil chemotaxis may also be due to altered level chemokines within inflammatory diseases. However, due to experimental restraints, primarily due to cell number available, it was not feasible to investigate the influence of IFNs on neutrophil chemotaxis against a full range of neutrophil chemokines. These experiments were conducted using an already optimised chemotaxis protocol with ideal fMLP and IL-8 concentrations, which were known to be strong neutrophil chemokines/chemoattractants. As at time of experimental investigation, the role of IFNs on neutrophil chemotaxis was unknown, at least towards fMLP and IL-8, in hindsight, GROα, MCP3 and RANTES may have been better, and more relevant candidates for this investigation.

Chemotaxis was measured using a transwell migration assay which is a standard assay for measuring chemotaxis, however the number of cells migrated were counted using a non-automated system, which can have increased variability and user bias compared to an automated cell counter. Other chemotaxis assays include using an under-agarose cell migration assay. However, this still requires manual counting by the user. Analysis of the chemotaxis receptors using flow cytometry would have been beneficial to see how IFN priming is affecting chemotaxis in this way.

Another difficulty noted was that priming of the neutrophils required extra incubations and centrifugation, which resulted in loss of cells. Therefore, cell yields used in the assay between donor repeats were varied and may have affected results. Possible assay improvements could include adding the IFNs into the inserts directly with the neutrophils. However, this may not allow the IFNs sufficient time to influence chemotaxis before the neutrophils migrate.

6.2.2. Phagocytosis

Neutrophils normally attack invading pathogens by phagocytosis, and subsequent ROS production. However, it has been shown that neutrophil phagocytosis in SLE and JSLE is reduced (112, 114, 115, 189) (Sections 1.3.2, 1.4.3, and 3.1). Thus, the next neutrophil function investigated in this present study was phagocytosis.

In published studies, IFNs have been shown to affect phagocytosis, but it should be noted that this dysregulation is very dependent on a variety of factors including type of immune cell and specific type of bacteria (183, 184) (Sections 1.4.3 and 3.1). Previously, it has been shown that IFNs can up-regulate neutrophil phagocytosis of both *S. aureus* bacteria and

bioparticles (184, 185). Although there is some indication that IFNγ may have some down-regulatory effect on macrophage phagocytosis, in general, IFNs up-regulated phagocytosis, particularly with regards to neutrophils (184, 185).

It is important to note that there are various receptors involved in phagocytosis, which in turn relies on how bacteria are opsonised. Published data has suggested that sheep erythrocytes opsonised with IgG were attached and internalised by mouse peritoneal macrophages via the Fc receptor, whereas C3b opsonised sheep erythrocytes were internalised by C3b receptors (183). Both processes were up-regulated by IFNα but not IFNγ (183). It has been shown that IFNγ induces an up-regulation of Fc receptors on human monocytes, and specifically Fcγ receptors are particularly augmented (215). IFNs also increased IgG binding to monocytes, although IFNα and IFNβ less so than IFNγ (215). IFNγ has also been shown to up-regulate FcγRI in circulating neutrophils, and FcγRI, FcγRII and FcγRIII in circulating monocytes (185). Integrin subunits CD11a, CD11b and CD18, and the human leukocyte antigen (HLA) class 2 antigen, HLA- antigen D Related (DR) was also increased on monocytes upon IFNγ stimulation (185). Within this present study, autologous serum was used to opsonise the *E.coli* bioparticles. However, due to the complexity of serum, the bioparticles may have been opsonised for several pathways, and thus any effect of IFNs on the phagocytosis of *E.coli* bioparticles may be masked by other, more prominent pathways. Alternatively, neutrophil phagocytosis could be activated by other pathways independent of IFNs. Therefore, it is difficult to discriminate any effect(s) due to the influence of IFNs alone.

This importance of selectively analysing the effect of IFNs on specific phagocytic pathways and not just broadly opsonising for all pathways was highlighted by a published study (185). The study showed that IFNγ increased neutrophil phagocytosis of *S. aureus*, but only when opsonised with complement depleted heat inactivated pooled human serum (HI-PHS) and not pooled human serum (185). It was thought that the increase in FcγRI on neutrophils by IFNγ increased FcR-mediated phagocytosis of this complement depleted opsonised *S. aureus* (185). However, whereas because complement-mediated phagocytosis of complete human serum opsonised *S. aureus* faster and is a preferable system than FcR-mediated phagocytosis, the complement in human serum (which may not be affected by IFNs) may mask the increase in FcR-mediated phagocytosis seen with complement depleted opsonised *S. aureus* (185). How bacteria are opsonised and therefore how neutrophils recognise bacteria is essential, and that it may be that complete serum-opsonised *E.coli* used in this study are phagocytosed by neutrophils via a receptor not influenced by IFNs, for example the complement system (185). CD11b/CD18 make up Mac-1, also known as complement receptor 3 or CR3 (96). This

complement receptor can recognise gram negative bacteria such as *E.coli*, and medicate the phagocytosis of such bacteria (232).

These data have shown that IFNs did not affect phagocytosis of *E. coli* in healthy neutrophils (Section 3.4.3). Previous studies have shown that IFNs increase phagocytosis and phagocytic receptors (183-185, 215) and therefore the observed IFN signature within JSLE, and heightened level of IFNs within SLE and JSLE serum could indicate that IFNs would have an increased effect on neutrophil phagocytosis in JSLE. For this reason, it was hypothesised that within JSLE, the decreased neutrophil phagocytosis reported is unlikely to be due to IFNs, and that the decreased neutrophil phagocytosis seen is probably due to other factors, either through altered expression of phagocytic genes, or factors within the serum (41, 68, 112, 113, 115, 171, 172).

There are other methods other than flow cytometry for analysis of phagocytosis. However, one benefit of flow cytometry is that it generates a reliable, quantitative estimate. Immunocytochemistry can be used to analyse phagocytosis, via the fluorescence of the *E.coli* bioparticles and staining of the nucleus of the neutrophils via DAPI. However, again this is not quantitative, and requires analysis on different planes to confirm that the particle is indeed within the neutrophil. The phagocytosis of the bioparticles can also be analysed using a fluorescence detecting plate reader, akin to that of enzyme-linked immunosorbent assays (ELISAs). This may be beneficial for analysing multiple samples at any given time, reduce the number of cells needed (as each experiment is conducted using a 96 well plate) and have the benefit of replication of samples to improve the repeatability and reliability of the results. It would also be beneficial to analyse not only the phagocytic receptor expression using flow cytometry, but also use ELISAs to analyse immune factors within serum, such as IgG or C3b that may be opsonising the *E.coli* bioparticles for phagocytic pathways (183). This may differ between donors, and also give insight into which phagocytic pathways are being stimulated (which may or may not be affected by IFNs). This analysis may allow discrimination, and therefore individual investigation, of the different phagocytosis pathways being influenced by the serum, and analysis into how opsonising immune complexes differ between paediatric controls and JSLE serum. However, as JSLE is a heterogeneous disease, and both serum and phagocytosis signalling are so complex, this type of analysis may offer limited interpretation.

The limitation of the phagocytosis assay used in these methods is that only opsonised *E.coli* bioparticles were analysed. There is evidence that there is a relationship between the means by which the bioparticles are opsonised and the effect on phagocytosis. Additionally, specific

bacteria type and whether bioparticles or bacteria were used in the assay may have a significant effect on phagocytic results achieved. Thus, further, more extensive study into how IFNs affect neutrophil phagocytosis is required.

6.2.3. NETosis

NETosis is a type of cell death, whereby neutrophils expel their contents to 'trap' bacterial products and has been shown to be up-regulated in SLE and JSLE (37, 163, 208). IFNs have been suggested to have a role in NETosis through priming and additionally, it has been shown that NETs induce the production of IFNα from dendritic cells (163, 175) (Sections 1.3.3, 1.4.3, and 3.1).

The data in this thesis have shown that IFN and TNFα priming induces some NETs from adult healthy neutrophils (Section 3.5.4). However, this was difficult to detect and was not consistently seen in all images and donors. In this study, two techniques were used to establish the role of IFNs in NET production. Using a confocal analysis, quantification was attempted by three independent operators; one confocal image from each donor was selected per condition and used for analysis. The three operators each counted both the number of viable neutrophils and number of neutrophils producing NETs and calculated the percentage of NETs within the population. For any experimental condition, the mean was calculated, and inter-operator variability was graphically analysed and any inter-operator variability calculated and compared. Although confocal microscopy gave a visual indication of NET formation, multiple operators' quantification comparisons showed that this technique showed high inter-operator variability within certain conditions, and therefore too subjective to be a quantifiable technique. Thus, quantification of DNA was also analysed as DNA is a constituent part of NET material (37).

The DNA quantification analysis only correlated well with analysis of the confocal images in regards to PMA stimulated neutrophils. This was the only condition that substantially increased the DNA release that is characteristic of NETs and was consistently detected by both DNA quantification and confocal analysis. Although these techniques have some validity for NET analysis, there are some experimental issues that were highlighted; staining with DAPI does show some, non-significant, release of DNA from the neutrophils (and thus is likely to be NET material). However, unless this is stained with MPO and NE, there was uncertainty whether the DNA material is: a) of neutrophil origin, and b) NET material. Unfortunately, time became limited at the late stage of laboratory work when these experiments were conducted, as otherwise additional staining would have been helpful to undertake further

validation experiments. Additionally, the DNA assay quantification assay may not be specific for DNA associated with NETs and that DNA from necrotic cells may compromise specificity. Minimal NET formation as observed using confocal microscopy for some of the other conditions in this experiment may not have produced enough DNA to be detected within the limits of the quantification assay. Due to the potential limitations of the DNA quantification technique, future work should include developing a protocol that allows more rigorous scoring of NETs, and therefore allow more consistent and improved inter-operator quantification of the confocal microscopy images. Computational methods of NET detection and quantification have been developed, which may be beneficial to analyse the data in this thesis, although this was not possible within the time frame of studies (216, 217).

Here, it was shown that the IFNs did prime neutrophils to induce NET formation as measured by confocal microscopy. However, this was not observed in all images and donors, and thus remains inconclusive as to whether IFN priming has a definitive role on NETs. However, another study has shown that IFNs prime neutrophils to undergo NETosis upon stimulation with C5a (using DNA staining of neutrophils and confocal microscopy); C5a was not used here (175). It has to be of note that Martinelli *et al.*, did quantify NETs by quantifying DNA release and MPO release using fluorescence and spectrometry quantification techniques respectively. Therefore, a wider investigation into the role of IFN priming on NETs, using a more extensive staining for confocal microscopy, variety of potential NETosis stimulants (such as C5a and HMGB1), and quantification of components such as MPO alongside DNA already used, may be necessary to conclude this IFN function (66, 175).

Contextually, however, NETs are important in inducing IFNα release from pDCs; within JSLE, this interaction could heighten the level of IFNα within the serum (163). As NETs are not disassembled effectively within SLE, chronic activation of pDCs and IFNα release (163, 208) prime neutrophils to undergo NETosis, but only in the presence of other stimuli, creating a feedback loop (68, 171, 172, 175). Additionally, although NET formation seen in this present study is minimal, this could be important as neutrophils are so large in number in the disease state, even a small percentage of NETs could be biologically relevant in the damage of joints or kidney within JSLE.

NETosis assays are traditionally analysed using confocal images, although a more extensive staining is typically used. MPO and NE staining can also be used in conjugation with DAPI to confirm the release of DNA is from NETs. Alongside this, ROS production analysis (ROS is linked to the induction of NETosis) can be conducted (63). It may be that although IFNs do

not induce NETosis, their priming function for NETosis may be due to alteration in ROS production. This may be another way to quantify the rate of NET induction.

The limitations of the assays used in these studies include confocal images with 'clumps' of cells. This made it difficult to differentiate between what was a viable cell and what was a NETing cell, and to discriminate between a large single NET, multiple smaller ones, or a combination. Confocal microscopy can be a very subjective method of NET quantification, and thus quantifying the NETs on these images has limitations. The DNA quantification assay, although superior for quantification than the confocal images, was not set up to detect specifically DNA from NET material and can detect any DNA present in the supernatant. Also, besides PMA, there was no indication of DNA in any of the experimental condition above that of unstimulated cells despite confocal images showing minimal NETs. Thus, the DNA assay may not be sensitive enough to detect small amounts of DNA released in these conditions.

6.3. IFNs and apoptosis

The involvement of the IFN subtypes in apoptosis is well established, and literature has indicated IFNs can have dual, opposing roles in influencing neutrophil apoptosis, which is very dependent on the phosphorylation state of pro-apoptotic STAT1 or anti-apoptotic STAT3 (138, 164, 165, 219, 233) (Sections 1.3.4, 1.4.3, 1.5.3.1, and 4.1). Neutrophils in JSLE undergo increased apoptosis, and this can be related to up-regulated extrinsic apoptotic factors in the serum, such as TRAIL and FasL, through which the serum can additively increase JSLE neutrophil apoptosis *in vitro* (72, 170). In JSLE, there can be changes in the balance of pro- and anti-apoptotic proteins within the neutrophil themselves (170). For example, JSLE neutrophils can demonstrate an increase in caspase 7, 8 and 9 mRNA, and decrease in IAP1, IAP2 and xIAP mRNA in JSLE neutrophils (170). This indicates that signalling pathways within JSLE neutrophils lean towards pro-apoptosis, and thus are likely to be an important contributor to the increased neutrophil apoptosis seen in JSLE (72, 170).

GMCSF has been shown to delay neutrophil apoptosis, and in particular has been shown to delay JSLE serum-induced neutrophil apoptosis via the stability of MCL1 (77, 110) (Section 1.3.4). Thus, GMCSF via the stability of MCL1 was used as an anti-apoptotic control in investigating the effect of IFNs on apoptosis. GMCSF was shown to significantly delay neutrophil apoptosis within this study, correlating well with other studies and was an

effective control for apoptosis assays, particularly in regards to investigating the anti-apoptotic effect of IFNs (77, 110, 141).

Neutrophils in SLE are activated, and it is likely that JSLE neutrophils are activated, which has been shown in this study via the increase in CD11b (83) (Sections 1.3.5, 4.5.8.1 and 5.5.2). In *in vitro* studies, priming of neutrophils with cytokines such as GMCSF, TNFα and IFNγ, changes the way neutrophils respond, with certain functions such as respiratory burst becoming enhanced (81, 90). Additionally, cell membrane and intracellular proteins can be up-regulated or activated upon priming, and these changes in protein constitution on and within primed neutrophils alter how other cytokines inflict their effect to naïve neutrophils (81, 85, 86, 95, 169). Activation of both SLE neutrophils and JSLE neutrophils may therefore alter the constitution of proteins on and within the neutrophils (81, 83, 85, 86, 95, 169). This may include the alteration of intrinsic apoptotic pathways within JSLE neutrophils described above (170). This may influence how IFNs effect apoptosis within JSLE neutrophils compared to that of healthy naïve neutrophils.

TNFα is a potent priming agent for neutrophils and was chosen over GMCSF and IFNγ as it was not an experimental condition used in this study downstream from priming (85, 90, 167) (Section 1.3.5). TNFα was used to prime neutrophils in order to try and model better the activated state neutrophils have been shown to be in SLE. The neutrophils in this present study were confirmed to be activated neutrophils through the up-regulation of CD11b (83) (Section 4.5.8.1 and 5.5.2). TNFα has also shown to be elevated in both the mesangial cells and plasma of patients with lupus nephritis and is associated with increased disease activity (67, 234). Therefore, TNFα is a physiologically relevant priming agent for the *in vitro* model, due to indication of its role in lupus nephritis and SLE (67, 234). It was therefore subsequently used to analyse IFN responses on neutrophils in an activating/inflammatory environment.

Initially, TNFα was used to prime neutrophils *in vitro* to model the activated neutrophils found in SLE and JSLE (83). These primed neutrophils were subsequently saturated with a high concentration of IFNs. Compared to naïve neutrophils, where a high concentration of IFNs reduced apoptosis, priming of neutrophils led to IFNs increasing the rate of apoptosis compared to unstimulated neutrophils This is an important consolidation of previous literature as this *in vitro* model suggests that a high level of IFNs in SLE and JSLE serum is not enough to induce apoptosis in JSLE neutrophils, and there is a need for other factors within the JSLE environment to activate the neutrophils for IFNs to induce apoptosis (138, 164, 165, 219, 233).

The concentration of IFNs used (1-10μg/ml) for above experiments is likely to be a supra-physiological concentration, thus a lower concentration was used for subsequent experiments (Section 4.5). Although 10ng/ml was still higher than that detected in JSLE serum (a range of 13.8 – 22.5 pg/ml of IFNα was detected in two observational studies), it is comparable to standard experimental concentrations of cytokines used *in vitro* (68, 77, 110, 172). Additionally, within JSLE serum levels of IFNs may be lower, and therefore not reflective, of IFN levels in areas of active disease or inflammation within organs (235). In these assays, IFNs again reduced naïve neutrophil apoptosis, and although IFNs reduced the amount of cleaved caspase 3, showing that they are, indeed, delaying apoptosis, it is not through MCL1. The anti-apoptotic STAT3 is likely involved in this delay in apoptosis and may up-regulate such anti-apoptotic proteins such as B-cell lymphoma extra-large (Bcl-X_L) and the IAPs (233, 236, 237). GCSF, STAT3 and cIAP2 have all been indicated to contribute anti-apoptosis in neutrophils and overexpression of cIAP2 contributes to neutrophilia in chronic neutrophilic leukaemia (78). Both type 1 and type 2 IFNs can delay neutrophil apoptosis via the activation of STAT3 and subsequent up-regulation of cIAP2 protein and mRNA transcripts (233). The pro-apoptotic STAT1 was also phosphorylated in these neutrophils (141). Thus, a fine balance between the activation of STAT1 and STAT3 may be important in how neutrophils respond to IFNs in regards to apoptosis, and thus needed more investigation within this study (138, 233).

Although this confirmation of the type 1 and 2 IFN role in reduced apoptosis, and elucidation of the pathway the IFNs may be signalling through to reduce apoptosis is important, this role in apoptosis was in naïve neutrophils. It was therefore also important to demonstrate the role of IFNs in activated or primed neutrophils; which may reflect better the status of neutrophils in inflammatory conditions such as JSLE (83). The investigation using the lower concentration of IFNs was repeated on TNFα-primed neutrophils; these primed neutrophils were used to model the activated JSLE neutrophils *in vitro*. Within this thesis, it was shown that the delay in apoptosis using 10ng/ml was abrogated in TNFα-primed neutrophils, which could potentially enable other factors, such as TRAIL and FasL (that have been shown to be elevated in JSLE serum) to induce apoptosis (72) (Section 4.5).

Cytokines rarely function alone, and as noted above, cytokines other than IFNs can have a priming effect that can influence the effect of IFNs on apoptosis. Importantly as stated above, the serum of JSLE patients has increased apoptotic factors, such as TRAIL and FasL amongst other pro-inflammatory factors which may also influence the effect of IFNs on apoptosis (72). As JSLE serum has been shown to increase neutrophil apoptosis (110) and that GMCSF can

reduce the level of apoptosis induced by JSLE serum (110), it was important to also investigate the effect IFNs may have on neutrophil apoptosis in the presence of other factors within JSLE serum.

Healthy control, inactive JSLE and two types of disease active JSLE serum (renal active and haematological active) were used to pre-treat adult healthy neutrophils before incubation with IFNs (Section 4.5.7). The rationale of the different types of JSLE serum was that in inactive patients, their disease is under control and least likely to induce neutrophil apoptosis compared to paediatric control serum. Within sera from patients with activity in the haematological domain, one of the clinical characteristics is a decreased white blood count, which includes a decreased level of neutrophils – these levels may reflect what is happening systematically and is an environment that would directly modulate the neutrophils, which are a primary interest of this thesis. Additionally, sera may contain increased factors, such as FasL and TRAIL, that lead to the increased neutrophil apoptosis that has been investigated greatly within this thesis (72). Patients with active renal manifestations was used as, although this was more localised to one organ, lupus nephritis is a major complication seen in JSLE, which occurs more frequently and more severely than on adult onset SLE patients, and may be more reflective of overall active JSLE disease than activity in the haematological domain (1, 238). Additionally, renal involvement in JSLE is a particular research interest of the UK JSLE cohort (239, 240). Given the complexity of JSLE, whereby classification of active disease can be organ domain specific, it was of interest to investigate how sera from inactive JSLE patients and JSLE patients with two different classifications of disease activity influences a) neutrophil activation, b) neutrophil apoptosis and c) the effect of the different subtypes of IFNs on apoptosis.

All patient sera groups were able to activate healthy adult control neutrophils. This observation did not differ between paediatric control sera and JSLE sera, or between the JSLE sera subtypes. The increase in adult neutrophil activation by the patient sera was a result of exposure to a foreign environment rather than a specific serum profile. Overall the serum did not significantly induce apoptosis, and neither IFNs nor GMCSF had any significant effect on this, in contrast with other published findings (72, 110). However, this study used adult healthy neutrophils, which may be less responsive to paediatric serum in regards to apoptosis, and in particular compared to JSLE neutrophils.

Future experiments investigating the compounded effect of serum and IFNs should include incubation of paediatric or JSLE neutrophils with paediatric control or JSLE serum. Unlike the

adult neutrophils here, published observations that both paediatric control neutrophils and JSLE have been shown to undergo increased apoptosis in response to JSLE serum (72, 110). These published studies could be expanded to investigate how JSLE sera of different disease states and organ involvement could affect paediatric control and JSLE neutrophil activation and subsequently how IFNs influence downstream apoptosis. These experiments would expand on published observations and the observations depicted in this thesis, and give a thorough overview on the effect of apoptosis on neutrophils from a variety of patient groups and environments which would reflect the complexity of JSLE

Within paediatric control neutrophils and JSLE neutrophils, GMCSF was able to delay apoptosis and this was consistent with previous data and literature (72, 110) (Sections 1.3.4, 4.1 and 4.5.8). Additionally, IFNγ was able to delay apoptosis in JSLE neutrophils, but not in control neutrophils. This is of interest as not only were control neutrophils more varied in response to cytokines, but there was also no overall difference in apoptosis between paediatric control and JSLE neutrophils. In the adult neutrophil model, the IFNs do induce apoptosis, but only at a very high IFN concentration (1-10μg/ml). Therefore, it would be of interest to expose JSLE neutrophils to higher concentrations of IFNs, with a particular focus on neutrophils from individuals with active JSLE disease. These neutrophils are likely to be more activated than the patient neutrophils used in this study and may be more likely to undergo IFN-induced apoptosis.

JSLE neutrophils have a more active intrinsic pro-apoptotic signalling pathway, via the up-regulation of caspase mRNA and the down-regulation of IAP mRNA (170). Thus, in theory, IFNs should be pro-apoptotic towards JSLE neutrophils if the neutrophils are already 'primed' for this effect. However, the data in this thesis shows that this is not necessarily the case and that neutrophils may need the pro-apoptotic environment found in JSLE and may have a 'healthier' response in *in vitro* conditions (such as in 10% FBS). The neutrophils used in these experiments were from JSLE patients with tight disease control and had a lower overall pBILAG score compared to other cohorts investigated in the literature, which may contribute to this response observed (72, 110). Additionally, although they have the apoptotic potential through the up-regulation of caspases, this seems to be insufficient for IFNs to induce apoptosis, and further investigation into the signalling pathway of IFNs was needed.

Neutrophil apoptosis involves the loss of the FcγRIII (an immunoglobulin receptor found on the surface of neutrophils) (241, 242). This loss of FcγRIII in neutrophil apoptosis also associates with annexin V binding sites, and therefore is possibly an important step in the

early stages of apoptosis (242). Importantly, a published study showed that there is an association between different aspects of SLE and polymorphic variants of FcγRIII genes (243). In particular, polymorphisms of the FcγRIIIa gene are particularly associated with lupus nephritis (243). The FcγRIIIa gene polymorphisms may dysregulate, in part, the FcγRIII protein in some SLE patients (and by association some JSLE patients) which may be contributing to increased SLE and JSLE neutrophil apoptosis (72, 230). It would be of interest to investigate whether any of the polymorphic variants and protein expression changes of FcγRIII, associate with the increased neutrophil apoptosis seen in JSLE, and how these changes influence any effect of IFNs on neutrophil apoptosis (72).

IFNγ signalling can be inhibited by immune complexes, and this inhibition is dependent on FcγRIII (244). Pre-incubation of macrophages with intravenous immunoglobulin (IVIG) for 16-24 hours abolished IFNγ-stimulated STAT1 phosphorylation (244). Downstream, IFNγ stimulated monokine induced by γ IFN (MIG), IP10 and IRF1 mRNAs were suppressed following IVIG pre-treatment, (244). Additionally, IVIG was able to down regulate the expression of IFNGR2 but not IFNGR1, , confirming that the IFNγ signalling pathway is supressed by the IgG immune complex (244). Within NK cells, that only express FcγRIII and no other Fcγ receptors, IVIG inhibited IFNγ signalling, but this was not seen in B cells that express only FcγRIIb, indicating FcγRIII is involved in IFNγ signalling (244). Additionally, within murine macrophages deficient in FcγRIII, IVIG did not supress IFNγ signalling (244). Therefore, it would be of interest in future work to investigate how IFNγ signalling and the FcγRIII are linked in regards to neutrophil apoptosis, and additionally if FcγRIII has a similar effect on type 1 IFN signalling.

Further analysis of the effect of IFNs on neutrophil apoptosis could involve the analysis of different apoptotic proteins or genes. For example, membrane associated apoptotic proteins such as Fas can be analysed via flow cytometry. Intracellular proteins such as caspase 8 and 9 can be analysed by Western blotting. Apoptotic genes such as IAPs can be analysed via qPCR. These analyses could be measured at baseline within all neutrophil types (adult healthy, paediatric control and JSLE) and upon TNFα-priming and IFN stimulation. The analysis of caspases would confirm which apoptotic pathway (extrinsic or intrinsic) the IFNs may influence. It would also be of interest to use the analysis described above to investigate how priming of neutrophils affects the composition of apoptotic proteins within neutrophils, and how these compare to JSLE and paediatric control neutrophils. The rationale of this analysis is that it is known that there are intrinsic differences of apoptotic proteins between paediatric control and JSLE neutrophils, and an appreciation of how this arises would

contribute to the knowledge base of the apoptotic process and highlight any potential therapeutic targets (170). It would also be of interest in how the IFNs, and their signalling pathways, influence apoptotic genes in regards to priming, and in both healthy and JSLE neutrophils. Additionally, the UK JSLE Study Group has shown a dysregulation of pro- and anti-apoptotic factors within JSLE serum, and thus it may be important to analyse the expression of the neutrophil receptors and subsequent signalling pathways for these factors in JSLE neutrophils and understand how these other factors may influence the IFN effect on apoptosis (72, 170).

Methods other than flow cytometry can be used to measure apoptosis. These include: (1) light microscopy, which can be used to analyse trypan blue stained, non-viable cells, which can be distinguished from other, viable cells. However, although a cheap way to analyse viability, this requires counting manually and introduces operator variability. (2) Western blotting, which can be used to measure the level of expression of apoptotic proteins. Although good for intracellular protein analysis, Western blots are only semi-quantitative, and the multiple steps need optimisation and allow for increased technical errors. They are also time-consuming compared to other techniques, and not as sensitive as techniques such as ELISAs. (3) Immunohistochemistry can also be used to visually analyse different apoptotic proteins within and on the cell. Immunohistochemistry is not quantifiable and can introduce operator subjectivity. However, this thesis used the annexin V/PI flow cytometry analysis, as this was consistently accurate, quantifiable assay that could distinguish viable cells from cells that are undergoing early and late apoptosis, and those cells undergoing necrosis (245). Dye based assays can be used to measure the number of viable cells. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay involves reducing the water soluble MTT compound to an insoluble formazan compound in the presence of viable cells. The colour goes from yellow to purple, and this colour change can be read and quantified using a plate reader. However this method would not necessarily differentiate cells in different stages of apoptosis or distinguish different types of cell death, which was an important analysis within this thesis (5). DNA fragmentation analysis could also be used to quantify apoptotic cell death by analysing DNA breaks that are characteristic of apoptosis. Terminal deoxynucleotidyl transferase (TUNEL) can catalyse the addition of 2´-deoxyuridine, 5´-triphosphate (dUTP) nucleotides to the free 3' ends of fragmented DNA. These dUTPs can contain fluorescent labels that can be detected by either microscopy or quantified using flow cytometry.

In regard to the analysis of MCL1 and caspase 3, Western blots are routinely used for intracellular protein analysis due to their reduced expense in comparison to other techniques

and the ability to analyse several proteins on the same blot, saving time, precious samples and additional expense. However, due to their limitations (see above), other techniques could be used to analyse these proteins, where some are fully quantitative. Flow cytometry can be used to analyse intracellular proteins and would require less time and less cells than Western blots, and also give a quantitative result. However, fixing of cells that would be required for flow cytometry can cause stable protein/protein interactions that prevent antibodies from binding, and thus skew results. This is not an issue in regards to Western blotting; the reducing and denaturing effect of the lysis buffer, with addition of the boil prep disrupts any protein/protein interactions and allows antibodies full access to the protein structures. ELISA can also give a fully quantitative analysis of intracellular proteins; however, they can be expensive. Immunocytochemistry can also be used to give a visual analysis of intracellular proteins; however, this is not necessarily quantifiable and signal can degrade if the slides are not punctually analysed

6.4. IFN signalling

IFNs bind to specific receptors (type 1 IFNs bind IFNAR and type 2 IFNs bind IFNGR) on the cell surface and downstream signalling is via the JAK/STAT pathway. IFNs phosphorylate STATs, whereby STAT1 is pro-apoptotic and STAT3 is anti-apoptotic (138, 141) (Sections 1.5.3.1 and 5.1). This has highlighted an important pathway to investigate in regards to IFNs and apoptosis within JSLE neutrophils.

The differences in signalling pathway, and thus how the different IFN subtypes influence apoptosis, may be dependent on whether neutrophils are activated. Here, it was investigated how activation of neutrophils influences the IFN signalling pathway (Section 5.5).

Data in this thesis showed that within TNFα-primed adult control neutrophils, there is a significant reduction of the IFNAR1 chain expression compared to naïve neutrophils, and there was indication that the IFNGR2 chain increased, although not significant (Section 5.5.1). This decrease in IFNAR1 expression correlates particularly well with the reduction in pSTAT3/STAT3 expression ratio also observed, indicating that activation of the STAT3 pathway is dependent on IFNAR1 expression (Section 5.5.3). It should be of note that blocking the IFNAR1 chain has been shown to be a beneficial therapeutic target for SLE patients (Section 6.7.2), and thus this pathway may need to be further investigation within JSLE (246). IFNGR2 was up-regulated (although the increase was not statistically significant) upon TNFα or IFNγ stimulation; however, no differences were seen in either chain of the IFNγ receptor between JSLE and paediatric control neutrophils (Sections 5.5.1 and 5.5.2). It has been shown

that IFNGR2 expression can influence whether IFNγ induces proliferation in T cells (when IFNGR2 expression is low) or apoptosis (when IFNGR2 expression is high) (162) (Sections 1.5.2 and 5.1). In immune cell lines the up-regulation of IFNGR2 on T cells, through serum starvation, can lead to apoptosis (161). However, this observation was not seen in the myeloid cell line (which was the closest cell line to neutrophils studied), which had a constitutionally high expression of the IFNGR2, as well as the constitutionally expressed IFNGR1 (161) (Sections 1.5.2 and 5.1). This may explain why no significant changes were observed within the activated neutrophil model or within patient neutrophils.

It has to also be noted that the activation state of the JSLE neutrophils was only shown through a non- significant increase in CD11b, and there was no difference in CD62L between JSLE and paediatric control neutrophils (Sections 4.5.8.1 and 5.5.2). This indicates that compared to the *in vitro* model, the JSLE neutrophils weren't as activated as the TNFα-primed neutrophils. However similar observations have been seen in SLE neutrophils (83). Thus, it may be that JSLE neutrophils need to be significantly activated for the IFN receptor expression to change (as shown with the TNFα-primed *in vitro* model), and affect downstream signalling, including apoptosis, as seen in a published study (161). Again, neutrophils used in this analysis were from JSLE patients whose disease was well managed. It would be of interest for future experiments to include JSLE patients with a more active disease and analyse receptor expression on JSLE neutrophils that may be more activated than the ones used here.

Experiments were conducted to investigate the balance of STAT1 and STAT3 phosphorylation between naïve and TNFα-primed neutrophils, and how this relates to the expression of the IFN receptor chains. Through Western blots, the study showed that the pSTAT3/STST3 expression ratio was increased more by type 1 IFNs than type 2 IFNs; whereas pSTAT1/β actin expression ratio was increased more by type 2 IFN, particularly in TNFα-primed neutrophils (Section 5.5.3). Within this thesis, it was also shown that pSTAT3/STAT3 expression ratios were reduced in primed neutrophils compared to naïve neutrophils, and pSTAT1/β actin expression ratios were increased in TNFα-primed neutrophils Section 5.5.3). It should be of note that a lower concentration (1ng/ml) of TNFα was used to prime neutrophils for STAT analysis. Different concentrations of TNFα can affect neutrophil responses and downstream protein signalling (221). In this context, data shown in this thesis suggest that STAT phosphorylation is more acutely influenced by TNFα priming than maybe that of apoptosis, and that the higher concentration of TNFα used in other assays may amplify the differences in phosphorylation of STAT1 and 3 between naïve and TNFα-primed neutrophils. It has been

shown that phosphorylation of the tyrosine within the protein structures in neutrophils is amplified dramatically by TNFα priming, and this amplification increases upon increasing doses of TNFα (247). In another study, priming of neutrophils with 10ng/ml TNFα for 1-2 minutes resulted in a varied increase in apoptosis upon subsequent stimulation by a variety of cytokines (248). An increase in apoptosis in TNFα-primed neutrophils stimulated with IFNγ compared to naïve neutrophils was observed, however this was not significant or as dramatic as the phosphorylation of tyrosine (247, 248). This small increase in apoptosis by TNFα priming was not consistently shown for all cytokines; apoptosis significantly increased in TNFα- primed neutrophils upon stimulation with factors such as PMA and fMLP (248). Therefore, the hypothesis is that intracellular signalling is more sensitive to the priming effects of TNFα than that of more generalised neutrophil functions, such as apoptosis, which may require a higher concentration of TNFα to be affected.

This difference in STAT phosphorylation seen in the *in vitro* model may be true of JSLE neutrophils. Literature has shown that in SLE, STAT1 is more highly expressed and pSTAT1 is up-regulated within PBMCs, and it was observed that the STAT1 pathway was activated in a mouse model of lupus nephritis, and thus it is likely that the pro-apoptotic STAT1 pathway may be heightened in JSLE neutrophils, contributing to the increased apoptosis seen in these cells (194, 196). Again, this may be due to the fact that SLE and JSLE neutrophils are activated, as activation can causes dysregulation of proteins, markers and functions (83, 85, 86, 90). It should be noted that in this study, the JSLE neutrophils were activated compared to paediatric control neutrophils, but not as significantly so as TNFα-primed adult neutrophils compared to naïve neutrophils. If JSLE neutrophils are investigated for STAT1 phosphorylation, the difference observed may not be as significant compared to that shown by the TNFα-primed models. Additionally, neutrophils from active JSLE patients may be more important to investigate the signalling pathways as they may be more activated and thus akin to the TNFα-primed neutrophils.

These data as well as previous studies suggest that STAT1 is important in IFN-related apoptosis, and possibly in SLE and JSLE pathogenesis (138, 194, 196). If IFNs are causing the observed increase in neutrophil apoptosis in JSLE, it is probably via STAT1, which has been suggested to be activated in SLE (72, 138, 194, 196). Thus, it was investigated whether inhibiting pSTAT1 may delay IFN-related pro-apoptosis and increase IFN-related anti-apoptosis through up-regulation of pSTAT3 in TNFα-primed neutrophils.

The initial STAT1 inhibitor used was fludarabine phosphate (Section 5.5.4). Fludarabine phosphate was selected as its mechanism of STAT1 inhibition has been described. Fludarabine phosphate functions through binding to the SH2 domain of STAT1 and preventing STAT1 phosphorylation of the tyrosine residues and subsequent dimerization of STAT1 with other STAT proteins (249). It had a promising function in delaying neutrophil apoptosis through STAT1 inhibition, as published data showed that fludarabine phosphate can inhibit STAT1 in PBMCs, which were still viable after fludarabine phosphate incubation (200). At the time of inhibitor selection, fludarabine phosphate was the only viable option, and it was already used as a chemotherapy agent with a known safety profile (200, 229). However, another report suggests that it has no effect on STAT1 (250). Data in this thesis additionally saw that although it did reduce pSTAT1/β actin expression ratios, indicated a possible inhibition of pSTAT1, fludarabine phosphate also reduced pSTAT3/STAT3 expression ratios, showing inhibition of STAT3 phosphorylation (Section 5.5.4). Thus, fludarabine phosphate was not deemed appropriate to investigate further.

S1495 was subsequently used as an STAT1 inhibitor (Section 5.5.5). S1495 was shown to reduce pSTAT1/β actin expression ratios, indicated a possible inhibition of pSTAT1, with no effect on pSTAT3/STAT3 expression ratios (Section 5.5.5). However, this inhibition was variable which in part maybe donor dependent, or due to problems with the solubility of S1495 in DMSO and its possible impact on inter-experimental variability in the final s1495 concentration (see Chapter 5). Interestingly S1495 had a consistently anti-apoptotic effect in TNFα-primed neutrophils, with IFNs having no additional effect, and additionally S1495 had no effect on naïve neutrophils (Section 5.5.5). From these observations, it was hypothesised that, although an increase in pSTAT1/β actin expression ratio in TNFα-primed neutrophils was not observed, TNFα may have some influence on the STAT1 pathway. TNFα has been previously shown to have an effect on STAT1, in in cell lines such as HeLa, A431 and 2fTGH, however this is inconsistent with the data in this thesis which did not show that TNFα had any effect on pSTAT1 expression in neutrophils, and thus it remains inconclusive (251, 252). To establish whether S1495 could be used as an inhibitor of STAT1 phosphorylation more investigation is required. However, due to its anti-apoptotic effect on TNFα-primed neutrophils, without IFN stimulation, and the fact that its inhibition of STAT1 was donor dependent, it may not be appropriate to fully investigate STAT1 role in JSLE neutrophil apoptosis.

Western blot analysis was used to measure the activation of STATs by measuring the expression of pSTATs. Additionally, due to issues with the pan STAT1 antibody (which did not

detect the protein even after multiple optimisation trials) changes in pSTAT1 relative to β actin were reported here. Flow cytometry again would give a more quantifiable analysis of the expression of STATs within the cell. However, again fixing of cells that would be required for flow cytometry can cause stable protein/protein interactions that prevent antibodies from binding, and thus skew results. Lysing of proteins for Western blot preparation disrupts protein/protein interactions and allows antibodies full access to the protein structures. A Luminex multiplex kit was investigated to analyse all STATs simultaneously, which would have increased reliability, consistency and given a more quantifiable result. However, at time of investigation, analysing both pan and phosphorylated STAT1 in the same sample was not possible, and the kits are very expensive compared to other techniques.

Although it was attempted, with some success, to inhibit STAT1 phosphorylation (although it remains inconclusive if this supposed decrease in pSTAT1 was due to a reduction in overall pan STAT1, or specifically pSTAT1), it was not consistently effective. Thus, other techniques that can be used to specifically inhibit STAT1 include use of small interfering RNA (siRNA) to inhibit the STAT1 at the gene transcription level. This would prevent protein translation and therefore expression of STAT1. However, published data have shown reduction of the STAT1 protein in DCs following 2-3 days incubation of the siRNA, and thus this may not be appropriate to use in neutrophils, which have a shorter life span (253, 254).

Within this section of the study, the experiments were limited mainly in how to rigorously analyse the STAT1 and STAT3 proteins, in that the Western blot could not be optimised for the pan-STAT1 antibody. Additionally, the specific inhibition of STAT1 was problematic, and thus limited the conclusions that could be made based on the limited results obtained. Western blots, in regards to the pan STAT1 antibody and new techniques in regards to STAT1 inhibition (as described above) would hopefully allow for a more in-depth investigation in the future.

6.5. Limitations

A limitation in this study was the access to JSLE patient samples in different disease states. JSLE patient numbers required for biosamples for the study are limited, and specifically the frequency of samples and the paediatric blood volume collected restrict the experiments that could be conducted. Analysis using flow cytometry is usually possible, due to a small yield of neutrophils required compared to the yields required for Western blotting. However, due to

the small volume of JSLE patient blood samples, and that the final neutrophil yield was frequently low, an appropriate yield of JSLE neutrophils to extract protein to analyse STAT1 and 3 phosphorylation states was not frequently possible. Consideration of the effect of treatment for clinical symptoms, whereby all patients are on to maintain their disease, on neutrophil protein expression is required. Possible approaches to address this consideration may include incubating neutrophils with common medication such as hydroxychloroquine and mycophenolate, or protein analysis of neutrophils from patients with a variety of medications.

Previous studies investigating neutrophil phagocytosis and chemotaxis had suggested a wide scope of signalling which IFNs may be affecting in these functions, such as affecting chemotactic receptors CCR1 and CCR3, and phagocytic receptor FcγRI (177-179, 183-185, 215). Although some of these may have been addressed, time and resources meant that this study was limited in what could be investigated. Therefore, in regards to phagocytosis and chemotaxis, analysis was conducted using an already optimised protocol, and conditions were chosen based on association with other experiments within this thesis (i.e. IFN priming) and also have been indicated to be functionally important in regards to neutrophils (such as IL-8). Chemotaxis and phagocytosis have also been indicated to be dysregulated in SLE and JSLE, and therefore were important to investigate. However, IFNs role in chemotaxis and phagocytosis could not be comprehensively concluded in this study due to only a snapshot of these functions being investigated. Therefore, it would be of importance to fully investigate these complex and diverse neutrophil functions outside the context of this thesis.

6.6. Future work

This study has highlighted new avenues for future work. From data obtained in this thesis, new questions have arisen through the investigation of the interaction of IFNs and neutrophils in JSLE. These are discussed below.

6.6.1. Neutrophil function

For phagocytosis assays, only opsonised *E.coli* phagocytosis were investigated. Although IFNs can affect phagocytosis, this is dependent on many factors including cell type, bacteria/pathogen type, and whether the bacteria or pathogen are opsonised (183-185, 215). However, literature on the role of IFNs in phagocytosis in general, (whereby generally IFNs increase phagocytosis) and phagocytosis in SLE and JSLE (in which neutrophil and immune cell phagocytosis is reduced) is unequivocal (112-115, 183-185, 189, 215). In this regard, it is important to consider any effect of disease-related medication on neutrophil

phagocytosis in JSLE. Thus, it would be important to investigate through flow cytometry: a) how IFNs influence neutrophil phagocytosis of other non-opsonised and specific opsonised pathogens and b) whether IFNs save the phagocytic function of JSLE neutrophils or reduce neutrophil phagocytosis function further.

Data in this thesis that IFNs do not induce any significant NET formation, although priming with TNFα or IFNs with additional stimulus minimally increased NETs, is in keeping with previously data (175). These published data showed that priming with IFNα, IFNγ or GMCSF, and subsequent stimulation with C5a-induced the formation of fibres, similar to NETs, in mature but not immature neutrophils (175). These immature neutrophils could be LDGs. These granulocytes have been detected within PBMC fractions of isolated blood, and Kaplan *et al.* have isolated these cells from patients with SLE using negative selection, and additionally have been shown to be increased in JSLE patients (40, 119). Phenotypically, LDGs, additionally known as low density neutrophils (LDNs), neutrophil markers; however CD15, CD11b and CD66b were higher on LDNs than on high density neutrophils (HDNs) (255). These LDGs have an enhanced ability to produce type 1 and type 2 IFNs (40, 256). However, Kaplan *et al.* have suggested that LDGs have an increased ability to undergo NETosis, and thus LDGs are unlikely to be the immature neutrophils investigated by Martinelli *et al*. (40, 175). It was shown the extracellular fibres contained DNA and MPO, confirming that IFN priming plus C5a stimulation resulted in NET formation (175). However, stimulation with IFNs, GMCSF or C5a alone did not induce NETs (175). Thus, it is likely that any role that IFNs have on NETosis, at least in healthy neutrophils, is purely through priming. These findings are important when considering IFNs role in JSLE neutrophil NETosis. Although IFNs probably do have an important priming role they may not be the most important mediator of NETosis induction, and thus it may be more important to investigate what is causing the IFN-primed neutrophils to NET. These mediators may include that of C5a and HMGB1 (66, 175).

Confocal microscopy methods used in this thesis to investigate induction of NETosis did not result in reliable data that always correlated well with DNA quantification data. The reliability, and therefore the validity, of the confocal image data interpretation would therefore need to be addressed before any solid conclusion can be made. Additional staining, through the staining of MPO and NE, may be beneficial to improve visual data outcome of these experiments. Additionally, conducting these experiments in JSLE neutrophils would add another important dimension; a combination of IFNs would replicate what JSLE neutrophils experience *in vivo* and would be of interest as cytokines rarely function alone.

6.6.2. Apoptosis

This study has shown that the IFNs can be both pro- and anti-apoptotic for neutrophils and this is dependent on whether the neutrophils are activated or naïve. However, cytokines rarely function alone, and observing the effects on neutrophil apoptosis of combinations of IFNs and in the presence of other pro and anti-apoptotic cytokines, such as FasL and GMCSF, would be paramount. FasL in particular has been shown to be increased in JSLE serum, and therefore is likely to not only have an effect on neutrophil apoptosis itself, but IFNs and FasL may influence each other's effect on apoptosis (72). If IFNs are acting as pro-apoptotic factors, a combination of FasL and IFNs may result in accelerated neutrophil apoptosis compared to the rate of neutrophil apoptosis seen with incubation of each cytokine alone. Alternatively, if IFNs lose their anti-apoptotic ability on JSLE or activated neutrophils, this may increase FasL-related apoptosis due to the IFNs inability to counteract this effect. In regards to GMCSF, it has been indicated that this cytokine is reduced in JSLE serum (72). it has also been demonstrated that GMCSF reduces JSLE serum-induced neutrophil apoptosis (110) If IFNs are acting as pro-apoptotic factors, GMCSF may counteract this effect and reduce the JSLE neutrophil apoptosis that may be comparable to healthy control neutrophils. Alternatively, if IFNs lose their anti-apoptotic ability on JSLE or activated neutrophils, GMCSF may not only be able to induce the anti-apoptotic effect in place of IFNs, it would most likely have a stronger anti-apoptotic effect and again reduce the JSLE neutrophil apoptosis to be comparable to healthy control neutrophils.

JSLE serum activates neutrophils and prevents IFNs from delaying apoptosis; however, it does not increase apoptosis as observed is some studies (72, 110). As paediatric control serum also had this effect, the results seen in this thesis may be due to healthy adult control neutrophils responding to homologous serum which is not specific to JSLE. It has been shown that both paediatric control neutrophils and JSLE neutrophils undergo increased apoptosis when primed with JSLE serum. However, the rate of increased apoptosis was greater in JSLE neutrophils than paediatric control neutrophils (72, 110). From this, it would be of interest to incubate paediatric control and JSLE neutrophils with IFNs in the presence of JSLE serum to see how IFNs might affect this increase in apoptosis (72, 110). Additionally, analysis of cytokines within sera, with a particular focus on IFNs may be beneficial; IFN expression within sera of healthy patients and volunteers may be increased if the patient/volunteer was recently exposed to a virus. Therefore, these IFN levels within the sera, along with other inflammatory cytokines, may affect neutrophil activation and apoptosis compared to sera with normal levels of IFNs and cytokines.

Additionally, data showed that there was no difference in baseline or cytokine influenced apoptosis between paediatric control and JSLE neutrophil apoptosis at 6hr, which does not correlate with previous literature (71, 72). However, the JSLE patients used had their disease well managed at time of blood sampling and this may be due to their steroid and disease-modifying anti-rheumatic drug (DMARD) medication. This may dampen any dysregulated neutrophil response, including increased apoptosis (72). It would be of interest to include patients who are flaring/have very active disease at time of sampling. This may show the increased apoptosis seen in other literature and show how IFNs affect this (71, 72).

6.6.3. IFN signalling

There are differences in the IFN/JAK/STAT signalling pathway between naïve and TNFα-primed neutrophils. Specifically, there is a down-regulation of STAT3 and an up-regulation of STAT1 phosphorylation within TNFα-primed neutrophils, and this may be due to the dysregulation of IFN receptor chains and may lead to the increase in apoptosis seen.

Although there was some indication of activation of JSLE neutrophils, the receptor analysis did not reflect the TNFα-primed, adult neutrophil *in vitro* model used in this thesis. This may be because the TNFα-primed adult neutrophils were more activated, or that again these patients' disease was inactive and well managed by medication. Thus, further experiments, that may include neutrophils from JSLE patients with active disease, may contribute to a better understanding of data, specifically in regards to IFN receptors. Additionally, full analysis of the sensitivity of IFN receptors, that go beyond the initial downstream signalling analysis conducted in this thesis (through STAT phosphorylation) may also be beneficial and add scope to the importance of altered expression levels seen.

The role of STATs in IFN signalling is complex, and additional investigations within JSLE and paediatric control neutrophils are needed to fully define their roles. It would be of interest to see if these data reflect that of naïve and TNFα-primed adult neutrophils, and whether inactive and active JSLE neutrophils have different pan and phosphorylated STAT expressions. Additionally, IFN/JAK/STAT-induced genes may confirm which STAT is more active within JSLE neutrophils and confirm how the IFNs affect the neutrophils and JSLE pathogenesis.

The experimental conditions used in this thesis were unable to provide confirmatory data on STAT1 inhibition to see if this prevents IFN-induced apoptosis, these experiments were not successful. S1495 may have potential if its dissolvability is improved; however, the ability of S1495 to delay apoptosis highlights a major issue that may not be resolved. Other options for inhibition of STAT1 in experimental cell models that could be investigated are siRNAs towards

the STAT1 gene. Alternatively, a STAT1 knock out mouse model could be used to investigate IFN-related effects on neutrophils and the immune system. These STAT1 knock out mice could also have lupus induced by pristine to see how the STAT1 knock out effects the development of SLE in regards to IFN signalling pathways, gene signatures, and neutrophil apoptosis.

6.6.4. LDGs

As described in the literature review, LDGs are a subset of granulocytes that are found in the PBMC layer of fractioned blood (39, 40). LDGs have been observed to be increased in SLE and JSLE blood compared to healthy controls and this increase correlates with active disease (42, 119). LDG NETosis has been indicated to contribute to inflammation and organ damage; in particular lupus nephritis (40). Additionally, LDGs have an increased ability to produce IFNα (40). Therefore, LDGs may be contributing to SLE and JSLE pathogenesis through directly causing organ damage, and contributing to the pro inflammatory environment by producing cytokines such as TNFα and IFNα.

Previous literature had stated that LDGs had decreased phagocytosis and increased NETosis compared to normal density LDGs which may contribute to decreased neutrophil phagocytosis and increased NETosis seen in SLE and JSLE (40). Within this study, there was no observed significant differences in neutrophil phagocytosis or with NETosis upon either IFN priming or IFN stimulation. Although as already discussed, there are other implications that may have resulted in lack of observed results, it may be that if IFNs are indeed affecting phagocytosis or NETosis, the affect may be on LDGs and not neutrophils. It would be therefore of interest to investigate the role of IFNs on LDG phagocytosis and NETosis, and how this reflect that of these functions in SLE and JSLE pathogenesis.

6.7. Therapies

6.7.1. IFN therapies and the induction of lupus-like symptoms

The importance of IFNs in SLE pathogenesis has been highlighted by the fact that IFN therapies are capable of inducing a lupus-like disease. IFNα has been shown to induce SLE in a patient treated for cryoglobulinaemic vasculitis associated with hepatitis C with IFNα therapy (257). SLE or lupus-like syndromes seems to be a common complication of IFNα treatment of hepatitis C (258-260). SLE or lupus like symptoms such as arthritis, arthralgia, leukopenia, fever and erythematosus maculopapular rash are also induced by IFNα treatment of malignancies and SLE like markers antibodies such as dsDNA and ANA were

increased (261, 262). These studies show the role of IFNα in clinical manifestations and symptoms that mirror that of SLE, and thus it is possible that IFNα is important in SLE pathogenesis.

6.7.2. Blocking IFNα as a potential therapeutic target in SLE

As the IFN subtypes, particularly IFNα, have been implicated to be involved in SLE and JSLE pathogenesis, a key step is to investigate whether blocking IFNα, and thus its signalling pathway, would be therapeutically beneficial to SLE patients (41, 68, 163, 171, 172, 174, 190). There are monoclonal antibodies (administered as biologic drugs) that target IFNα and its signalling pathways. Some of these are now being trialled in adults within phase 1 and 2 clinical trials in the treatment of SLE (246, 263-267). For example, two block the IFNα protein itself (sifalimumab and rontalizumab) and another blocks the IFNAR (anifrolumab) (246, 263-267).

A fully human IgG1κ monoclonal antibody has been developed that binds to the majority of IFNα subtypes, and by this can inhibit IFN signalling within a phase 1 clinical trial (263). 21 genes that form part of the type 1 IFN gene signature were investigated in a phase 1a clinical trial as pharmacodynamics biomarkers to determine if the antibody effectively blocks IFNα activity (263). It was shown that there was a dose dependent increase in the neutralisation of IFN gene signature within whole blood by using the monoclonal antibody in SLE patients (263). It was also shown that the increased type 1 gene signature within skin lesions and associated proteins could also be neutralised by the antibody (263).

This human IgG1κ monoclonal antibody, re-marketed as sifalimumab, which can bind to IFNα with high affinity and prevents IFNα binding to the IFNAR, has been tested in other clinical trials (264). One study, looking at sifalimumab in a phase 1a clinical trial, showed that most adverse effects (AEs) in this study were mild or moderate, incidence of infection was similar between sifalimumab and placebo populations and no patients developed antibodies towards sifalimumab (264). Within the sifalimumab group, patients had a dose dependent inhibition of the type 1 IFN signature in whole blood, and two type 1 IFN inducible proteins, ubiquitin specific peptidase 18 (USP18) and epithelial stromal interaction 1 (ESI1) were reduced in paired skin biopsies upon 10ng/kg administration of sifalimumab (264). Disease activity also decreased in patients administered sifalimumab compared to placebo, defined by SLEDAI score, SLEDAI flare and BILAG flare (264).

Another phase 1b clinical trial of use of sifalimumab in SLE, suggested that multiple doses of sifalimumab were safe and tolerable, and similar rates of AEs were found in placebo and

treatment groups (265). It was shown that SLE flares decreased when higher sifalimumab doses were administered (265). Multiple-dose pharmacokinetics (PK) of sifalimumab was linear and proportional to the dose administered, and had a long half-like of 20-29 days, and therefore the antibody was long lasting and was not systematically cleared quickly (265). On initial inspection, the effect of sifalimumab on disease activity, through analysis of Safety of Estrogens in Lupus Erythematosus National Assessment - Systemic Lupus Erythematosus Disease Activity Index (SELENA-SLEDAI) and BILAG scores, was no different than that of the placebo group (265). However, *post hoc* analysis indicated a positive trend in the sifalimumab group as a whole and the subgroup of SLE patients with a high IFN gene signature treated with sifalimumab (265). Additionally, sifalimumab had a normalising effect on C3 and C4 complement levels in those patients with low levels of C3 and C4 at baseline (265). This suggests that the sifalimumab, through IFN signalling blockade, had a positive benefit on important immunological factors that correlate with SLE, and thus is a good potential therapeutic for SLE patients with a high IFN gene score. However, a subset of JSLE patients with a low IFN gene signature may not respond well or at all to IFN therapy. Therefore, personalised therapies, based on IFN gene signatures, may be particularly useful for JSLE patients.

Another antibody has been tested as a therapeutic agent in SLE. Rontalizumab is a humanised IgG1 monoclonal antibody, which, unlike sifalimumab, can bind all subtypes of IFNα (266). Within a phase 1 clinical trial, it was shown that rontalizumab was well tolerated and most AEs were mild or moderate (266). There was some reporting of more severe adverse effects (SAE) in the rontalizumab group compared with placebo. However, there was no definitive rontalizumab dose versus AE demonstrated (266). The majority of infections were deemed mild or moderate, and their frequency did not differ between groups (266). Similar to sifalimumab, the PK of rontalizumab was proportional to dose, and had a long half-life 18.8 days (266). It was shown that at 3 and 10mg/kg of intravenous (IV) rontalizumab reduced interferon regulatory genes, at both a single dose and repeated dose, and in particular this gene expression was maintained at this low level for more than 100 days after 10mg/kg IV dose (266). However, rontalizumab did not change the protein expression level of IFN inducible genes such as 1P10, MCP1 or BAFF in whole blood, or any autoantibodies such as anti-dsDNA, anti-RNP or anti-Ro/Sjögren's syndrome type A (SSA) in patients deemed to have a high interferon signature matrix (ISM) (266).

In the phase 2 trial of rontalizumab it was shown that although disease activity did not differ between patients with a high ISM and patients with a low ISM, according to SELENA-SLEDAI

or BILAG, high ISM patients were more likely to have increased anti-dsDNA antibodies, and low C3 and C4 levels (267). It was shown that there was no differences in improvement in BILAG score between treatment and placebo, or any differences between ISM high and ISM low patients (267). It was shown that the SLE response index (SRI)-4 was also not different between pooled placebo or pooled rontalizumab treated patients, or comparing placebo vs rontalizumab in the ISM high SLE sub group (267). However, it was shown that ISM low patients did have a low SRI-4 score upon rontalizumab administration compared to placebo (267). It was shown that there were fewer flares in the rontalizumab treated patient group compared to placebo, which were predominantly seen in the ISM low patients (267). AEs were comparable between placebo and rontalizumab treated groups (267). Thus, rontalizumab may have some benefit in patients with a low IFN gene score whereby patients with a high IFN gene score received little benefit from the antibody. Thus, it may be beneficial only as a supplementary treatment in patients with a low IFN gene score. The fact that rontalizumab binds to all IFNα subtypes and sifalimumab doesn't may contribute to their different effects on IFN high and IFN low patients.

Anifrolumab is a human, IgG1κ monoclonal antibody that can bind to the IFNAR1 and thus inhibits the receptor and signalling by type 1 IFNs (268). Within a phase 2b study, it was shown that the SRI-4 (defined as sustained reduced oral corticosteroids at week 24) was more frequently obtained in the group taking anifrolumab than the placebo, and within the treatment group, the (genetically) IFN-high sub-population reached this SRI-4 more frequently than the IFN-low sub-population (246). Anifrolumab reduced the SLEDAI-2K and BILAG score more than placebo group, indicating disease activity improved (246). Organ disease specific disease measurements were also improved with anifrolumab (246). Anifrolumab also reduced anti-dsDNA and increased complement C3 levels, and neutralised the type 1 IFN gene signature (246). AEs were similar across all treatment groups, although upper respiratory infections occurred more frequently in patients treated with anifrolumab (246).

From these studies, it is evident that there is some clinical, demonstrable benefit from using IFNα blocking antibodies. This is both by blocking IFNα itself and blocking the receptors. Clinical trials have shown significant changes in the type 1 IFN signature, proteins associate with type 1 IFNs were reduced, and improvement in disease activity seen. However, the IFNα blocking antibodies weren't always effective. The antibodies did not consistently reduce every aspect of the IFN signalling (e.g. no differences seen in protein expression of IFN inducible genes with rontalizumab treatment) and disease activity did not always improve.

Additionally, sifalimumab did not improve disease activity in those patients with a low IFN gene score. Thus, alternative treatments must be investigated. Anifrolumab had the most benefits, with the most improvement in disease activity observed in the clinical trials discussed here.

However, IFNα is not the only IFN involved in SLE and JSLE pathogenesis, and the role of IFNγ has been demonstrated in the published literature and this present study to be important in SLE and JSLE (171, 174, 190). Its role is particularly related to the up-regulation of the STAT1 pathway, and its involvement in apoptosis, where both functions are indicated to be important in SLE, with apoptosis important in JSLE (71, 72, 138, 194). Thus, it would be important to investigate how an IFNγ monoclonal antibody works as a therapeutic. There is a distinct type 2 IFN genetic signature found in SLE patients, indicating a strong IFNγ signalling pathway in SLE, and so an antibody specific for IFNα may not always be effective in these patients (174). Additionally, as mentioned below (6.8), IFNλ is also increased and therefore likely to be involved in SLE (and thus JSLE) pathogenesis and can up-regulate a subset of type 1 IFN-induced genes, and thus can induce a type 1 IFN like response (269-272). Thus, it would be of interest to investigate the role how an IFNλ monoclonal antibody may work as a potential therapeutic, and how a combination of these treatments may be beneficial to those patients that may have a genetic signature induced by a combination of type 1, type 2 and type 3 IFNs, and thus have a multitude of IFN signalling pathways that do not respond to just one treatment.

6.7.3. Neutrophils as therapeutic targets

Neutrophils themselves may be an important cell of the immune system to target therapeutically in JSLE. This is reflected in their increased apoptosis and that they can be a potential source of autoantigens (72). RP3 is a monoclonal antibody found to deplete neutrophils (273). Rats with adjuvant arthritis were shown to have increased leukocyte numbers, in particular with neutrophils (274). RP3 was found to significantly reduce the number of neutrophils within rats with arthritis to that of normal levels (274). RP3 treatment, and subsequent neutrophil depletion, decreased adjuvant arthritis, including reduced joint swelling and reduced synovial fluid cell influx (274).

Neutrophils have been implicated in chronic obstructive pulmonary diseases (COPD), in particular, the increase in neutrophil inflammation, and have been shown to be involved in the significant lung tissue damage within COPD (275). Neutrophil targeted nanoparticles have been developed for COPD (276). A published study used polyethylene glycol (PEG)ylated

immune-conjugated Polylactide-coglycolide (PLGA) nanoparticle (PINP), loaded with ibuprofen (IBF) and the anti-neutrophil antibody NIMP-R14 ($PINP^{NIMP\text{-}IBF}$) to investigate how neutrophil targeted therapies could help chronic obstructive lung diseases (276). Within wild type mice, LPS induced inflammatory lung disease. Additionally, the $PINP^{NIMP\text{-}IBF}$ decreased MPO levels and NFκB activity in bronchoalveolar lavage fluid (BALF) neutrophils, and decreased LPS-induced Fas expression compared to LPS treated mice (276). It was also shown that $PINP^{NIMP\text{-}IBF}$ decreased NFκB expression, neutrophil infiltration and Fas expression in mice exposed to chronic smoke and thus used as an emphysema model (276).

It was investigated whether targeting neutrophils, through the inhibition of STAT1, may prevent possible IFN related apoptosis, which may reduce the production of autoantigens within JSLE, and thus may act as a therapeutic. Although not successful here, other studies shown here that it is possible to target neutrophils as a therapeutic target, resulting in clinical and aetiological benefits. Although this approach did not produce any unequivocal conclusions, the concept, along with other literature, does still stand in that neutrophil targeted therapeutics may be of benefit to JSLE patients, particularly via the inhibition of neutrophil apoptosis and thus production of the autoantigens that is characteristic of JSLE pathogenesis.

6.8. IFNλ

IFNλ has been more recently characterised alongside other known IFNs and is deemed the only subtype 3 IFN (121, 277). Specifically, there are 3 types of IFNλ; IFNλ1, IFNλ2 and IFNλ3, previously known as IL29, IL28A and IL28B respectively (120). IFNλ is secreted by pDCs upon viral infection such as influenza (278). IFNλ has a role in modulating Th1/Th2 responses and functions through a distinct receptor and the initiation of the JAK/STAT pathway, phosphorylating STAT1, STAT2 and STAT3, which can induce antiviral responses (120, 122, 269). IFNλ also can induce a type 1 IFN like response via the activation of the JAK/STAT pathway (via STAT1 and STAT2) and MAPKs (269). It is important to note that IFNλ increased a subset of the type 1 IFN-induced gene profile, albeit more weakly than IFNα, and thus there is not a unique IFNλ gene profile (269).

A published study developed a colorectal adenocarcinoma p53 deficient HT29 cell line with a FLAG (aspartic acid-tyrosine-lysine-aspartic acid-aspartic acid-lysine [DYKDDDDK] epitope)-IL-10 receptor 1 (FLIL10R1)/ λ receptor 1 (λR1) chimeric receptor, as they have found that

HT29 cells transfected with a FLAG epitope-tagged IFNλR1 did not express any detectable level of the receptor (120, 279). As IL-10 and IFNλ share this receptor, and IL-10 could induce an IFNλ signalling pathway in HT29 cells with this chimeric receptor, this was subsequently used to characterise the effect of IFNλ signalling on apoptosis, through IL-10 stimulation of the FLIL10R1/λR1 chimeric receptor (120, 279). IFNλ signal induction through the IL-10 stimulation of the chimeric receptor lead to the externalisation of PS (through detection with annexin V) and DNA fragmentation (through detection with terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) assay) (279). The signalling also induced cleavage of caspase 8, 9 and 3, confirming that IFNλ signalling induces apoptosis in these HT29 cells (279). Thus, it may be, like with type 1 and 2 IFNs, IFNλ may have a role in apoptosis in other cells, and would be interesting to see its effect on neutrophil apoptosis.

Research has also shown that IFNλ is possibly involved in SLE pathogenesis. IFNλ1 mRNA and its serum protein levels were elevated in SLE patients compared to healthy controls and were shown to be higher in active SLE patients compared to inactive SLE patients (270). It was also shown that IFNλ1 serum protein levels positively correlated with SLEDAI scores and laboratory markers such as dsDNA, and negatively correlated with C3 levels (270) IFNλ1 protein was also shown to be higher in patients with renal disease and arthritis (270). IFNλ1 also induced chemokine production from PBMCs, such as IP10 and MIG, with more secretion from SLE PBMCs compared to healthy control PBMCs (270). Interestingly IFNλ1 reduced IL-8 secretion from SLE PBMCs compared to control PBMCs, which can relate back to the reduced neutrophil chemotaxis seen in other studies (116, 117, 270).

IFNλ3 was particularly shown to be increased in the serum of SLE patients compared to healthy controls, but not IFNλ1 or IFNλ2 (272). Additionally, IFNλ3 was shown to be affected by medication, whereby patients on glucocorticoids had 3 times higher IFNλ3 serum levels than those patients not on glucocorticoids (272). Prednisone also affected IFNλ3, with those patients on a high dose of prednisone having the highest serum level of IFNλ3 (272). High IFNλ3 levels also correlated with patients with serositis and cutaneous involvement, positively correlated with Ro/SSA and ds-DNA antibodies and negatively correlated with C3 and C4 levels (272).

It has been shown that IFNλ is increased in serum of SLE patients with active nephritis at baseline compared to healthy controls, which did not change at follow up (271). IFNλ was found to be more detectable in biopsies from patients with membranous nephritis than in patients with proliferative nephritis (271). In particular staining of IFNλ in these biopsies was

more prominent in the glomeruli with cellular crescent formations, in inflammatory infiltrates of $CD3^+$ T cells and in tubular cells (271).

These studies confirm that IFNλ functions like type 1 and 2 IFNs in regard to anti-viral action, and there are crucial similarities in the downstream signalling, whereby genes are activated by both IFNα and IFNλ. It may be that IFNλ may have a dual role in apoptosis that is akin to other IFNs, and this may be important in regards to the increased neutrophil apoptosis seen in JSLE. Studies have also shown that IFNλ has a possible important in lupus nephritis pathogenesis, which suggests that it may be important in JSLE (270-272). To date, there was no published literature investigating the role of IFNλ in JSLE. However, based on findings with IFNα and IFNγ within literature and within this thesis, it would be of interest to investigate whether the increase in IFNλ mRNA expression and serum protein concentration present within adult SLE patients is also true of JSLE patients. Furthermore, it the potential role of IFNλ in SLE and JSLE pathogenesis should be investigated to see if it compares or contrasts with the roles of the type 1 and 2 IFNs. From this, it would be important to analyse the genetic signature and serum levels of IFNλ within JSLE and investigate its functional role in JSLE pathogenesis in relation to type 1 and 2 IFNs, particularly in regard to any potential interaction with neutrophils.

6.9. Conclusion

In this study, and in previous literature, it was shown that IFNs may have a potential priming role in NETosis, however it was not conclusively shown here. Additionally, evidence from the literature indicates that the IFN subtypes can have roles in neutrophil phagocytosis and chemotaxis (albeit not demonstrated clearly here), and these may also be important in JSLE pathogenesis. IFNs have shown both a pro- and anti-apoptotic effect on neutrophils; however, this is dependent on the activation of neutrophils and the subsequent IFN concentration. These findings may be relevant in particularly JSLE patients with active disease. It was also shown that the pro-apoptotic STAT1 has increased phosphorylation in type 2 IFN-stimulated TNFα-primed neutrophils compared to naïve neutrophils, and the anti-apoptotic STAT3 has decreased phosphorylation in type 1 IFN-stimulated, TNFα-primed neutrophils compared to naïve neutrophils, which may relate to differences in the IFN receptor chain expression. These findings may link to the differential effect of IFNs on apoptosis, and thus again may be important in the increased apoptosis found in JSLE neutrophils. However, it is important to note that a reduction of IFNAR1 was only found in

the *in vitro* model and did not extrapolate to JSLE neutrophils. Additionally, an antibody which blocks IFNAR has recently been shown in published studies to have therapeutic benefit in SLE, correlating with the observation that the IFNAR chains are well expressed on JSLE neutrophils. It was investigated whether STAT1 inhibition delays any potential IFN-induced apoptosis, and although here, it did not have consistent or appropriate inhibitory results, it still remains a potential therapeutic target in preventing neutrophil apoptosis within JSLE. Neutrophil therapeutic targets have been shown to be beneficial in other diseases, and thus neutrophils as a target for therapy may be beneficial in JSLE, due to neutrophil apoptosis being a potential source of autoantigens. Lastly, results from this study have also provided the foundation for further studies that may wish to investigate the effects of other IFN subtypes such as IFNλ.

In conclusion, it was demonstrated that type 1 and type 2 IFNs do have an important role in modulating neutrophil function. In particular, this was demonstrated through dual, opposing apoptotic effects on neutrophil apoptosis via the JAK/STAT pathway. IFNs may be involved in the increased neutrophil apoptosis seen in JSLE; IFNs may have a direct, pro-apoptotic effect on neutrophils or that the pro-inflammatory environment, and subsequent activation of JSLE neutrophils, may abrogate the anti-apoptotic IFN effect on neutrophils. In the latter scenario, neutrophil apoptosis may be induced by other pro-inflammatory factors that are increased in the JSLE serum. Additionally, the JAK/STAT pathway was shown to be altered when neutrophils are primed to be activated, through the dysregulation of IFNAR in particular, an increase in IFNγ-related STAT1 phosphorylation, and decrease in IFNα/β related STAT3 phosphorylation. This may be true of JSLE neutrophils and highlights a potential therapeutic target. Inhibiting the pro-apoptotic STAT1 may lead to increase in IFN-induced up-regulation of anti-apoptotic STAT3, which may reduce the neutrophil apoptosis which in turn may contribute to the increased autoantigens that are fundamental to JSLE pathogenesis.

Appendix A. Research Ethics Approval

A1. JSLE patient and paediatric control sample ethics

NHS
Health Research Authority

North West - Liverpool East Research Ethics Committee

Barlow House
3rd Floor
4 Minshull Street
Manchester
M1 3DZ

Tel: 0207 104 8002

09 February 2016

Professor Michael W. Beresford
Professor in Child Health
University of Liverpool
Institute of Child Health
Alder Hey Children's NHS Foundation Trust
Eaton Road
Liverpool
L12 2AP

Dear Professor Beresford

Study title:	**UK Juvenile Systemic Lupus Erythematosus Cohort Study & Repository: "Clinical characteristics and immunopathology of juvenile-onset systemic lupus erythematosus"**
REC reference:	**06/Q1502/77**
EudraCT number:	**N/A**
Amendment number:	**Substantial Amendment 4**
Amendment date:	**01 January 2016**
IRAS project ID:	

The above amendment was reviewed by the Sub-Committee in correspondence.

Ethical opinion

Approval was sought for updates made to the protocol.

The Sub-Committee commented that page 11 of the protocol stated that you are proposing to include healthy controls, aged 13-15 from friends and siblings and they requested further information on how these controls would be approached, who will approach them, etc.

You replied that you have provided more detailed information on how you plan to approach these healthy controls in the protocol on page 11 with the following text:

"Visitors aged 13 – 15 years attending the hospital may also be approached where appropriate for example through asking patients to identify friends/siblings who may be willing to take part in the study. Visitors will only be approached in an appropriate, confidential, quiet environment by GCP trained staff on the delegation log. Staff will only approach potential participants where they feel comfortable that it is appropriate timing and the family may be interested. They will provide an appropriate patient information leaflet and offer time to take the information leaflet away and read it in their own time. They will be clear that the family may say no. They will typically be approached by a doctor or nurse during a

A Research Ethics Committee established by the Health Research Authority

clinic setting when the family are attending for another reason, for example, asking patients / parents if they have siblings / other children who may be interested in taking part in the study."

The Sub-Committee also sought further information on where the adult controls would come from, how and who will be approaching them.

You replied that more detailed information on how you plan to approach adult healthy controls in the protocol on page 11 with the following text:

"Adults aged sixteen years or over, without any significant past medical history of inflammatory disease and freely able to consent will be recruited to assist with experiment optimisation at AHCH. Persons working in the Trust or the University (e.g. medical students / administration staff etc.), will be approached and provided with an information sheet. Staff will be approached by GCP trained staff who are familiar with the study and on the study delegation log. They will only be approached in an appropriate, confidential and quiet environment and provided with an information sheet. They will be offered time to take the information leaflet away and read it in their own time. Staff will be clear that they can say no and there is no requirement for them to take part in the study. Only staff that are willing to take part in the study will be asked to sign the consent form."

The Sub-Committee commented that on page 14 of the protocol it stated that the only identifiable details to be stored electronically are email address; the Sub-Committee sought further clarification as to why you can't store these separately and just access them when required.

You replied that you agree that email addresses can be stored separately and accessed when required. You have removed this from the protocol on page 14.

The Sub-Committee asked for clarification on how much blood is to be taken, for example, will it be 5ml/1 teaspoon. They requested that the PILs are changed to state the amount of blood to be taken.

You replied that the volume of blood to be taken is 2-3 teaspoons. You have revised the PILs and resubmitted them.

The Sub-Committee requested that in the section *"Do I have to help"* in the PILs, it should start with "no". They noted that some of the PILs already do this, but not all of them.

You updated all the PILs and resubmitted.

In the PILs for the inflammatory controls for <6 and 6-12, the Sub-Committee noted that it does not state that participants have been chosen because they have arthritis, but does in the PILs for 13-15 year olds and sought further clarification as to why this is the case.

You updated the PILs accordingly and resubmitted.

The Sub-Committee commented that the PILs do not mention being flagged with the HSCIC, or informing the GP and sought further clarification for this.

You replied that you are only using HSCIC flagging on JSLE recruits and this was already included on the PILs for the 13-15 year olds, >16 year olds and parents. You said that you would not be using the HSCIC service for any of the control recruits. You also said that informing the GP was already included in all the PILs except for the adult healthy controls, which you amended and resubmitted.

The Sub-Committee commented that there was no contact information provided for complaints in the PILs.

You replied that you have updated the PILs to include the following in the parent and participant >16 years PILs:

"What do we do if there is a problem?
If you have a problem you can speak to any member of the study team and we will try to help. If the problem is not resolved or you remain unhappy you can make a complaint by contacting the research team at research@alderhey.nhs.uk or by contacting your hospital's PALS team."

The Sub-Committee sought further clarification as to whether participants can still take part if they do not agree to flagging with the HSCIC and/or if they do not want DNA analysis.

You replied that patients can still be involved in the study if they don't agree to flagging with the HSCIC and/or if they don't want DNA analysis by not initialling the appropriate boxes on the consent form. Each line of the consent form needs to be initialled specifically, and any not included such as these are noted. You said that to date, none have not wanted to take part in all of the components, but it remains always an option. You also mentioned that this is already included in the protocol for HSCIC on page 16.

You went to say that you have updated the protocol regarding DNA analysis on page 18 with the following sentence:

"Participation in this section of the study requires the patient to specifically initial a box saying 'I agree that DNA can be extracted and stored from blood that I provide. I understand that no result on my genes will be fed back to me or anyone else'. Patients who do not want their DNA to be analysed can still participate in the rest of the study."

You went on further to state that you have included an additional point on the assent/consent forms regarding DNA analysis:

"I agree that DNA can be extracted and stored from blood that I provide. I understand that no result on my genes will be fed back to me or anyone else."

The members of the Committee taking part in the review gave a favourable ethical opinion of the amendment on the basis described in the notice of amendment form and supporting documentation.

Approved documents

The documents reviewed and approved at the meeting were:

Document	*Version*	*Date*
Covering letter on headed paper		29 December 2015
Notice of Substantial Amendment (non-CTIMP)	Substantial Amendment 4	01 January 2016
Other [Response to Committee Queries]		05 February 2016
Participant consent form	4	01 January 2016
Participant consent form [Controls]	4	01 January 2016

Participant information sheet (PIS) [Controls]	4	01 January 2016
Participant information sheet (PIS)	4	01 January 2016
Research protocol or project proposal	4	01 January 2016

Membership of the Committee

The members of the Committee who took part in the review are listed on the attached sheet.

R&D approval

All investigators and research collaborators in the NHS should notify the R&D office for the relevant NHS care organisation of this amendment and check whether it affects R&D approval of the research.

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

We are pleased to welcome researchers and R & D staff at our NRES committee members' training days – see details at http://www.hra.nhs.uk/hra-training/

06/Q1502/77: Please quote this number on all correspondence

Yours sincerely

On behalf of
Mrs Glenys J Hunt
Chair

E-mail: nrescommittee.northwest-liverpooleast@nhs.net

Enclosures: List of names and professions of members who took part in the review

North West - Liverpool East Research Ethics Committee

Attendance at Sub-Committee of the REC meeting

Committee Members:

Name	*Profession*	*Present*	*Notes*
Mrs Glenys J Hunt Chair	Solicitor	Yes	
Dr Peter Walton	Lay Member	Yes	

Also in attendance:

Name	*Position (or reason for attending)*
Miss Ewa Grzegorska	REC Assistant

A2. Adult volunteer donor ethics

From: Ethics
Sent: 26 January 2015 17:10
To: Flanagan, Brian
Subject: RE: RETH000773: request for review of study ethcs application.

Dear Dr Flanagan,

I am pleased to inform you that the Sub-Committee has approved your application for ethical approval for your study. Details and conditions of the approval can be found below.

Reference:	RETH000773
Sub-Committee:	Physical Interventions
Review type:	Full committee review
Principle Investigator:	Dr Brian Flanagan
Department:	Women's and Children's Health
Title:	Analysis of normal blood leukocyte function using cells from healthy volunteers.
First Reviewer:	Professor Graham Kemp
Date of initial review:	11/12/2014
Date of Approval:	26/01/2015

The application was APPROVED subject to the following conditions:

Conditions

All serious adverse events must be reported to the Sub-Committee within 24 hours of their occurrence, via the Research Integrity and Governance Officer (ethics@liv.ac.uk).

This approval applies for the duration of the research. If it is proposed to extend the duration of the study as specified in the application form, the Sub-Committee should be notified. If it is proposed to make an amendment to the research, you should notify the Sub-Committee by following the Notice of Amendment procedure outlined at http://www.liv.ac.uk/media/livacuk/researchethics/notice%20of%20amendment.doc. If the named PI / Supervisor leaves the employment of the University during the course of this approval, the approval will lapse. Therefore please contact the Research Integrity and Governance Officer at ethics@liverpool.ac.uk in order to notify them of a change in PI / Supervisor.

Kind regards

Matthew Billington
Research Integrity and Governance Officer

Research Support Office
University of Liverpool
Waterhouse Building (2nd Floor, Block C)
3 Brownlow Street
Liverpool
L69 3GL

Email: ethics@liverpool.ac.uk
Telephone: 0151 794 8290
Website: Research Integrity & Ethics

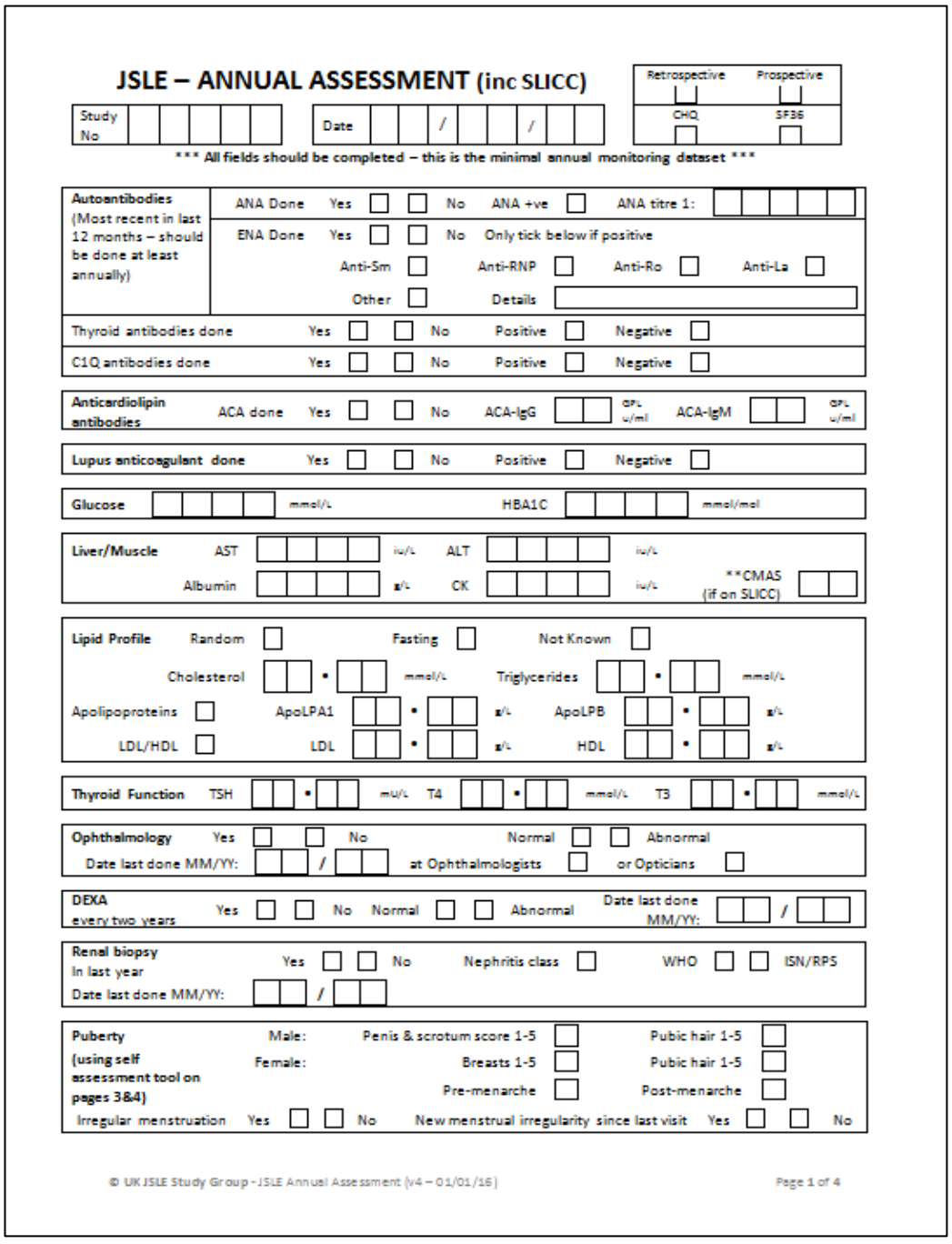
Please note: My working hours are Monday to Friday, **8:00am - 4:00pm.**

Please ensure you are familiar with the Research Integrity Concordat

Appendix B. Clinical and consent forms

B1. JSLE clinical and BILAG forms

B1.1. JSLE annual assessment form



JSLE – ANNUAL ASSESSMENT (inc SLICC)

Retrospective ☐ Prospective ☐ CHQ ☐ SF36 ☐

Study No ☐☐☐☐☐ Date ☐☐/☐☐/☐☐

*** All fields should be completed – this is the minimal annual monitoring dataset ***

Autoantibodies (Most recent in last 12 months – should be done at least annually)
ANA Done Yes ☐ ☐ No ANA +ve ☐ ANA titre 1: ☐☐☐☐☐
ENA Done Yes ☐ ☐ No Only tick below if positive
Anti-Sm ☐ Anti-RNP ☐ Anti-Ro ☐ Anti-La ☐
Other ☐ Details ☐

Thyroid antibodies done Yes ☐ ☐ No Positive ☐ Negative ☐

C1Q antibodies done Yes ☐ ☐ No Positive ☐ Negative ☐

Anticardiolipin antibodies ACA done Yes ☐ ☐ No ACA-IgG ☐☐ GPL u/ml ACA-IgM ☐☐ GPL u/ml

Lupus anticoagulant done Yes ☐ ☐ No Positive ☐ Negative ☐

Glucose ☐☐☐☐ mmol/L HBA1C ☐☐☐☐ mmol/mol

Liver/Muscle AST ☐☐☐☐ iu/L ALT ☐☐☐☐ iu/L
Albumin ☐☐☐☐ g/L CK ☐☐☐☐ iu/L **CMAS (if on SLICC) ☐☐

Lipid Profile Random ☐ Fasting ☐ Not Known ☐
Cholesterol ☐☐.☐☐ mmol/L Triglycerides ☐☐.☐☐ mmol/L
Apolipoproteins ☐ ApoLPA1 ☐☐.☐☐ g/L ApoLPB ☐☐.☐☐ g/L
LDL/HDL ☐ LDL ☐☐.☐☐ g/L HDL ☐☐.☐☐ g/L

Thyroid Function TSH ☐☐.☐☐ mU/L T4 ☐☐.☐☐ mmol/L T3 ☐☐.☐☐ mmol/L

Ophthalmology Yes ☐ ☐ No Normal ☐ ☐ Abnormal
Date last done MM/YY: ☐☐/☐☐ at Ophthalmologists ☐ or Opticians ☐

DEXA every two years Yes ☐ ☐ No Normal ☐ ☐ Abnormal Date last done MM/YY: ☐☐/☐☐

Renal biopsy In last year Yes ☐ ☐ No Nephritis class ☐ WHO ☐ ☐ ISN/RPS
Date last done MM/YY: ☐☐/☐☐

Puberty (using self assessment tool on pages 3&4)
Male: Penis & scrotum score 1-5 ☐ Pubic hair 1-5 ☐
Female: Breasts 1-5 ☐ Pubic hair 1-5 ☐
Pre-menarche ☐ Post-menarche ☐
Irregular menstruation Yes ☐ ☐ No New menstrual irregularity since last visit Yes ☐ ☐ No

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JSLE – ANNUAL ASSESMENT (Page 2 of 2)

To be used with Glossary A blank box will be assumed to = 0. Please tick to confirm	☐ Yes ☐ No

Parameters	SLICC Damage Criteria	0	1	2	3
Ocular (either eye by clinic assessment)	Ocular cataract EVER				
	Retinal change OR optic atrophy				
Neuropsychiatric	Cognitive impairment OR major psychosis				
	Seizures requiring therapy for 6 months				
	Cerebral vascular accident ever (Score 2 if >1), or resection not for malignancy				
	Cranial or peripheral neuropathy (excluding optic)				
	Transverse myelitis				
Renal	Estimated or measured GFR <50%				
	Proteinuria 24h, >=3.5g OR ACR > 1000mg/mm OR > 10mg/mm				
	End stage renal disease (regardless of dialysis or transplantation)				
Pulmonary	Pulmonary hypertension (right ventricular prominence, or loud P2)				
	Pulmonary fibrosis (physical and x-ray)				
	Shrinking lung (x-ray)				
	Pleural fibrosis (x-ray)				
	Pulmonary infarction (x-ray) OR resection not for malignancy				
Cardiovascular	Angina OR coronary artery bypass				
	Myocardial infarction ever (score 2 if >1)				
	Cardiomyopathy (ventricular dysfunction)				
	Valvular disease (diastolic murmur, or a systolic murmur > 3/6)				
	Pericarditis x 6 months or pericardectomy				
Peripheral Vascular	Claudication x 6 months				
	Minor tissue loss (pulp space)				
	Significant tissue loss ever (e.g. loss of digit or limb, resection) (Score 2 if > 1)				
	Venous thrombosis with swelling, ulceration, OR venous stasis				
Gastrointestinal	Infarction or resection of bowel (below duodenum), spleen, liver or gall bladder (Score 2 if > 1)				
	Mesenteric insufficiency				
	Chronic peritonitis				
	Stricture OR upper gastrointestinal tract surgery ever				
	Pancreatic insufficiency requiring enzyme replacement or pseudocyst				
Musculoskeletal	Atrophy or weakness (**if yes please record CMAS on page 1 of this form)				
	Deforming or erosive arthritis (including reducible deformities, excluding avascular necrosis)				
	Osteoporosis with fracture or vertebral collapse (excluding avascular necrosis)				
	Avascular necrosis (Score 2 if > 1)				
	Osteomyelitis				
	Ruptured tendons				
kin	Alopecia				
	Extensive scarring of panniculum other than scalp and pulp space				
	Skin ulceration (not due to thrombosis) > 6 months				
Other	Diabetes (regardless of treatment)				
	Malignancy (excluding dysplasia) (Score 2 if > 1)				
	Premature gonadal failure / secondary amenorrhoea				

PUBERTY SELF ASSESSMENT BOYS

UK JSLE COHORT STUDY NUMBER:

.....

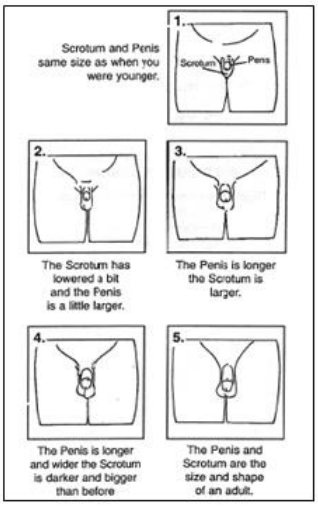
DATE:

.....

PUBERTY SELF ASSESSMENT – BOYS

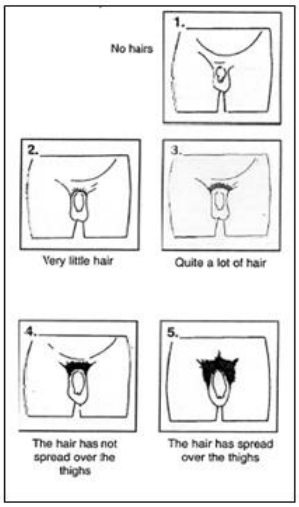
Please look at the **Penis** and **Scrotum** only in these pictures

- Please tick the box that looks most like you now



Please look at the Pubic Hair only in these pictures

- Please put a tick in the box that looks most like you now



PUBERTY SELF ASSESSMENT GIRLS

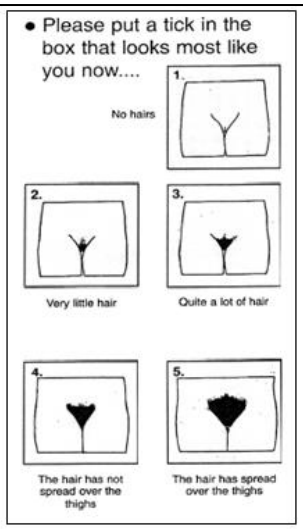
UK JSLE COHORT STUDY NUMBER:

.....

DATE:

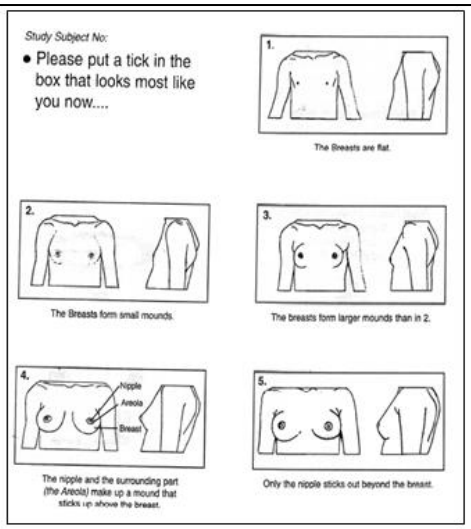
.....

- Please put a tick in the box that looks most like you now....



Study Subject No:

- Please put a tick in the box that looks most like you now....



Please answer the following questions:

1. Have you started having your period yet? Y / N
2. If yes, at what age did it start:
3. Do you have irregular periods? Y / N
4. If yes, is this a new problem since your last clinic appointment? Y/N

B1.2. JSLE ACR and SLICC form

SLICC CLASSIFICATION CRITERIA AT ANNUAL REVIEW

JSLE – ACR CLASSIFICATION CRITERIA AT ANNUAL REVIEW

Study No ☐☐☐☐☐ Date ☐☐/☐☐/☐☐ Retrospective ☐ Prospective ☐

These are cumulative. Please add new criteria only or tick the box if no new criteria since last review ☐

Criterion		Present	Subtype		Definition
1	Malar Rash	☐			Fixed erythema, flat or raised, over malar eminences, tending to spare the nasolabial folds
2	Discoid Lupus	☐			Erythematous raised patches with adherent keratotic scaling and follicular plugging; atrophic scarring may occur in older lesions
3	Photosensitivity	☐			Skin rash as a result of the unusual reaction to sunlight, by patient history or physician observation
4	Oral/nasal ulcerations	☐			Oral or nasopharyngeal ulceration, usually painless, observed by physician
5	Non-erosive Arthritis	☐			Non-erosive arthritis involving 2 or more peripheral joints , characterised by tenderness, swelling or effusion
6	Serositis	☐	A	☐	Pleuritis-convincing history of pleuritic pain or rub heard, or evidence of pleural effusion
		☐	B	☐	Pericarditis–documented ECG or rub, or evidence of pericardial effusion
7	Nephritis	☐	A	☐	Persistent proteinuria > 0.5g/day or > +++ (on protein dipstix) if quantification not performed
			B	☐	Cellular casts – may be red cell, haemoglobin, granular, tubular or mixed
8	Neurologic	☐	A	☐	Seizures in the absence of offending drugs or metabolic derangements (e.g. uraemia, ketoacidosis, electrolyte imbalance)
			B	☐	Psychosis in the absence of offending drugs or metabolic derangements (e.g. uraemia, ketoacidosis, electrolyte imbalance)
9	Haematological Disorder	☐	A	☐	Haemolytic anaemia with reticulocytes
			B	☐	Leucopenia < 4,000/mm³ total on 2 or more occasions
			C	☐	Lymphopenia < 1,500/mm³ on 2 or more occasions
			D	☐	Thrombocytopenia < 100,000/mm³ in absence of offending drugs
10	Immunological Disorder	☐	A	☐	Anti-DNA: antibody to native DNA in abnormal titre
			B	☐	Anti-Sm: presence of antibody to Sm nuclear antigen
			C	☐	+ve finding of anti-phospholipid antibodies based on:
			C1	☐	Abnormal level of IgG or IgM anti-cardiolipiantibody
			C2	☐	Positive test result for lupus anticoagulant (standard method)
			C3	☐	False +ve serologic result for syphilis > 6 mths
11	ANA	☐			Abnormal titre ANA at any time point in absence of drugs known to be associated with "drug-induced lupus"

If less than 4 criteria why evolving Lupus?	

Study No								Date			/			/		
These are cumulative. Please add new criteria only or tick the box if no new criteria since last review																☐

		Criterion	Present	Subtype		Definition
Clinical Criteria	1	Acute cutaneous lupus *in the absence of dermatomyositis*	☐	A	☐	Lupus malar rash (do not count if malar discoid)
				B	☐	Bullous lupus
				C	☐	Toxic epidermal necrolysis variant of SLE
				D	☐	Maculopapular lupus rash
				E	☐	Photosensitive lupus rash
					☐	Subacute cutaneous lupus (Nonindurated psoriaform and/or annular polycyclic lesions that resolve without scarring, although occasionally with postinflammatory dyspigmentation or telangiectasias)
	2	Chronic cutaneous lupus	☐	A	☐	Classic discoid rash localised (above the neck)
				B	☐	Classic discoid rash generalised (above and below the neck)
				C	☐	Hypertrophic (verrucous lupus)
				D	☐	Lupus panniculitis (profundus)
				E	☐	Mucosal lupus
				F	☐	Lupus erythematosus tumidus
				G	☐	Chilblains lupus
				H	☐	Discoid lupus / lichen planus overlap
	3	Ulcers	☐	A	☐	Oral ulcers (Palate/Buccal/Tongue) *in absence of other causes, such as vasculitis, Behcet's disease, infection (herpesvirus), inflammatory bowel disease, reactive arthritis and acidic foods*
				B	☐	Nasal ulcers - *in the absence of other causes, such as vasculitis, Behcet's disease, infection (herpesvirus), inflammatory bowel disease, reactive arthritis and acidic foods*
	4	Nonscarring alopecia	☐			Diffuse thinning or hair fragility with visible broken hairs *in the absence of other causes such as alopecia areata, drugs, iron deficiency and androgenic alopecia*
	5	Synovitis	☐			Involving 2 or more joints, characterised by swelling or effusion OR tenderness in 2 or more joints and at least 30 minutes of morning stiffness
	6	Serositis	☐	A	☐	Typical pleurisy for more than 1 day OR pleural effusions OR pleural rub
				B	☐	Typical pericardial pain (pain with recumbency improved by sitting forward) for more than 1 day OR pericardial effusion OR pericardial rub OR pericarditis by ECG *in the absence of other causes such as infection, uraemia, and Dressler's pericarditis*
	7	Renal	☐	A	☐	Urine protein-to-creatinine ratio (or 24-hour urine protein) representing 500mg protein/24 hours
				B	☐	Red blood cell casts
	8	Neurological	☐	A	☐	Seizures
				B	☐	Psychosis
				C	☐	Mononeuritis multiplex *in the absence other known causes such as primary vasculitis*
				D	☐	Myelitis
				E	☐	Peripheral or cranial neuropathy *in the absence of other known causes such as primary vasculitis, infection, and diabetes mellitus*
				F	☐	Acute confusional state *in the absence of other causes, including toxic/metabolic, uraemia, drugs*
	9	Haemolytic anaemia	☐			
	10	Leukopenia or lymphopenia	☐	A	☐	Leukopenia (<4,000/mm³ at least once) *in the absence of other known causes such as Felty's syndrome, drugs and portal hypertension*
				B	☐	Lymphopenia (<1,000/mm³ at least once) *in the absence of other known causes such as corticosteroids, drugs and infection*
	11	Thrombocytopenia	☐			Thrombocytopenia <100,000/mm³ at least once *in the absence of other known causes such as drugs, portal hypertension , and thrombotic thrombocytopenic purpura*
Immunologic criteria	1	ANA	☐			ANA level above laboratory reference range
	2	Anti-dsDNA	☐			Anti-dsDNA antibody level above laboratory reference range (or >2-fold the reference range if tested by ELISA)
	3	Anti-Sm	☐			Presence of antibody to Sm nuclear antigen
	4	Antiphospholipid antibody positivity	☐	A	☐	Positive test result for lupus anticoagulant
				B	☐	False-positive test result for rapid plasma reagin
				C	☐	Medium- or high-titer anticardiolipin antibody level (IgA, IgG, or IgM)
				D	☐	Positive test result for anti-β2-glycoprotein I (IgA, IgG, or IgM)
	5	Low complement	☐	A	☐	Low C3
				B	☐	Low C4
				C	☐	Low CH50
	6	Direct Coombs' test	☐			*In the absence of haemolytic anaemia*

B.1.3 JSLE pBILAG form

JSLE – BILAG Form

Routine ☐ Flare ☐ Retrospective ☐ Prospective ☐

Study No | | | | | | Date | | | / | | | / | | |

For all questions use codes: 1 = improving; 2 = same; 3 = worse; 4 = new;; 0 = not present (except for Y/N and numeric questions).

Height cm | | | | | **Weight kg** | | | | | **Systolic BP mmHg** | | | **Diastolic BP mmHg** | | |

Urinalysis (score 0, trace, 1+, 2+, 3+).
If abnormal urinalysis send urine for Microscopy, culture, sensitivities (MCS) Not Done ☐

Proteinuria ☐ Haematuria ☐ Leucocytes ☐ Nitrites ☐ Menstruating Y / N

NB. The following bloods should be sent as part of BILAG: FBC, ESR, CRP, C3, C4, dsDNA, total Igs, Creatinine, urinary protein/Cr or Alb/Cr ratio. **Please tick to confirm done** ☐ Yes ☐ No

To be used with Glossary: * = Definition in Glossary
Only features attributable to JSLE to be recorded and refer only to last 4 week compared with previous 4 weeks

For all questions use codes: 1 = improving; 2 = same; 3 = worse; 4 = new; 0 = not present (except for Y/N and numeric questions). **A blank box will be assumed to = 0 or No. Please tick to confirm** ☐ Yes ☐ No

	General	
* 1	Pyrexia (documented >37.5°C)	
* 2	Weight Loss – unintentional; >5%	
* 3	Lymphadenopathy/splenomegaly	
4	Fatigue/malaise/lethargy	
5	Anorexia	
	Mucocutaneous	
* 6	Skin eruption – severe active (not discoid/bullous/panniculitis)	
* 7	Skin eruption – mild	
* 8	Active discoid lesions: generalised/extensive	
* 9	Active discoid lesions: localised include lupus profundus	
* 10	Alopecia (severe, active)	
* 11	Alopecia (mild)	
* 12	Panniculitis/bullous lupus (severe)	
*13	Panniculitis/bullous lupus (mild)	
* 14a	Angio-oedema (severe)	
* 14b	Angio-oedema (mild)	
* 15	Mucosal ulceration (severe)	
*16	Mucosal ulcers (mild)	
* 17	Malar erythema	
18	Subcutaneous nodules	
19	Perniotic skin lesions	
*20	Peri-ungual erythema / chillblains	
21	Swollen fingers	Y ☐ ☐ N
22	Sclerodactyly	Y ☐ ☐ N
23	Calcinosis	Y ☐ ☐ N
24	Telangiectasia	Y ☐ ☐ N
25	Splinter haemorrhages	Y ☐ ☐ N
	Neurological	
26	Impaired level of consciousness	
* 27	Cognitive dysfunction	
* 28	Acute psychosis or delirium or confusional state	
* 29	Psychosis	
* 30	Seizure disorder	
* 31	Status epilepticus	
* 32	Cerebral vascular disease (not due to vasculitis)	
* 33	Cerebral vasculitis	
* 34	Aseptic meningitis	
* 35	Mononeuropathy (single/multiplex)	
36	Ascending or transverse myelitis	
* 37	Demyelinating syndrome	
* 38	Myelopathy	

JSLE – BILAG Form

Study No | | | | | | | Date | | | / | | | / | | |

For all questions use codes: 1 = improving; 2 = same; 3 = worse; 4 = new;; 0 = not present (except for Y/N and numeric questions).

	Neurological continued	
* 39	Acute inflammatory demyelinating polyradiculoneuropathy	
40	Peripheral neuropathy	
* 41	Cranial neuropathy	
* 42	Plexopathy	
* 43	Polyneuropathy	
* 44	Autonomic disorder	
* 45	Disc swelling	
* 46	Chorea	
* 47	Cerebellar ataxia (isolated)	
* 48	Movement disorder	
* 49	Lupus headache (severe, unremitting)	
* 50	Episodic migrainous headaches	
* 51	Tension headache	
* 52	Cluster headache	
* 53	Headache from IC hypertension	
* 54	Organic depressive illness	
* 55	Mood disorder (depression/mania)	
* 56	Anxiety disorder	
* 57	Organic brain syndrome	
	Musculoskeletal	
* 58	Definite myositis (severe)	
* 59	Myositis with incomplete criteria	
* 60	Myositis (mild)	
* 61	Myalgia	
* 62	Severe polyarthritis – with loss of function	
* 63	Moderate arthritis	
* 64	Arthralgia	
* 65	Tendonitis/tenosynovitis	
66	Tendon contractures and fixed deformity	Y / N
67	Aseptic necrosis	Y / N
	Cardiovascular & Respiratory	
* 68	Pleuropericardial pain	
* 69	Dyspnoea	
* 70	Cardiac failure	
* 71	Friction rub	
* 72	Effusion (pericardial or pleural)	
* 73	Mild or intermittent chest pain	
74	Progressive CXR changes – lung fields	Y / N
75	Progressive CXR changes – heart size	Y / N
76	ECG evidence of pericarditis/ myocarditis/endocarditis	Y / N
* 77	Cardiac arrhythmia including tachycardia (>100 no fever)	Y / N
* 78	Pulmonary function fall by >20%	Y / N
79	Cytohistological evidence of inflammatory lung disease	Y / N
* 80	Myocarditis - mild	
* 81	New valvular dysfunction	
* 82	Cardiac tamponade	
* 83	Pleural effusion with dyspnoea	
* 84	Pulmonary haemorrhage/vasculitis	
* 85	Interstitial alveolitis/pneumonitis	
* 86	Shrinking lung syndrome	
* 87	Aortitis	
* 88	Coronary vasculitis	
	Vasculitis	
* 89	Major cutaneous vasculitis including ulcers	
* 90	Major abdominal crisis due to vasculitis	
91	Recurrent thromboembolism (excluding strokes)	
92	Raynaud's	
93	Livido reticularis	

JSLE – BILAG Form

Study No | | | | | | | Date | | | / | | | / | | |

For all questions use codes: 1 = improving; 2 = same; 3 = worse; 4 = new; 0 = not present (except for Y/N and numeric questions).

	Vasculitis continued				
94	Superficial phlebitis				
* 95	Minor cutaneous vasculitis (nailfold, digital, purpura, urticaria)				
96	Thromboembolism (excl stroke) 1st episode	Y			N
	Renal				
* 99	Severe hypertension	Y			N
103	Newly documented proteinuria (level 4)	Y			N
* 104	Nephrotic syndrome	Y			N
* 108	Histological evidence of active nephritis within 3 months	Y			N
	Gastrointestinal				
* 109	Lupus peritonitis				
* 110	Abdominal serositis or ascites				
* 111	Lupus enteritis/colitis				
* 112	Malabsorption				
* 113	Protein losing enteropathy				
* 114	Intestinal pseudo-obstruction				
* 115	Lupus hepatitis				

* 116	Acute lupus cholecystitis	
* 117	Acute lupus pancreatitis	
	Ophthalmic	
* 118	Orbital inflammation with myositis and/or proptosis	
* 119	Keratitis – severe	
* 120	Keratitis – mild	
121	Anterior uveitis	
* 122	Posterior uveitis/retinal vasculitis – severe	
* 123	Posterior uveitis/retinal vasculitis – mild	
124	Episcleritis	
* 125	Scleritis – severe	
* 126	Scleritis – mild	
* 127	Retinal / choroidal vaso-occlusive disease	
* 128	Isolated cotton-wool spots (cytoid bodies)	
* 129	Optic neuritis	
* 130	Anterior ischaemic optic neuropathy	

Function

Patient CHAQ | | • | | |

Global assessment on CHAQ (0: Very well – 100: Very ill) | | | | |

Physicians clinical global score — Very well — 0 ——— 100 — Very ill

Important assessment of current clinical status

In relation to the patients LUPUS ACTIVITY at this time point, please tick the box that best describes your opinion as to their current flare status:

1. Clinical improvement – no clinical flare ☐
2. No change in Lupus activity ☐
3. Minor flare (may not require specific therapy) ☐
4. Moderate flare ☐
5. Major flare ☐

A flare of JSLE has been defined as "a measurable worsening of SLE disease activity in at least one organ system, involving new or worse signs of disease that may be accompanied by new or worse JSLE symptoms; depending on the severity of the flare, more intensive therapy may be required" [1]

1. Brunner HI, Klein-Gitelman MS, Higgins GC, et al. Arthritis Care Res. 2010;62(6):811-20.

JSLE – BILAG Form

Study No | | | | | | | Date | | | / | | | / | | |

For all questions use codes: 1 = improving; 2 = same; 3 = worse; 4 = new; 0 = not present (except for Y/N and numeric questions).

Treatment	Current dose	Revised dose
Hydroxychloroquine (mg/day)		
Azathioprine (mg/day)		
Mycophenolate (mg/day)		
Cyclosporin (mg/day)		
Prednisolone (mg/day)	.	.
Methotrexate (mg/week)	.	.
Oral		
Subcut		

	Current dose	Total number of pulses since last visit
IVIG (g/pulse)	.	

Rituximab:		**Cyclophosphamide:** IV ☐ Oral ☐	
Total dose per cycle (mg)		Total dose since last visit (mg)	
Number of infusions per cycle		Number of infusions since last visit	
Number of cycles since last visit		Cumulative dose	
IV methyl-prednisolone in last 3 months		<5 pulses ☐	>=5 pulses ☐

Other Drugs

Aspirin ☐	Bisphosphonate ☐	Oral contraceptive pill ☐
Angiotensin receptor blocker ☐	Ca** blockers ☐	Statins ☐
Other biological DMARDS ☐	ACEi ☐	Diuretic ☐	Anticoagulant ☐

Clinicians Intention re Medication

Please tick which of the following best describes your intention to change treatment, and explain why:

1) Decrease in treatment ☐
- Disease improvement ☐
- Side effects of treatment ☐
- Compliance problems ☐
- Weaning regimen ☐

2) Increase in treatment ☐
- Disease worsening ☐
- Standard dose increment ☐
- Dose increment due to weight ☐

3) Change in DMARD ☐
- Concerns over efficacy ☐
- Planned maintenance treatment ☐
- Side effects of treatment ☐
- Compliance problems ☐

4) No change in treatment ☐
- Active disease (induction phase) ☐
- Stable, not yet decrease ☐
- Patients choice ☐
- Not on Medication ☐

JSLE – BILAG Form

Study No | | | | | | | Date | | | / | | | / | | |

For all questions use codes: 1 = improving; 2 = same; 3 = worse; 4 = new; 0 = not present (except for Y/N and numeric questions).

Results Page

Microscopy & culture results

MCS results	1. Proven UTI (>1x10^5 growth (cfu) of a single organism)	Y / N	2. Mixed growth / contamination	Y / N
	3. Microscopy results: a) White cell count	(per hpf)	b) Red cell count	(per hpf)
	c) Red cell casts	Y / N	d) White cell casts	Y / N

Renal function

* 102a	Urinary Alb/Cr ratio (mg/mmol Cr)		105	Creatinine (plasma/serum)	
* 102b	Urinary Protein/Cr Ratio (mg/mmol Cr)		106a	GFR:EDTA clearance (exact) (mls/min. 1.73m^2)	
102c	24hr/Urinary Protein(g)		106b	GFR: ht/creat ratio (estimate)	
	Not done		107	Active urinary sediment	Y N

Haematology

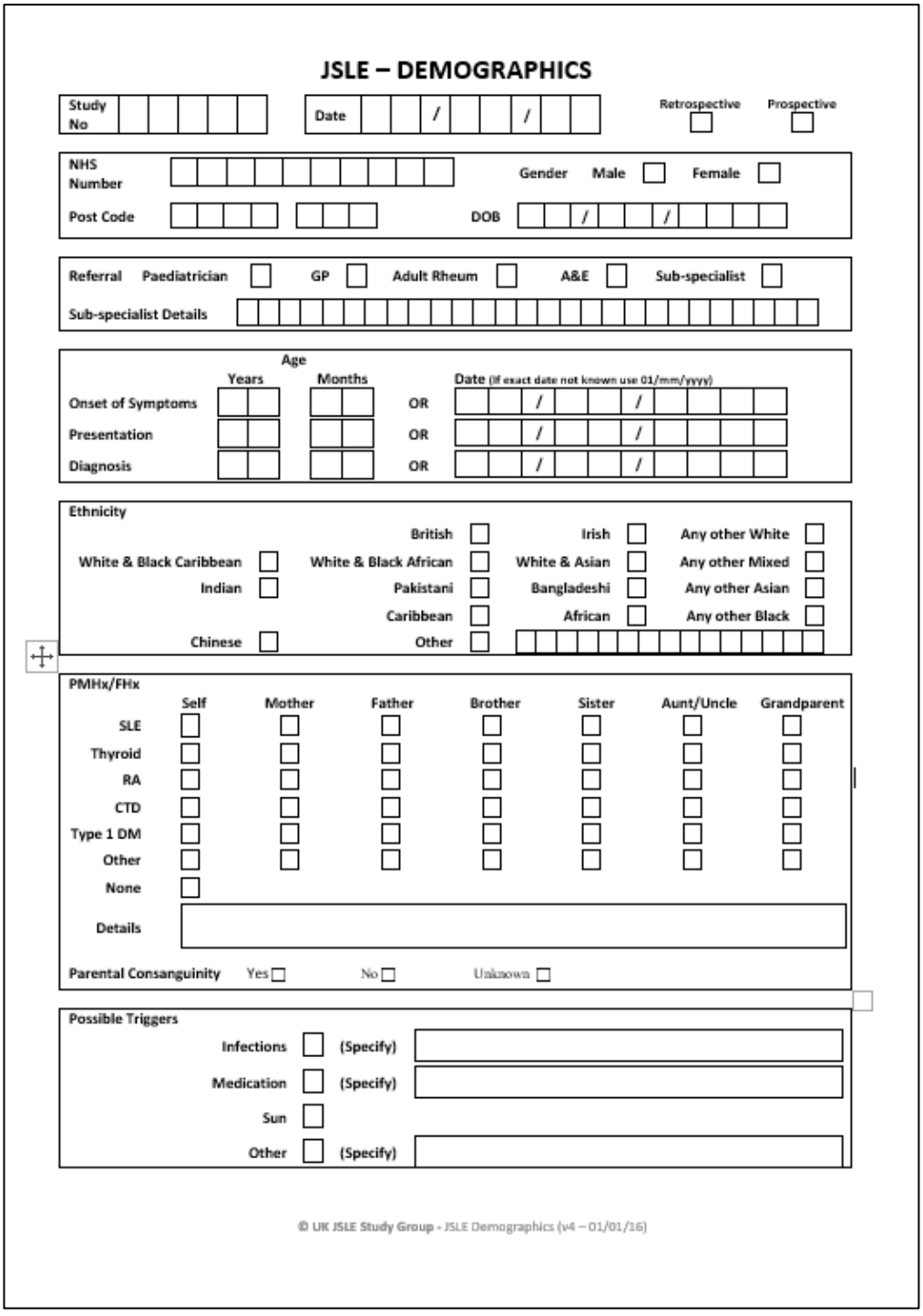
* 131	Haemoglobin g/dl		* 135	Platelets x 10^9/l	
* 132	Total white cell count x 10^9/l		* 136	Evidence of active haemolysis	Y N
* 133	Neutrophils x 10^9/l		137	Coomb's test positive	Y N
134	Lymphocytes x 10^9/l		* 138	TTP	Y N

Other measures of disease activity

ESR (mm/hr)		dsDNA	
CRP		IgG	
C3 (g/l)		IgA	
C4 (g/l)		IgM	
Viscosity (mPa.s) If no ESR		Ferritin (µg/L)	

B2: Demographic forms

B2.1. JSLE patient demographic form



JSLE – DEMOGRAPHICS

Study No | Date / / | Retrospective ☐ | Prospective ☐

NHS Number | Gender Male ☐ Female ☐

Post Code | DOB / /

Referral Paediatrician ☐ GP ☐ Adult Rheum ☐ A&E ☐ Sub-specialist ☐

Sub-specialist Details

	Age Years	Months		Date (If exact date not known use 01/mm/yyyy)
Onset of Symptoms			OR	/ /
Presentation			OR	/ /
Diagnosis			OR	/ /

Ethnicity

	British ☐	Irish ☐	Any other White ☐
White & Black Caribbean ☐	White & Black African ☐	White & Asian ☐	Any other Mixed ☐
Indian ☐	Pakistani ☐	Bangladeshi ☐	Any other Asian ☐
	Caribbean ☐	African ☐	Any other Black ☐
Chinese ☐	Other ☐		

PMHx/FHx

	Self	Mother	Father	Brother	Sister	Aunt/Uncle	Grandparent
SLE	☐	☐	☐	☐	☐	☐	☐
Thyroid	☐	☐	☐	☐	☐	☐	☐
RA	☐	☐	☐	☐	☐	☐	☐
CTD	☐	☐	☐	☐	☐	☐	☐
Type 1 DM	☐	☐	☐	☐	☐	☐	☐
Other	☐	☐	☐	☐	☐	☐	☐
None	☐						

Details

Parental Consanguinity Yes ☐ No ☐ Unknown ☐

Possible Triggers

Infections ☐ (Specify)

Medication ☐ (Specify)

Sun ☐

Other ☐ (Specify)

© UK JSLE Study Group - JSLE Demographics (v4 – 01/01/16)

B2.2. Paediatric control patient demographic form

CONTROL DEMOGRAPHICS

Study Number: _____ Gender: Male ☐ Female ☐

DOB: __ / __ / ____ Post Code: _____ Date Consented: __/__/____

Ethnicity (please tick)

White British		White & Black Caribbean		Indian		Caribbean		Chinese	
White Irish		White & Black African		Pakistani		African		Other: _____	
Any other white background		White & Asian		Bangladeshi		Any other Black background			
		Any other mixed background		Any other Asian background					

Type of Control

JIA		Renal		Non Inflammatory	

Diagnosis (see list on next page)

_____

(JIA only)

	Age (Years/Months)		Date
Onset of Symptoms	_____	OR	__/__/____
Presentation	_____	OR	__/__/____
Diagnosis	_____	OR	__/__/____

Has there been a family history of SLE, Thyroid problems, RA, CTD, IDDM, renal disease or any other related diseases? Yes ☐ No ☐

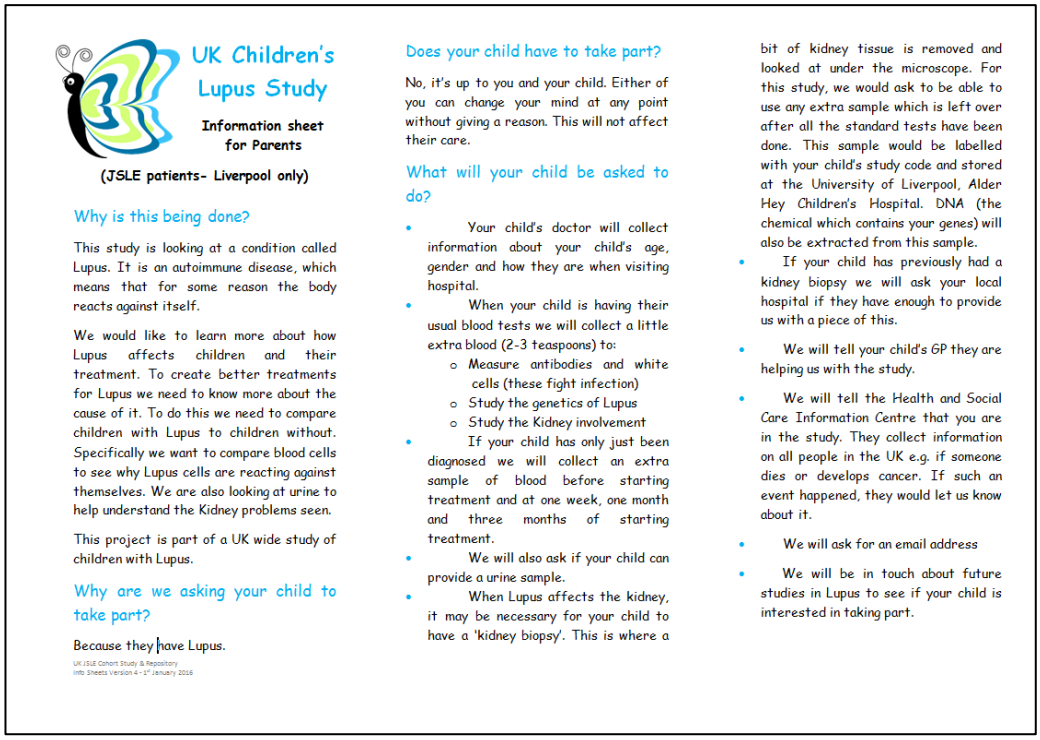
	Renal Disease	Autoimmune Disease	Other
Mother			
Father			
Brother			
Sister			
Aunt / Uncle			
Grandparents			

Diagnosis

JIA:	Systemic Oligoarthritis Oligoarthritis (Extended) Oligoarthritis (Persistent) Polyarthritis Polyarthritis (Rheumatoid Factor Positive) Polyarthritis (Rheumatoid Factor Negative) Psoriatic Enthesitis related Other – meeting more than one criteria Other – not meeting other JIA criteria Undetermined
Renal:	Diabetic nephropathy Nephrotic syndrome HSP nephritis Renal dysplasia Renal scarring Other
Non-Inflammatory	Minor operation Dental surgery Infusion Other

B3: Consent forms and information sheets

B3.1. JSLE patient/parent information sheet



UK Children's Lupus Study

Information sheet for Parents

(JSLE patients- Liverpool only)

Why is this being done?

This study is looking at a condition called Lupus. It is an autoimmune disease, which means that for some reason the body reacts against itself.

We would like to learn more about how Lupus affects children and their treatment. To create better treatments for Lupus we need to know more about the cause of it. To do this we need to compare children with Lupus to children without. Specifically we want to compare blood cells to see why Lupus cells are reacting against themselves. We are also looking at urine to help understand the Kidney problems seen.

This project is part of a UK wide study of children with Lupus.

Why are we asking your child to take part?

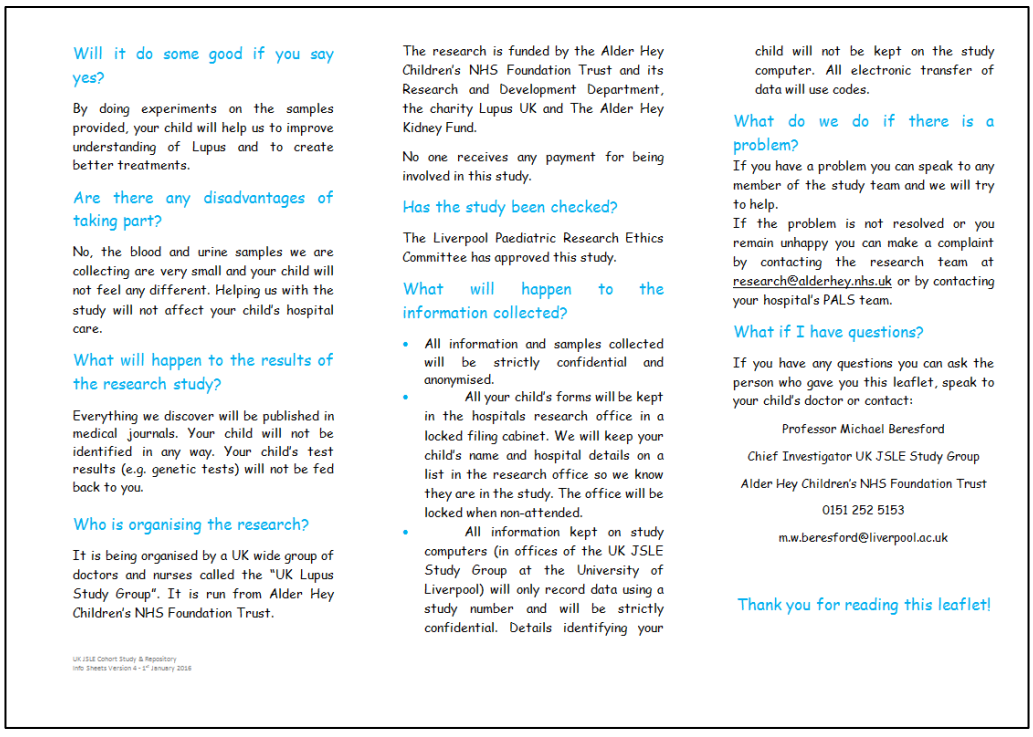
Because they have Lupus.

Does your child have to take part?

No, it's up to you and your child. Either of you can change your mind at any point without giving a reason. This will not affect their care.

What will your child be asked to do?

- Your child's doctor will collect information about your child's age, gender and how they are when visiting hospital.
- When your child is having their usual blood tests we will collect a little extra blood (2-3 teaspoons) to:
  - Measure antibodies and white cells (these fight infection)
  - Study the genetics of Lupus
  - Study the Kidney involvement
- If your child has only just been diagnosed we will collect an extra sample of blood before starting treatment and at one week, one month and three months of starting treatment.
- We will also ask if your child can provide a urine sample.
- When Lupus affects the kidney, it may be necessary for your child to have a 'kidney biopsy'. This is where a bit of kidney tissue is removed and looked at under the microscope. For this study, we would ask to be able to use any extra sample which is left over after all the standard tests have been done. This sample would be labelled with your child's study code and stored at the University of Liverpool, Alder Hey Children's Hospital. DNA (the chemical which contains your genes) will also be extracted from this sample.
- If your child has previously had a kidney biopsy we will ask your local hospital if they have enough to provide us with a piece of this.
- We will tell your child's GP they are helping us with the study.
- We will tell the Health and Social Care Information Centre that you are in the study. They collect information on all people in the UK e.g. if someone dies or develops cancer. If such an event happened, they would let us know about it.
- We will ask for an email address
- We will be in touch about future studies in Lupus to see if your child is interested in taking part.



Will it do some good if you say yes?

By doing experiments on the samples provided, your child will help us to improve understanding of Lupus and to create better treatments.

Are there any disadvantages of taking part?

No, the blood and urine samples we are collecting are very small and your child will not feel any different. Helping us with the study will not affect your child's hospital care.

What will happen to the results of the research study?

Everything we discover will be published in medical journals. Your child will not be identified in any way. Your child's test results (e.g. genetic tests) will not be fed back to you.

Who is organising the research?

It is being organised by a UK wide group of doctors and nurses called the "UK Lupus Study Group". It is run from Alder Hey Children's NHS Foundation Trust.

The research is funded by the Alder Hey Children's NHS Foundation Trust and its Research and Development Department, the charity Lupus UK and The Alder Hey Kidney Fund.

No one receives any payment for being involved in this study.

Has the study been checked?

The Liverpool Paediatric Research Ethics Committee has approved this study.

What will happen to the information collected?

- All information and samples collected will be strictly confidential and anonymised.
- All your child's forms will be kept in the hospitals research office in a locked filing cabinet. We will keep your child's name and hospital details on a list in the research office so we know they are in the study. The office will be locked when non-attended.
- All information kept on study computers (in offices of the UK JSLE Study Group at the University of Liverpool) will only record data using a study number and will be strictly confidential. Details identifying your child will not be kept on the study computer. All electronic transfer of data will use codes.

What do we do if there is a problem?

If you have a problem you can speak to any member of the study team and we will try to help.
If the problem is not resolved or you remain unhappy you can make a complaint by contacting the research team at research@alderhey.nhs.uk or by contacting your hospital's PALS team.

What if I have questions?

If you have any questions you can ask the person who gave you this leaflet, speak to your child's doctor or contact:

Professor Michael Beresford

Chief Investigator UK JSLE Study Group

Alder Hey Children's NHS Foundation Trust

0151 252 5153

m.w.beresford@liverpool.ac.uk

Thank you for reading this leaflet!

B3.2. JSLE patient/parent consent form

Alder Hey Children's NHS
NHS Foundation Trust

Parental Consent Form

(Liverpool only)

UK Juvenile SLE Cohort Study and Repository

		Please INITIAL box
1.	I have read and understand the information sheet (Version 4 – 1st January 2016) for the above study and have had the chance to ask questions	
2.	I understand my child's taking part is voluntary and that I am free to withdraw at any time, without giving any reason, without my child's medical care or legal rights being affected	
3.	I understand that relevant sections of my child's medical notes and data collected during the study may be looked at by responsible individuals from the UK JSLE Cohort Study & Repository research team, from regulatory authorities, or from the NHS Trust where it is relevant to my taking part in this research but understand strict confidentiality will be maintained. I give permission for these individuals to have access to my records.	
4.	I agree that a small amount of my child's blood may be used to investigate the immune system	
5.	I agree that a small amount of my child's blood may be collected and then gifted to the "UK JSLE Study Group." It will be stored for future genetic studies. I understand that no result on my child's genes will be fed back to them or anyone else.	
6.	I agree that a small amount of my blood and urine samples may be used to look into ways of detecting kidney damage in lupus.	
7.	I agree that if my child were to require a kidney biopsy in the future, then a small amount of left over kidney tissue could be stored as an anonymised coded sample at the University of Liverpool, Alder Hey Children's Hospital	
8.	**To be completed if your child has had a kidney biopsy in the past:** I agree that a small amount of left over kidney tissue from my child's previous kidney biopsy can be stored as an anonymised coded sample at the University of Liverpool, Alder Hey Children's Hospital.	
9.	I agree that DNA can be extracted and stored from any kidney biopsy sample my child provides. I understand that no result on my genes will be fed back to me or anyone else.	
10.	I agree to allow researchers to make contact with me and my child about other studies or a follow-up of this study through my child's doctors and my child's NHS number	
11.	I give permission for my child's GP to be informed that information about my child is to be held on the study database	

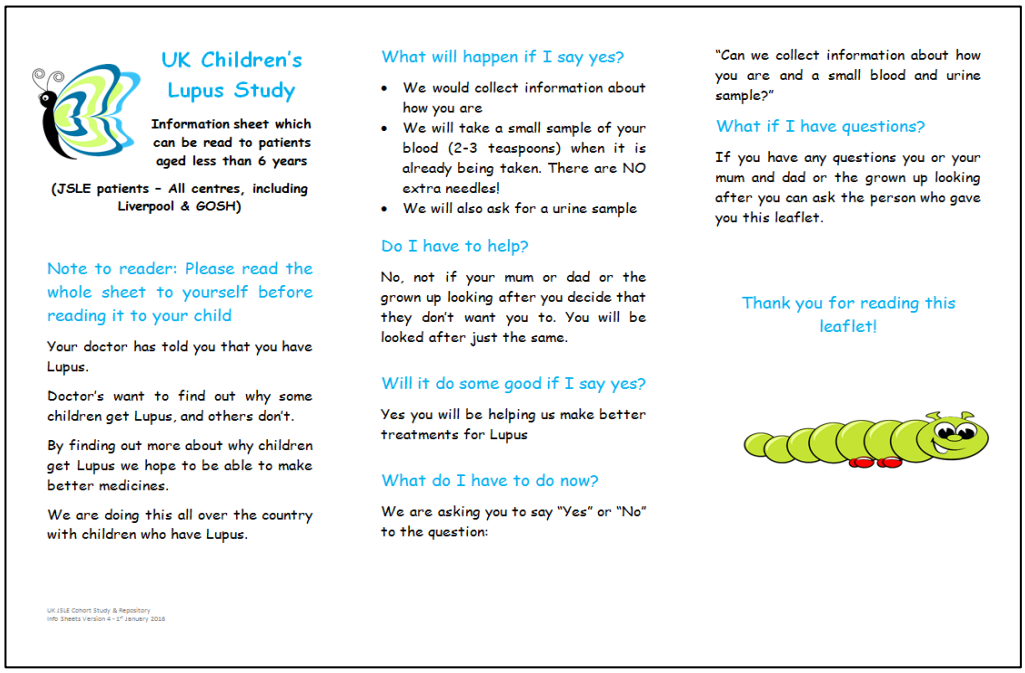
12.	I agree to my child being flagged with the Health and Social Care Information Centre. I understand that information held and managed at the Health and Social Care Information Centre and other central UK NHS bodies may be used in order to help contact me or provide information about my health status.	
13.	I agree that DNA can be extracted and stored from blood that I provide. I understand that no result on my genes will be fed back to me or anyone else.	
14.	I agree to provide an email address. I understand that this will be stored securely. I understand it will only be used to provide information about the study such as the newsletter and to provide a way for the study group to contact us for follow up in the future.	
15.	I agree for my child to take part in the above study	

Name of patient		
Name of person with parental responsibility for patient	Date	Signature
Name of person taking consent (if different from researcher)	Date	Signature
Researcher	Date	Signature

1 copy for patient and person with parental responsibility, 1 for researcher, 1 to be kept with hospital notes

B3.3. JSLE patient information sheets

B3.3.1. JSLE patient information sheet for under 6



UK Children's Lupus Study

Information sheet which can be read to patients aged less than 6 years

(JSLE patients – All centres, including Liverpool & GOSH)

Note to reader: Please read the whole sheet to yourself before reading it to your child

Your doctor has told you that you have Lupus.

Doctor's want to find out why some children get Lupus, and others don't.

By finding out more about why children get Lupus we hope to be able to make better medicines.

We are doing this all over the country with children who have Lupus.

What will happen if I say yes?

- We would collect information about how you are
- We will take a small sample of your blood (2-3 teaspoons) when it is already being taken. There are NO extra needles!
- We will also ask for a urine sample

Do I have to help?

No, not if your mum or dad or the grown up looking after you decide that they don't want you to. You will be looked after just the same.

Will it do some good if I say yes?

Yes you will be helping us make better treatments for Lupus

What do I have to do now?

We are asking you to say "Yes" or "No" to the question:

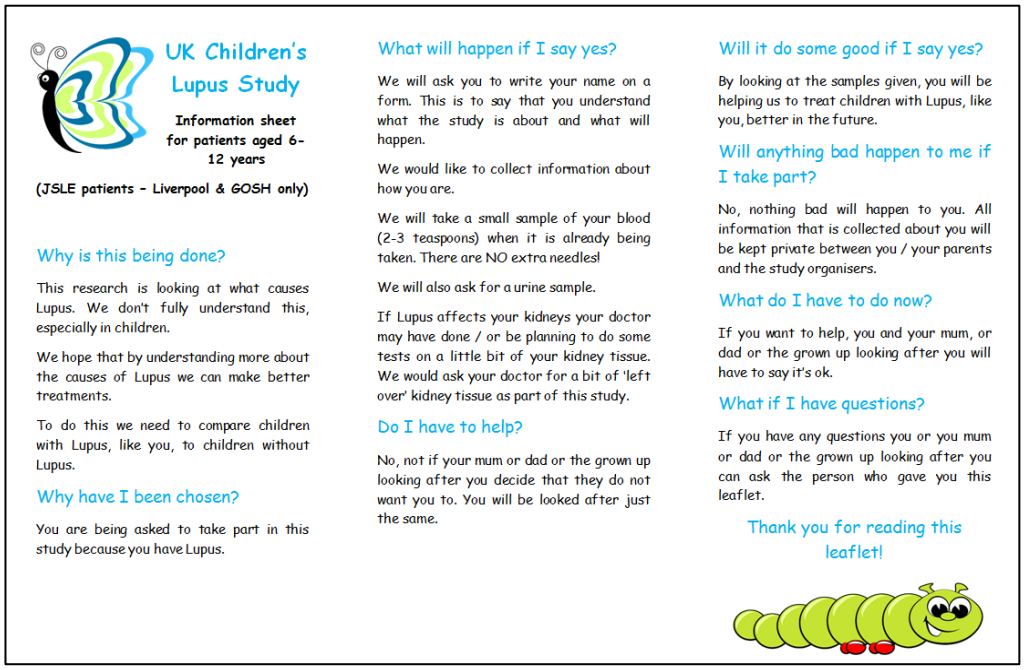
"Can we collect information about how you are and a small blood and urine sample?"

What if I have questions?

If you have any questions you or your mum and dad or the grown up looking after you can ask the person who gave you this leaflet.

Thank you for reading this leaflet!

B3.3.2. JSLE patient information sheet for 6-12



UK Children's Lupus Study

Information sheet for patients aged 6-12 years

(JSLE patients – Liverpool & GOSH only)

Why is this being done?

This research is looking at what causes Lupus. We don't fully understand this, especially in children.

We hope that by understanding more about the causes of Lupus we can make better treatments.

To do this we need to compare children with Lupus, like you, to children without Lupus.

Why have I been chosen?

You are being asked to take part in this study because you have Lupus.

What will happen if I say yes?

We will ask you to write your name on a form. This is to say that you understand what the study is about and what will happen.

We would like to collect information about how you are.

We will take a small sample of your blood (2-3 teaspoons) when it is already being taken. There are NO extra needles!

We will also ask for a urine sample.

If Lupus affects your kidneys your doctor may have done / or be planning to do some tests on a little bit of your kidney tissue. We would ask your doctor for a bit of 'left over' kidney tissue as part of this study.

Do I have to help?

No, not if your mum or dad or the grown up looking after you decide that they do not want you to. You will be looked after just the same.

Will it do some good if I say yes?

By looking at the samples given, you will be helping us to treat children with Lupus, like you, better in the future.

Will anything bad happen to me if I take part?

No, nothing bad will happen to you. All information that is collected about you will be kept private between you / your parents and the study organisers.

What do I have to do now?

If you want to help, you and your mum, or dad or the grown up looking after you will have to say it's ok.

What if I have questions?

If you have any questions you or you mum or dad or the grown up looking after you can ask the person who gave you this leaflet.

Thank you for reading this leaflet!

B3.3.3. JSLE patient information sheet for 13-15

UK Children's Lupus Study

Information sheet for patients aged 13-15 years |

(JSLE patients - Liverpool only)

Why is this study being done?

This study is looking at a condition called Lupus. It is an autoimmune disease, which means that for some reason the body reacts against itself.

We would like to learn more about how Lupus affects children and their treatment. To create better treatments for Lupus we need to know more about the cause of it. To do this we need to compare the blood cells and urine of children with and without Lupus. This will give us information on why Lupus cells are reacting against themselves and the kidney problems seen in Lupus.

Why am I being asked to take part?

Because you have Lupus.

Do I have to take part?

No, it's up to you. You can change your mind at any point without giving a reason. This will not affect your hospital care.

What will happen if I say yes?

- Your doctor will collect information about your age, gender and how you are.
- When you are having your usual blood tests we will collect a little extra blood (2-3 teaspoons). We will use this to:
  - Measure your autoantibodies and white cells (these usually fight infection)
  - Study the genetics of Lupus
  - Study the kidney involvement
- If you have only just been diagnosed we will collect an extra sample of blood before you start treatment and after one week, one month and three months of starting treatment.
- We will also ask if you can give us a urine sample.
- When Lupus affects the kidney, it may be necessary to have a 'kidney biopsy'. This is where a bit of kidney tissue is removed and looked at under the microscope. We would like to have any extra sample, which is left over after all the standard tests have been done. This sample would be stored at the University of Liverpool. DNA (contained in your genes) in the kidney sample will also be tested.
- If you have had a kidney biopsy in the past we will ask your local hospital if they have enough to provide us with a small piece of this.
- We will tell your GP you are in the study.
- We will tell the Health and Social Care Information Centre that you are in the study. They collect health information on all people in the UK e.g. if someone dies or develops cancer.
- We will ask for an email address
- We will be in touch about any future studies in Lupus to see if you are interested in taking part.

Will it do some good if I say yes?

By doing experiments on your samples you will help us to improve understanding of Lupus.

Are there any disadvantages of taking part?

No, the blood and urine samples we are collecting are very small. Helping us with the study will not affect your hospital care.

What will happen to the results of the research study?

Everything we discover will be presented in scientific meetings and published in medical journals. This may take several years.

Individual test results (e.g. genetic tests) will not be fed back to you.

Who is organising the research?

It is being organised by a UK wide group of doctors and nurses called the "UK Lupus Study Group".

The research is being funded by Alder Hey Children's NHS Foundation Trust, it's Research and Development Department, Lupus UK and The Alder Hey Kidney Fund.

No one, including your doctor receives any payment for being involved.

UK JSLE Cohort Study & Repository
Info Sheets Version 4 - 1st January 2016

Has the study been checked?

The Liverpool Research Ethics Committee has approved this study.

What will happen to the information collected about me?

All information and samples collected from you will be strictly confidential and anonymised.

All your study forms will be kept in your hospitals research office, stored in a locked filing cabinet. Your name and hospital details will be kept on a list in the research office so we know you are in the study. This office is locked when not-attended, for confidentiality.

All information kept on study computers (at the University of Liverpool) will only record data using your study number and be strictly confidential. Details identifying who you are will not be kept on the study computer. All electronic transfer of data will use codes.

What if I have questions?

If you have any questions you, your mum / dad or the grown up looking after you can speak to the person who gave you this leaflet, speak to your doctor or contact:

Professor Michael Beresford

Chief Investigator UK JSLE Study Group

Alder Hey Children's NHS Foundation Trust

0151 252 5153

m.w.beresford@liverpool.ac.uk

Thank you for reading this leaflet!

B3.3.4. JSLE patient information sheet for 16 and over

UK Children's Lupus Study

Information sheet for patients aged 16 years and older

(JSLE patients - Liverpool only)

Why is this study being done?

This study is looking at a condition called Lupus. It is an autoimmune disease, which means that for some reason the body reacts against itself.

We would like to learn more about how Lupus affects children and their treatment. To create better treatments for Lupus we need to know more about the cause of it. To do this we need to compare the blood cells of children with and without Lupus, to see why Lupus cells are reacting against themselves. We are also looking at urine to better understand the kidney problems seen in Lupus.

Why am I being asked to take part?

Because you have Lupus.

Do I have to take part?

No, it's up to you. You can change your mind at any point without giving a reason. This will not affect your hospital care.

What will happen if I say yes?

- Your doctor will collect information about your age, gender and how you are when you visit the hospital.
- When you are having your usual blood tests we will collect a little extra blood (2-3 teaspoons). We will use this to:
  - Measure your autoantibodies and white cells (these usually fight infection)
  - Study the genetics of Lupus
  - Study the kidney involvement
- If you have only just been diagnosed we will collect an extra sample of blood before you start treatment and after one week, one month and three months of starting treatment.
- We will also ask if you can give us a urine sample.
- When Lupus affects the kidney, it may be necessary to have a 'kidney biopsy'. This is where a bit of kidney tissue is removed and looked at under the microscope. We would like to have any extra sample which is left over after all the standard tests have been done. This sample would be stored at the University of Liverpool, Alder Hey Children's Hospital. DNA (contained in your genes) in the kidney sample will also be tested.
- If you have had a kidney biopsy in the past we will ask your local hospital if they have enough to provide us with a small piece of this.
- We will tell your GP you are in the study.
- We will tell the Health and Social Care Information Centre that you are in the study. They collect information on all people in the UK e.g. if someone dies or develops cancer. If such an event happened, they would let us know about it.
- We will ask for an email address
- We will be in touch about any future studies in Lupus to see if you are interested in taking part.

Will it do some good if I say yes?

By doing experiments on your samples you will help us to improve understanding of Lupus and create better treatments.

Are there any disadvantages of taking part?

No, the blood and urine samples we are collecting are very small. Helping us with the study will not affect your hospital care.

What will happen to the results of the research study?

Everything we discover will be presented in scientific meetings and published in medical journals. This may take several years. Individual test results (e.g. genetic tests) will not be fed back to you.

Who is organising the research?

It is being organised by a UK wide group of doctors and nurses called the "UK Lupus Study Group". It is run from Alder Hey Children's Hospital.

The research is being funded by Alder Hey Children's NHS Foundation Trust, it's Research and Development Department, Lupus UK and The Alder Hey Kidney Fund.

No one, including your doctor receives any payment for being involved.

Has the study been checked?

The Liverpool Research Ethics Committee has approved this study.

What will happen to the information collected about me?

All information and samples collected from you will be strictly confidential and anonymised.

All your study forms will be kept in your hospitals research office, stored in a locked filing cabinet. Your name and hospital details will be kept on a list in the research office so we know you are in the study. This office is locked when not-attended, for confidentiality.

All information kept on study computers (at the University of Liverpool) will only record data using your study number and be strictly confidential. Details identifying who you are will not be kept on the study computer. All electronic transfer of data will use codes.

What do we do if there is a problem?

If you have a problem you can speak to any member of the study team and we will try to help.
If the problem is not resolved or you remain unhappy you can make a complaint by contacting the research team at research@alderhey.nhs.uk or by contacting your hospital's PALS team.

What if I have questions?

If you have any questions you can ask the person who gave you this leaflet, speak to your doctor or contact:

Professor Michael Beresford

Chief Investigator UK JSLE Study Group

Alder Hey Children's NHS Foundation Trust

0151 252 5153

m.w.beresford@liverpool.ac.uk

Thank you for reading this leaflet!

UK JSLE Cohort Study & Repository
Info Sheets Version 4 - 1st January 2016

B3.4. JSLE patient assent form

Alder Hey Children's NHS
NHS Foundation Trust

Assent Form – Patients

(Liverpool and GOSH only)

UK Juvenile SLE Cohort Study and Repository

		Please INITIAL box
1.	I have been told about the study and given the information sheet (Version 4 – 1st January 2016) for the above study and have had the chance to ask questions	
2.	I understand taking part is voluntary and I can stop taking part at any time, without giving any reason, without this making any difference to my medical care or legal rights	
3.	I understand that relevant sections of my medical notes and data collected during the study may be looked at by responsible individuals from the UK JSLE Cohort Study & Repository research team, from regulatory authorities, or from the NHS Trust, where it is relevant to my taking part in this research but understand strict confidentiality will be maintained. I give permission for these individuals to have access to my records	
4.	I agree that a small amount of my blood and in some cases urine may be used for research.	
5.	I agree that if I need a kidney biopsy in the future, then a small amount of left over kidney tissue could be stored as an anonymised coded sample at the University of Liverpool, Alder Hey Children's Hospital, to look at how the kidney is affected by Lupus.	
6.	**To be completed by patients who have had a kidney biopsy in the past:** I agree that a small amount of left over kidney tissue from my previous kidney biopsy can be stored as an anonymised coded sample at the University of Liverpool, Alder Hey Children's Hospital.	
7.	I agree that DNA can be extracted and stored from any kidney biopsy samples that I provide. I understand that no result on my genes will be fed back to me or anyone else.	
8.	I agree that I will take part in the above study	
9.	I agree to allow researchers to make contact with me about other studies or a follow-up of this study through my doctors and NHS number	
10.	I give permission for my GP to be informed that information about me is to be held on the study database	
11.	I agree to being flagged with the Health and Social Care Information Centre. I understand that information held and managed at the Health and Social Care Information Centre and other central UK NHS bodies may be used in order to help contact me or provide information about my health status.	
12.	I agree that DNA can be extracted and stored from blood that I provide. I understand that no result on my genes will be fed back to me or anyone else.	

13.	I agree to provide my email address. I understand that this will be stored securely. I understand it will only be used to provide me with information about the study such as the newsletter and to provide a way for the study group to contact me for follow up in the future.	

Name of patient	Date	Signature
Name of person taking consent (if different from researcher)	Date	Signature
Researcher	Date	Signature

1 copy for patient, 1 for researcher, 1 to be kept with hospital notes

B3.5 JSLE patient consent form

Alder Hey Children's NHS
NHS Foundation Trust

Patient's Consent Form

(Liverpool only)

UK Juvenile SLE Cohort Study and Repository

Please INITIAL box

1.	I have read and understand the information sheet (Version 4 -1st January 2016) for the above study and have had the chance to ask questions	
2.	I understand taking part is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected	
3.	I understand that relevant sections of my medical notes and data collected during the study may be looked at by responsible individuals from the UK JSLE Cohort Study & Repository research team, from regulatory authorities, or from the NHS Trust where it is relevant to my taking part in this research but understand strict confidentiality will be maintained. I give permission for these individuals to have access to my records	
4.	I agree that a small amount of my blood may be used to investigate the immune system	
5.	I agree that a small amount of my blood may be collected and gifted to the "UK JSLE Study Group." It will be stored for future genetic studies. I understand that no result on my genes will be fed back to me or anyone else.	
6.	I agree that a small amount of my blood and urine samples may be used to look into ways of detecting kidney damage in lupus.	
7.	I agree that if I was to need a kidney biopsy in the future, then a small amount of left over kidney tissue could be stored as an anonymised coded sample at the University of Liverpool, Alder Hey Children's Hospital, to look at how the kidney is affected by Lupus.	
8.	**To be completed by patients who have had a kidney biopsy in the past:** I agree that a small amount of left over kidney tissue from my previous kidney biopsy can be stored as an anonymised coded sample at the University of Liverpool, Alder Hey Children's Hospital.	
9.	I agree that DNA can be extracted and stored from any kidney biopsy samples that I provide I understand that no result on my genes will be fed back to me or anyone else.	
10.	I agree to allow researchers to make contact with me about other studies or a follow-up of this study through my doctors and my NHS number	
11.	I give permission for my GP to be informed that information about me is to be held on the study database	

12.	I agree to being flagged with the Health and Social Care Information Centre. I understand that information held and managed at the Health and Social Care Information Centre and other central UK NHS bodies may be used in order to help contact me or provide information about my health status.	
13.	I agree that DNA can be extracted and stored from blood that I provide. I understand that no result on my genes will be fed back to me or anyone else.	
14.	I agree to provide my email address. I understand that this will be stored securely. I understand it will only be used to provide me with information about the study such as the newsletter and to provide a way for the study group to contact me for follow up in the future.	
15.	I agree that I will take part in the above study	

Name of patient	Date	Signature
Name of person taking consent (if different from researcher)	Date	Signature
Researcher	Date	Signature

1 copy for patient, 1 for researcher, 1 to be kept with hospital notes

B3.6 Paediatric control patient/parent information sheet

UK Children's Lupus Study

Information sheet for Parents

(Paediatric controls - Liverpool only)

Why is this being done?

This study is looking at a condition called Lupus. It is an autoimmune disease, which means that for some reason the body reacts against itself.

To create better treatments for Lupus we need to know more about the cause of it. To do this we need to compare children with Lupus to children without Lupus, like your child. Specifically we need to compare blood cells to see why Lupus cells are reacting against themselves and urine to detect inflammation of the kidney, a common complication of Lupus.

This project is part of a UK wide study of children with Lupus.

Why are we asking your child to take part?

Because they do not have Lupus.

Does your child have to take part?

No, it's up to you and your child. You and your child can change your mind at any point without giving a reason. This will not affect the care they receive.

What will your child be asked to do?

- We will take a sample of your child's blood (2-3 teaspoons). If your child needs a blood test for another reason we will take it at the same time.
- We will also ask if your child can provide a urine sample. We use this to look at how the kidneys work in Lupus.
- We will collect information about your child's age, gender and your child's general health. Your child's participation in the study will be kept confidential.
- We will keep your child's name and hospital details on a list in the hospitals locked research office so we know you are in the study.
- We will tell your GP you are helping us with the study

Will it do some good if we say yes?

By doing experiments on the samples given by your child, this will help us create better treatments for children with Lupus.

Are there any disadvantages of taking part?

No, the blood and urine samples we are collecting are very small. Helping us with the study will not affect their care.

What will happen to the results of the research study?

Everything we discover will be presented at scientific meetings and published in medical journals. Your child will not be identified in any way.

Who is organising the research?

It is being organised by a group of doctors and nurses from around the country called the "UK Lupus Study Group". It is run from Alder Hey Children's NHS Foundation Trust.

The research is being funded by Alder Hey Children's NHS Foundation Trust, it's Research and Development Department at Alder Hey, the charity Lupus UK and The Alder Hey Kidney Fund.

No one, including your doctor receives any payment for being involved in this study.

Has the study been checked?

The Liverpool Paediatric Research Ethics Committee has approved this study.

UK JSLE Cohort Study & Repository
Info Sheets Version 4 - 1st January 2016

What will happen to the information collected?

All information and samples collected will be strictly confidential and anonymised.

Forms will be kept in the hospitals research office. All forms will be stored in locked filing cabinets in rooms that are locked when non-attended.

All information kept on study computers (in offices of the UK JSLE Study Group at the Institute Child Health, University of Liverpool) will only record data using a study number and will be strictly confidential. Details identifying your child will not be kept on the study computer. All electronic transfer of data will use codes.

What do we do if there is a problem?

If you have a problem you can speak to any member of the study team and we will try to help.
If the problem is not resolved or you remain unhappy you can make a complaint by contacting the research team at research@alderhey.nhs.uk or by contacting your hospital's PALS team.

What if I have questions?

If you have any questions you can ask the person who gave you this leaflet, speak to your doctor or contact:

Professor Michael Beresford

Chief Investigator UK JSLE Study Group

Alder Hey Children's NHS Foundation Trust

0151 252 5153

m.w.beresford@liverpool.ac.uk

Thank you for reading this leaflet!

B3.7. Paediatric control patient/parental consent form

Alder Hey Children's NHS
NHS Foundation Trust

Parental Consent Form
(Paediatric Controls - Liverpool only)
UK Juvenile SLE Cohort Study and Repository

Please INITIAL box

1.	I have read and understand the information sheet (Version 4 - 1st January 2016) for the above study and have had the chance to ask questions.	
2.	I understand my child's taking part in voluntary and that I am free to withdraw at any time, without giving any reason, without my child's medical care or legal rights being affected.	
3.	I understand that relevant sections of my child's medical notes and data collected during the study may be looked at by responsible individuals from the UK JSLE Cohort Study & Repository research team, from regulatory authorities, or from the NHS Trust where it is relevant to my taking part in this research but understand strict confidentiality will be maintained. I give permission for these individuals to have access to my records.	
4.	I agree that a small amount of my child's blood may be used to investigate Lupus.	
5.	I agree that a small amount of my child's urine may be used to investigate the kidney.	
6.	I agree that DNA can be extracted and stored from blood that I provide. I understand that no result on my genes will be fed back to me or anyone else.	
7.	I give permission for my GP to be informed that information about me is to be held on the study database.	
8.	I agree for my child to take part in the above study	

Name of patient

Name of person with parental responsibility for patient	Date	Signature
Name of person taking consent (if different from researcher)	Date	Signature
Researcher	Date	Signature

1 copy for patient and person with parental responsibility, 1 for researcher, 1 to be kept with hospital notes

B3.8. Paediatric control patient information sheet

B3.8.1. Paediatric control patient information sheet for under 6

UK Children's Lupus Study

Information sheet for patients aged less than 6 years

(Paediatric controls – Liverpool only)

Note to reader: Please read the whole sheet to yourself before reading it to your child

Some children are sick because they have an illness called Lupus.

Doctor's want to find out why some children get Lupus and others don't

To do this we need to compare children with Lupus to children without Lupus, like you

UK JSLE Cohort Study & Repository
Info Sheets Version 4 - 1st January 2016

Why have I been chosen?

Because you do not have Lupus

What will happen if I say yes?

- We would like to collect information about how you are.
- We will take a small sample of your blood (2-3 teaspoons) when it is already being taken or you are going for an operation. There are NO extra needles!
- We will also ask for a urine sample.

Do I have to help?

No, not if you or your mum / dad or the grown up looking after you decide you do not want to. You will be looked after just the same.

Will it do some good if I say yes?

Yes, you will be helping us invent better treatments for sick children with Lupus

What do I have to do now?

We are asking you to say "Yes" or "No" to the question:

"Can we collect information about how you are and a small blood and urine sample?"

What if I have questions?

If you have any questions you or you mum or dad, or the grown up looking after you can ask the person who gave you this leaflet.

Thank you for reading this leaflet!

B3.8.2.Paediatric control patients' information sheet for 6-12

UK Children's Lupus Study

Information sheet for patients aged 6-12 years

(Paediatric controls – Liverpool only)

Why is this research being done?

This research looks at a condition called "Lupus". We want to understand more about what causes it and the best ways to treat it.

To do this we need to compare children with Lupus to children without Lupus.

Why have I been chosen?

You are being asked to take part in this study because you do not have Lupus.

UK JSLE Cohort Study & Repository
Info Sheets Version 4 - 1st January 2016

Do I have to help?

No, not if you, your mum / dad or the grown up looking after you do not want to. You will be looked after just the same.

What will happen if I say yes?

You will be asked to write your name on a form. This is to say that you understand the study and what will happen. You will be given your own copy of the form to keep as well as this leaflet.

We will take a sample of your blood (2-3 teaspoons) when you are going for your operation. There are NO extra needles!

We will also ask if you can give us a urine sample. We use this to look at how the kidneys work in Lupus.

Will it do some good if I say yes?

Using your samples, we will work on ways to us create better treatments for children with Lupus.

Will anything bad happen to me if I take part?

No, nothing bad will happen to you.

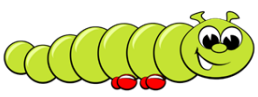
What do I have to do now?

If you want to help, you and your mum or dad or the grown up looking after you will have to say it's ok.

What if I have questions?

If you have any questions you or you mum or dad or the grown up looking after you can ask the person who gave you this leaflet.

Thank you for reading this leaflet!



B3.8.3 Paediatric control patient information sheet for 13-15

UK Children's Lupus Study

Information sheet for patients aged 13-15 years

(Paediatric controls – Liverpool only)

Why is this study being done?

This study is looking at a condition called "Lupus". We don't know a lot about what causes it or the best ways to treat it. We hope that by understanding more about what causes Lupus we can create better treatments.

To do this we need to compare children with Lupus to children without Lupus, like you.

Why have I been chosen?

You are being asked to take part in this study because you do not have Lupus.

Do I have to help?

No, it's up to you and your parents or the adult looking after you. You can change your mind at any time during the research without giving a reason. This will not affect the care you receive.

What will happen if I say yes?

- We will take a blood sample (2-3 teaspoons). If you need blood tests for another reason we will take it at the same time.
- We will also ask if you can give us a urine sample.
- We will collect information about your age, gender and your general health.
- We will keep your name and hospital details on a list in the hospitals locked research office so we know you are in the study.
- We will tell your GP you are helping us with the study.

Will it do some good if I say yes?

By doing experiments on your samples, you will be helping us create better treatments for children with Lupus in the future.

Are there any disadvantages of taking part?

No, the blood and urine samples we are collecting are very small and you will not feel any different. Helping us with the study will not affect your hospital care

Who is organising the research?

It is being organised by a group of doctors and nurses from around the country called the "UK Lupus Study Group". It is run from Alder Hey Hospital.

Has the study been checked?

The Liverpool Paediatric Research Ethics Committee has approved this study.

What do I have to do now?

If you are happy to take part you will be asked to write your name on a form. This is to say that you understand the study and what will happen. You will be given your own form to keep as well as this leaflet.

What if I have questions?

If you have any questions you or you mum or dad or the grown up looking after you can ask the person who gave you this leaflet, speak to your doctor or contact:

Professor Michael Beresford

Chief Investigator UK JSLE Study Group

Alder Hey Children's NHS Foundation Trust

0151 252 5153

m.w.beresford@liverpool.ac.uk

Thank you for reading this leaflet!

B3.8.4. Paediatric control patient information sheet for 16 and over

UK Children's Lupus Study

Information sheet for patients aged 16 years and older

(Paediatric controls - Liverpool only)

What is a study? Why is this being done?

This study is looking at a condition called Lupus. It is an autoimmune disease, which means that for some reason the body attacks itself. To create better treatments for Lupus we need to know more about the cause of it. To do this we need to compare the blood cells of children with Lupus and without Lupus to see why Lupus cells are reacting against themselves. In addition we need to look at urine to detect inflammation of the kidney, a common complication of Lupus.

This project is part of a UK wide study of children with Lupus.

Why am I being asked to take part?

Because you do not have Lupus.

Do I have to take part?

No, it's up to you. You can change your mind at any point without giving a reason. This will not affect the care you receive.

What will happen if I say yes?

- We will take a sample of your blood (2-3 teaspoons). If you are having blood tests for another reason we will take it at the same time.
- We will also ask if you can give us a urine sample. We use this to look at how the kidneys work in Lupus.
- We will collect information about your age, gender and your general health.
- We will keep your name and hospital details on a list in your hospitals research office so that we know you are in the study. Your participation will be kept strictly confidential.
- We will tell your GP you are helping us with the study

Will it do some good if I say yes?

By doing experiments on the samples given you will be helping us create better treatments for children with Lupus in the future.

Are there any disadvantages of taking part?

No, the blood and urine samples we are collecting are very small and you will not feel any different. Helping us with the study will not affect your hospital care.

What will happen to the results of the research study?

Everything we discover will be presented at scientific conferences and published in medical journals. You will not be identified in any way.

Who is organising the research?

It is being organised by a group of doctors and nurses from around the country called the "UK Lupus Study Group". It is run from Alder Hey Children's NHS Foundation Trust.

The research is being funded by Alder Hey Children's NHS Foundation Trust, it's Research and Development Department at Alder Hey, the charity Lupus UK and The Alder Hey Renal Fund.

No one, including your doctor receives any payment for being involved in this study.

Has the study been checked?

The Liverpool Paediatric and Research Ethics Committee have approved this study.

What will happen to the information collected about me?

All information and samples collected from you will be strictly confidential and anonymised. This means that no-one will know it belongs to you.

UK JSLE Cohort Study & Repository
Info Sheets Version 4 - 1st January 2016

Forms will be kept in your hospitals research office. They will be stored in locked filing cabinets at all times, in rooms that are locked when non-attended.

All information kept on study computers (in offices of the UK JSLE Study Group at the Institute Child Health, University of Liverpool) will only record data using your study number and be strictly confidential. Details identifying who you are will not be kept on the study computer. All electronic transfer of data will use codes.

What do we do if there is a problem?

If you have a problem you can speak to any member of the study team and we will try to help.
If the problem is not resolved or you remain unhappy you can make a complaint by contacting the research team at research@alderhey.nhs.uk or by contacting your hospital's PALS team.

What if I have questions?

If you have any questions you can ask the person who gave you this leaflet, speak to your doctor or contact:

Professor Michael Beresford

Chief Investigator UK JSLE Study Group

Alder Hey Children's NHS Foundation Trust

0151 252 5153

m.w.beresford@liverpool.ac.uk

Thank you for reading this Leaflet!

B3.9. Paediatric control patient assent form

Alder Hey Children's NHS
NHS Foundation Trust

Assent Form – Controls (on local centre headed paper)

(Paediatric Controls – Liverpool only)

UK Juvenile SLE Cohort Study and Repository

Please INITIAL box

1.	I have been told about the study and given the information sheet (Version 4, 1st January 2016) for the above study and have had the chance to ask questions.	
2.	I understand taking part is voluntary and I can stop taking part at any time, without giving any reason, without this making any difference to my medical care or legal rights.	
3.	I understand that relevant sections of my medical notes and data collected during the study may be looked at by responsible individuals from the UK JSLE Cohort Study & Repository research team, from regulatory authorities, or from the NHS Trust where it is relevant to my taking part in this research but understand strict confidentiality will be maintained. I give permission for these individuals to have access to my records.	
4.	I agree that a small amount of my blood may be used to investigate Lupus.	
5.	I agree that a small amount of my urine may be used to investigate the kidney.	
6.	I agree that DNA can be extracted and stored from blood that I provide. I understand that no result on my genes will be fed back to me or anyone else.	
7.	I give permission for my GP to be informed that information about me is to be held on the study database.	
8.	I agree that I will take part in the above study.	

Name of patient	Date	Signature
Name of person taking consent (if different from researcher)	Date	Signature
Researcher	Date	Signature

1 copy for patient, 1 for researcher, 1 to be kept with hospital notes

B3.10. Paediatric control patient consent form

Alder Hey Children's NHS
NHS Foundation Trust

Patient's Consent Form

(Paediatric Controls – Liverpool only)

UK Juvenile SLE Cohort Study and Repository

Please INITIAL box

1.	I have read and understand the information sheet (Version 4 – 1st January 2016) for the above study and have had the chance to ask questions.	
2.	I understand taking part is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected.	
3.	I understand that relevant sections of my medical notes and data collected during the study may be looked at by responsible individuals from the UK JSLE Cohort Study & Repository research team, from regulatory authorities, or from the NHS Trust where it is relevant to my taking part in this research but understand strict confidentiality will be maintained. I give permission for these individuals to have access to my records.	
4.	I agree that a small amount of my blood may be used to investigate Lupus.	
5.	I agree that a small amount of my urine may be used to investigate the kidney.	
6.	I agree that DNA can be extracted and stored from blood that I provide. I understand that no result on my genes will be fed back to me or anyone else.	
7.	I give permission for my GP to be informed that information about me is to be held on the study database.	
8.	I agree that I will take part in the above study.	

Name of patient	Date	Signature
Name of person taking consent (if different from researcher)	Date	Signature
Researcher	Date	Signature

1 copy for patient, 1 for researcher, 1 to be kept with hospital notes

B.11. Adult volunteer information sheet

UNIVERSITY OF LIVERPOOL

PARTICIPANT INFORMATION SHEET

1. **Study Title:** Analysis of normal blood leukocyte function using cells from healthy volunteers.
2. **Version Number and date.** Version 3, January 2015
3. **Invitation.** You are being invited to participate in a research study. Before you decide whether to participate, it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and feel free to ask us if you would like more information or if there is anything that you do not understand. Please also feel free to discuss this with your friends, relatives and GP if you wish. We would like to stress that you do not have to accept this invitation and should only agree to take part if you want to.
4. **What is the purpose of the study?** The purpose of the study is to derive baseline data for how specialised white blood cells in the blood, called leucocytes, behave in normal healthy donors. These cells normally help prevent infection as part of the normal immune response. This will involve measuring protein expression, cell function and/or analysis of genes related to immune function as part of a series of studies in the Dept of Women's and Children's Health, University of Liverpool.
5. **Why have I been chosen?** You have been chosen because you are a normal healthy person who has indicated they are willing to give blood.
6. **Do I have to take part?** No. It is up to you to decide whether or not to take part. If you decide to take part you are still free to withdraw at any time and without giving a reason.
7. **What will happen if I take part?** You will be asked to give 5-30ml blood. We may ask to take blood again, so that we can reproduce our results, but no more frequently than 10 times per year. However, we will only do this if you are well and completely happy to give the extra samples. You are free to say no and opt out of the study at any stage. Blood samples will be tested in the laboratory at the department of Women's and Children's health. All tests will be performed by the recognised researchers concerned with this project. Samples taken will not be identified by a person's name after collection but will be anonymised and only identified to the research worker by a code number. Samples will be tested immediately not stored in department refrigerators or freezers until tests are performed. Only the investigators will have access to this material. Samples will be destroyed after analysis and will not be used for any other research studies.
8. **Expenses and payments**. We do not offer any payments for participants.

9. **What are the risks in taking part?** There are no risks additional to those normally encountered when giving a blood sample for clinical purposes. You may experience slight bruising at the needle site.

10. **Are there any benefits in taking part?** There are no benefits intended for participants but you will be contributing to research which may eventually lead to the development of improved therapies.

11. **What if i am unhappy or if there is a problem?** If you are unhappy, or if there is a problem, please feel free to let us know by contacting Dr. Brian Flanagan (0151 282 4732) and we will try to help. If you remain unhappy or have a complaint which you feel you cannot come to us with then you should contact the Research Governance Officer on 0151 794 8290 (ethics@liv.ac.uk). When contacting the Research Governance Officer, please provide them with name of this study and the researcher(s) involved, and the details of the complaint you wish to make.

12. **Will my taking part in the study be kept confidential?** Yes, the results of the study will be anonymised and any information about you kept confidential. The data may be kept for up to five years on password protected computers.

13. **What will happen to the results of this study?** The results of the research may ultimately be published in scientific papers but you will not be identified in any report.

14. **What will happen if I want to stop taking part?** You are free to withdraw at anytime, without explanation. Results up to the period of withdrawal may be used, if you are happy for this to be done. Otherwise you may request that they are destroyed and no further use is made of them.

15. **Who can I contact if I have further questions?** For any further questions or concerns about participating in this study please contact:

    Dr Brian Flanagan
    Department of Women's and Children's health,
    University of Liverpool,
    Alder hey Hospital,
    Eaton Road,
    Liverpool L12 2AP
    Tel. 0151 282 4732
    email fla1@liv.ac.uk

16. **Duty of care to research participants.** Occasionally research studies can reveal significant unexpected abnormalities which require medical follow-up, either for further investigation or (more rarely) treatment. You will be asked to agree that if any significant abnormality is found, we will send the report to your GP, who will be able to take it further with you. Should this be needed the study PI will discuss this with you.

Thank you for taking the time reading this information sheet, please complete the attached consent form if you wish to participate.

B3.12. Adult volunteer consent form

UNIVERSITY OF LIVERPOOL

Committee on Research Ethics

PARTICIPANT CONSENT FORM

Title of Research Project: Analysis of normal blood leukocyte function using cells from healthy volunteers.

Researcher(s): Dr BF Flanagan

	Please initial box
1. I confirm that I have read and have understood the information sheet version 3 dated January 2015 for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily.	☐
2. I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason, without my rights being affected.	☐
3. Data will be stored electronically in a anonymised form and will not be identified using my name, only a sample code number. I understand that, under the Data Protection Act, I can at any time ask for access to the information I provide and I can also request the destruction of that information if I wish.	☐
4. I understand that confidentiality and anonymity will be maintained and it will not be possible to identify me in any publications.	☐
5. If an unexpected significant abnormality is discovered I consent to my GP being contacted	☐
6. I agree to take part in the above study.	☐

Participant Name		Date	Signature
Name of Person taking consent		Date	Signature
Researcher		Date	Signature

Principal Investigator:
Name: Dr BF Flanagan
Work Address: Department of Women's and Children's Health, Univ. of Liverpool
Work Telephone: 0151 282 4752
Work Email: fla1@liv.ac.uk

Student Researcher:
Name
Work Address
Work Telephone
Work Email

Appendix C. Reagents and equipment

C1. Reagents

1640 RPMI media with L-glutamine	Lonza, Switzerland/SLS, UK
Acetic acid	P&R lab supplies, UK
Ammonium chloride	Sigma-Aldrich, UK
Annexin V FITC	Sigma-Aldrich, UK
Anti – B actin mouse polyclonal antibody (pAB)	Abcam, UK
Anti – caspase 3 rabbit pAB	Cell Signalling, USA - New England Biolabs, UK
Anti - human CD11b (ICRF44) PE mouse IgG1κ monoclonal antibody (mAB)	eBioscience, UK
Anti-human CD62L (DREG56) APC mouse IgG1κ mAB	eBioscience, UK
Anti-human IFNAR1 PE mouse IgG1 antibody (AB)	Sino Biological, China – Stratech, UK
Anti-human IFNAR2 (REA124) APC human mAB	Miltenyi Biotec, UK
Anti-human IFNGR1 (GIR208) PE mouse IgG1 mAB	eBioscience, UK
Anti-human IFNGR2 APC goat IgG pAB	R&D systems, UK
Anti-pSTAT1 (Y701) (58D6) rabbit mAB	Cell Signalling - New England Biolabs, UK
Anti-pSTAT3 (Y705) (3E2) mouse mAB	Cell Signalling - New England Biolabs, UK
Anti – STAT3 (79D7) rabbit mAB	Cell Signalling - New England Biolabs, UK
APC isotype control, mouse IgG1 AB	Beckman Coulter, UK
APC isotype control, mouse IgG1κ AB (p3.6.2.8.1)	eBioscience, UK
APC isotype control, REA control (S) (REA293) human IgG1 mAB	Miltenyi Biotech, UK
APC isotype control, goat IgG pAB	R&D systems, UK
Bromophenol blue	Sigma-Aldrich, UK
Calcium chloride	Sigma-Aldrich, UK
Crystal violet	Sigma-Aldrich, UK

DMEM	Sigma-Aldrich, UK
DTT	Sigma-Aldrich, UK
DMSO	Sigma-Aldrich, UK
Ethanol	Chemistry Department, University of Liverpool, UK
EDTA	Fisher, UK
FBS	Life Technologies, UK
fMLP	Sigma-Aldrich, UK
Formaldehyde	Sigma-Aldrich, UK
Gentamicin	Sigma-Aldrich, UK
Glycerol	BDH, UK
Glycine	Fisher, UK
Goat anti-mouse IgG HRP conjugated pAB	R&D systems, UK
Goat anti-Rabbit IgG HRP conjugated pAB	R&D systems, UK
Fludarabine phosphate	Sigma-Aldrich, UK
Hanks' balanced salt solution (HBSS)	Sigma-Aldrich, UK
Heat-inactivated FBS	Life Technologies, UK
4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)	Sigma-Aldrich, UK
HetaSep	StemCell, UK
Histopaque 1077	Sigma-Aldrich, UK
Hydrogen chloride	BDH, UK
Ionic detergent compatibility reagent for Pierce™ 660nm protein assay	ThermoFisherScientific, UK
LPS	Sigma-Aldrich, UK
Marvel semi-skimmed milk	Sainsbury's, UK
12% mini-PROTEAN® TGX™ precast protein gels, 12-well, 20 µl	BioRAD, UK
fMLP	Sigma-Aldrich, UK
PBS	Sigma-Aldrich, UK Fisher, UK
PE isotype control, IgG1 AB	Beckman Coulter, UK
PE isotype control, anti-mouse Rat mIgG1 AB (M1-14D12)	eBioscience, UK
Pierce™ 660nm protein assay reagent	Life Technologies, UK

pHrodo red *E.coli* bioparticles conjugate for phagocytosis	Life Technologies, UK
PI	Sigma-Aldrich, UK
PMA	Sigma-Aldrich, UK
Poly(HEMA)	Sigma-Aldrich, UK
Polymorph prep	Axis Shield, Norway - Alere Ltd, UK
Potassium bicarbonate	Sigma-Aldrich, UK
Precision plus protein western c standards	BioRAD, UK
Precision protein streptactin HRP conjugate	BioRAD, UK
Prolong gold antifade reagent with DAPI	Molecular Probes by Life Technologies, UK
100x protease and phosphatase inhibitor cocktail	ThermoFisherScientific, UK
Quant-iT™ PicoGreen™ dsDNA assay kit	Invitrogen - ThermoFisherScientific, UK
Recombinant human GMCSF	R&D systems, UK
Recombinant human IFNα	PeproTech, UK
Recombinant human IFNβ	PeproTech, UK
Recombinant human IFNγ	PeproTech, UK
Recombinant human IL-8	PeproTech, UK
Recombinant human TNFα	PeproTech, UK
Sodium azide	Sigma-Aldrich, UK
Sodium chloride	Sigma-Aldrich, UK
Sodium dodecyl (lauryl) sulphate	BioRAD, UK
Sodium hydroxide	BDH, UK
Trans-Blot® Turbo™ mini PVDF transfer packs	BioRAD, UK
Tris base	Fisher, UK
Trypsin	Sigma-Aldrich, UK
Tween 20	Fisher, UK
WesternSure® PREMIUM chemiluminescent substrate	LI-COR, UK

C2. Equipment

C-DiGit® blot Scanner	LI-COR, UK
DM2500 confocal microscope	Leica, UK
Evos microscope w/camera	ThermoFisherScientific, UK

Flow cytometer F500	Beckman Coulter, UK
Guava EasyCyte	Merck Millipore, UK
Light microscope	Leica, UK
TECAN GENios Plus plate reader	Tecan, UK
Western blot electrophoresis tank	BioRAD, UK
Western blot transfer blot	BioRAD, UK
Varioskan flash plate reader	ThermoFisherScientific, UK

Appendix D. Extended patient demographics tables

Appendix D, Table 1. Extended clinical and demographic information at time of blood collection and serum isolation, whereby serum was used in experimental apoptosis analysis (for shortened table, see Table 4.1, Chapter 4)

Variable	Active Haematological pBILAG (n=3)	Active Renal pBILAG[a] (n=5)	Inactive pBILAG[a] (n=5)	Control (n=5)
Age (years)[b]	13.60 [13.12-15.32]	16.59 [11.85-17.97]	15.52 [12.22-17.98]	15.00 [14.78 – 15.36]
Duration of disease (months)[b]	25 [4-109]	13 [0-24]	18 [7-43]	-
Female[c]	2 (66.6)	5 (100)	4 (80)	4 (80)
Total pBILAG score[d]	9 [7-10]	9 [8-13]	0	-
Ethnicity				
White British	-	3 (60)	4 (80)	4 (80)
Any other White background	-	1 (20)	-	-
Bangladeshi	1 (20)	-	-	-
Pakistani	1 (20)	-	1 (20)	-
Chinese	-	1 (20)	-	-
Latin American	-	-	-	1 (20)
Other	1 (20)	-	-	-
Family history				
Autoimmune diseases	-	3	3	-
Hemophagocytic Lymphohistiocytosis	-	1	-	-
Psoriasis/psoriatic arthritis	-	1	-	-
Rheumatoid Arthritis	-	-	3	-
Type 1 Diabetes	-	-	1	-
Vitiligo	-	1	-	-
Wegner's Granulomatosis	-	1	-	-
Other	1	1	1	2
Arthritis (undefined)	-	-	-	2
Synthetic Aortic Valve	-	1	-	-
Thyroid Problems (undefined)	1	-	1	-
None	2	2	1	3
Medication[e]				
Steroids	2	5	2	-
Prednisolone	2	5	2	-
DMARDs	3	5	5	-
Hydroxichloriquine	3	4	5	-
Mycophenolate	1	3	3	-
Infliximab	1	-	-	-
Azathioprine	1	1	-	-
Rituximab	1	1	1	-
Other	2	4	2	1
Omeprazole	-	2	-	-
Diclofenic	-	1	-	-
Aspirin	2	-	-	-
Bisphosphonate	-	1	-	-
OCP	-	-	1	-
Sunsence	2	2	1	-
Antihistamine	-	-	-	1
Cotrimox	1	-	-	-
Ranitidine	1	-	-	-
Statin	-	-	1	-
Sunblock	-	1	-	-
Novorapid	-	1	-	-
dsDNA (IU/L)	0	0	0 [0-7]	-
C3 (g/L)	1.13 [0.95-1.36]	1.07 [0.65-1.32]	1.11 [1.03-1.33]	-
ESR (mm/h)	1 [1-4]	2 [1-23]	8 [2-16]	-

Data are expressed as median and interquartile range [square brackets] or as total numbers with percentages (round brackets). pBILAG. Paediatric British Isles Lupus Assessment Group; DMARDs, disease-modifying anti-rheumatic drugs; dsDNA, anti-double stranded DNA antibody; C3, complement component 3; ESR, erythrocyte sedimentation rate.

[a]Classification of patients into active disease in haematological domain (scored A/B), active disease in the renal domain (scored A/B) and inactive disease in all domains (D/E) was determined using the pBILAG.

[b]Age and disease duration at time of serum sample collection.

[c]Gender of patients.

[d]Total pBILAG score determined by the numeric scoring of disease classifications of the organ domains.

[e]Medication taken at time of serum sample collection.

Appendix D, Table 2. Extended clinical and demographic information of individuals providing blood for neutrophil activation and IFN receptor experimental analysis (for shortened table, see Table 4.2, Chapter 4, and Table 5.1, Chapter 5).

Variable	JSLE (n=5)	Control (n=6)
Age (years)[a]	17.65 [14.50-18.91]	13.87 [8.21-15.42]
Duration of disease (months)[a]	40 [22-101]	-
Female[b]	5 (100)	2 (33.3)
Total pBILAG score[c]	3 [0-5]	-
Ethnicity		
White British	5 (100)	5 (100)
Family history		
Autoimmune diseases	2	1
Hemophagocytic Lymphohistiocytosis	1	-
JSLE	1	-
Psoriasis	-	1
Other	2	1
Kidney problems (undefined)	-	1
Raynaud's	1	-
Thyroid problems (undefined)	1	-
None	2	3
Medication[d]		
Corticosteroids	2	-
Prednisolone	2	-
DMARDs	5	-
Hydroxychloroquine	4	-
Mycophenolate Mofetil	5	-
C3 (g/L)	1.1 [1.05-1.47]	-
C4 (g/L)	0.19 [0.07-0.24]	-
ESR (mm/h)	4 [3-12]	-

Data are expressed as median and interquartile range [square brackets] or as total numbers with percentages (round brackets).

pBILAG. Paediatric British Isles Lupus Assessment Group; C3, complement component 3; C4, complement component 4; DMARDs, disease-modifying anti-rheumatic drugs; ESR, erythrocyte sedimentation rate.

[a]Age and disease duration time of neutrophil isolation and analysis.

[b]Gender of patients.

[c]Total pBILAG score determined by the numeric scoring of disease classifications of the organ domains.

[d]Medication taken at time of serum sample collection.

Appendix D, Table 3. Extended clinical and demographic information of individuals providing blood for neutrophil apoptosis experimental analysis. (For shortened table, see Table 4.3, chapter 4)

Variable	JSLE (n=5)	Control (n=5)
Age (years)[a]	14.88 [14.24-18.45]	10.35 [7.74-14.77]
Duration of disease (months)[a]	41 [30-125]	-
Female[b]	4 (80)	2 (40)
Total pBILAG score[c]	1 [0-8]	-
Ethnicity		
White British	4 (80)	5 (100)
Bangladeshi	1 (20)	-
Family history		
Autoimmune diseases	2	1
Hemophagocytic Lymphohistiocytosis	1	-
JSLE	1	-
Rheumatoid Arthritis	-	1
Other	1	2
Arthritis (undefined)	-	2
Raynaud's	1	-
Thyroid problems (undefined)	-	1
Type 2 Diabetes	-	1
None	3	2
Medication[d]		
Corticosteroids	4	-
Prednisolone	4	-
DMARDs	5	-
Hydroxychloroquine	5	-
Mycophenolate	4	-
Other	1	-
OCP	1	-
dsDNA (IU/L)	7 [0-202]	-
C3 (g/L)	1.17 [1.06-1.27]	-
ESR (mm/h)	3 [1-25]	-

Data are expressed as median and interquartile range [square brackets] or as total numbers with percentages (round brackets).

pBILAG. Paediatric British Isles Lupus Assessment Group; DMARDs, disease-modifying anti-rheumatic drugs; dsDNA, anti-double stranded DNA antibody; C3, complement component 3; ESR, erythrocyte sedimentation rate.

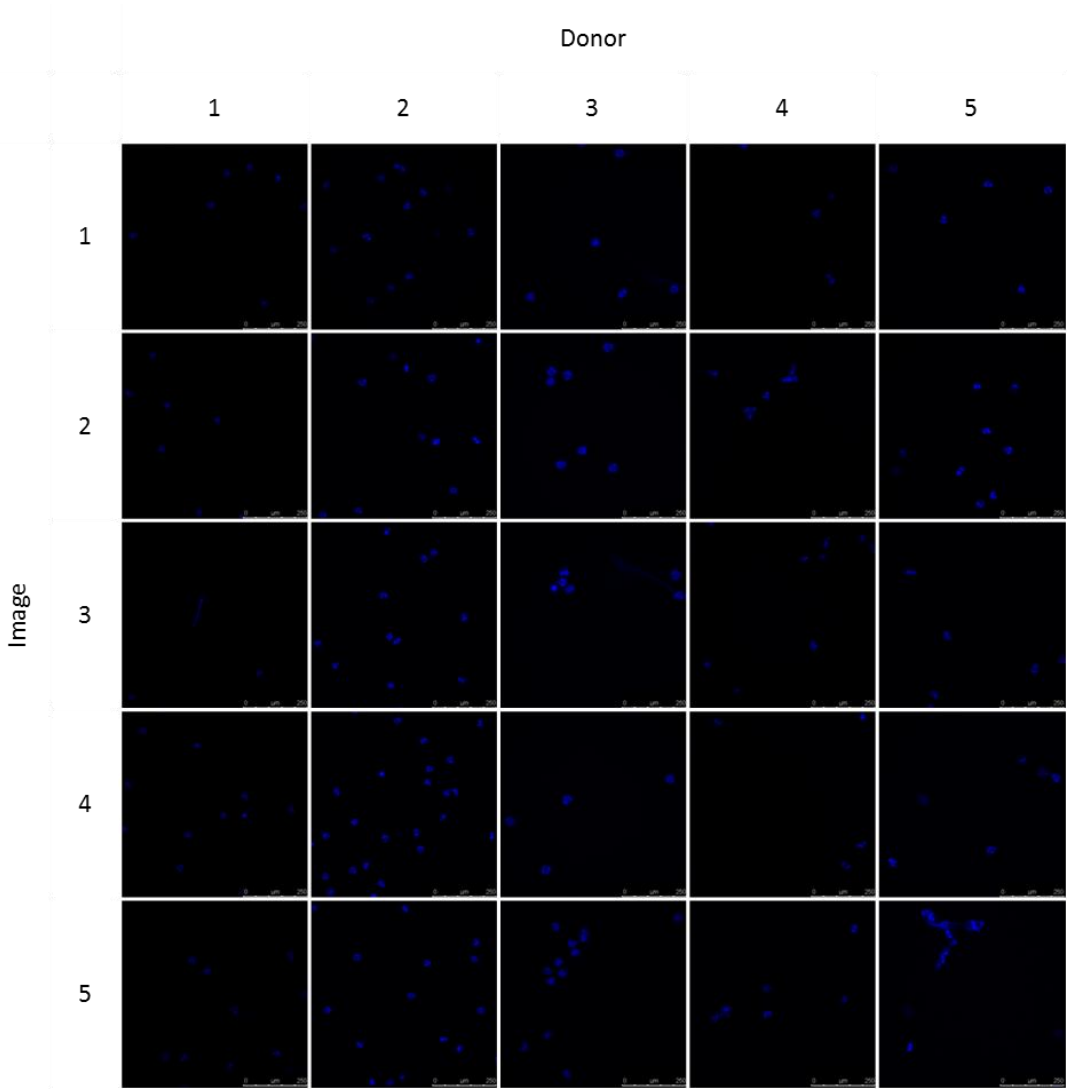
[a]Age and disease duration time of neutrophil isolation and analysis.

[b]Gender of patients.

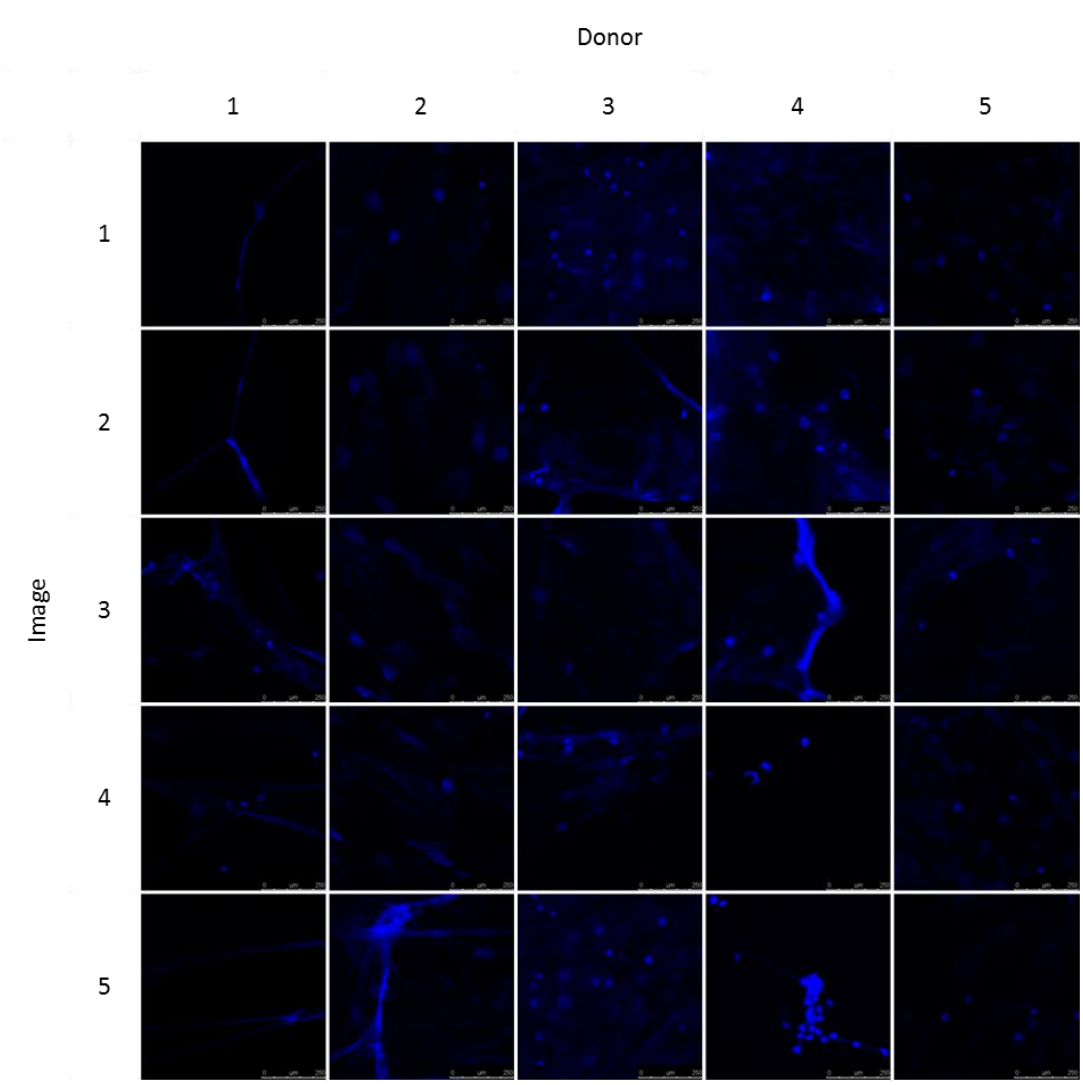
[c]Total pBILAG score determined by the numeric scoring of disease classifications of the organ domains.

[d]Medication taken at time of serum sample collection.

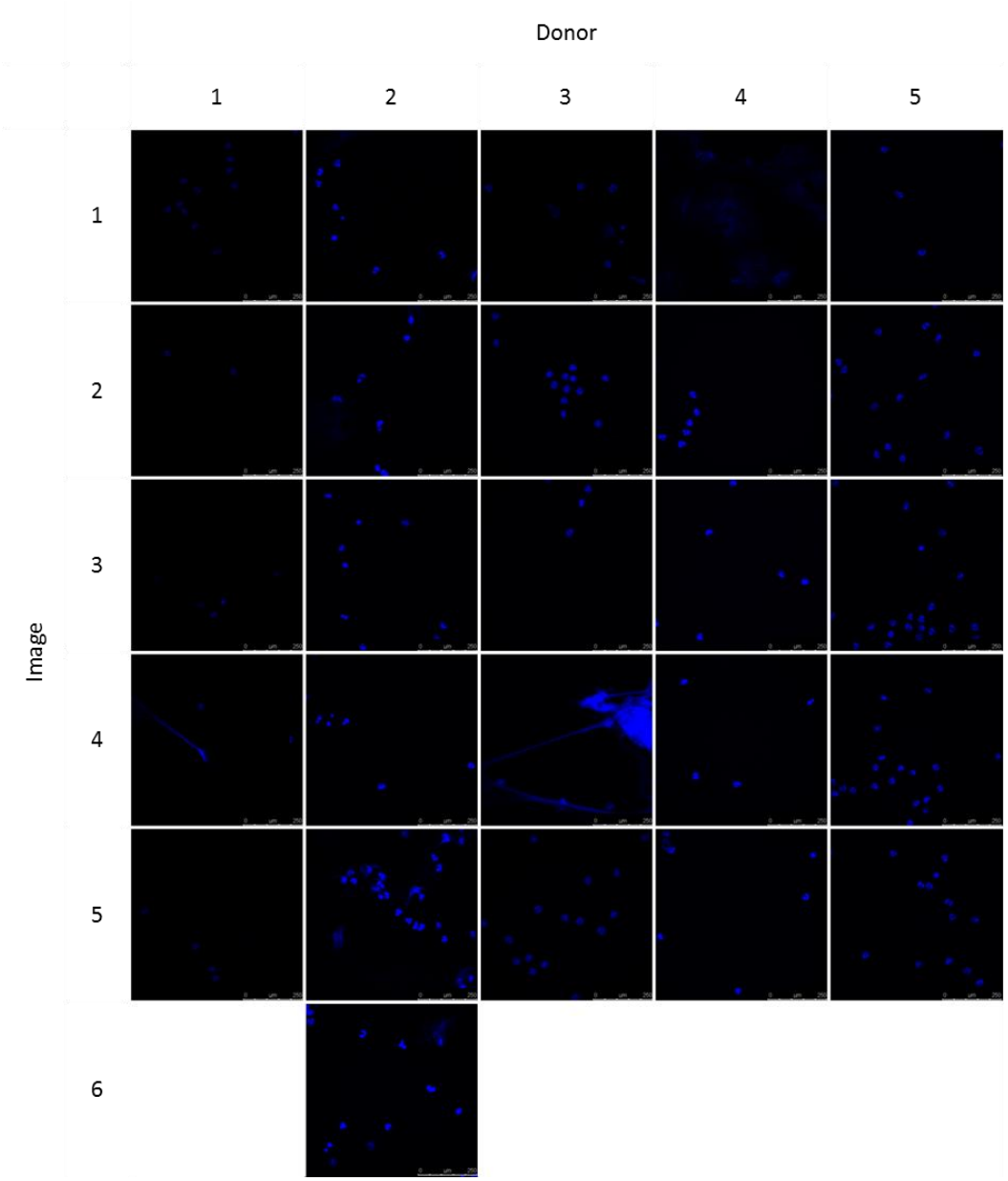
Appendix E. All confocal microscopy images obtained for NETosis analysis (chapter 3)



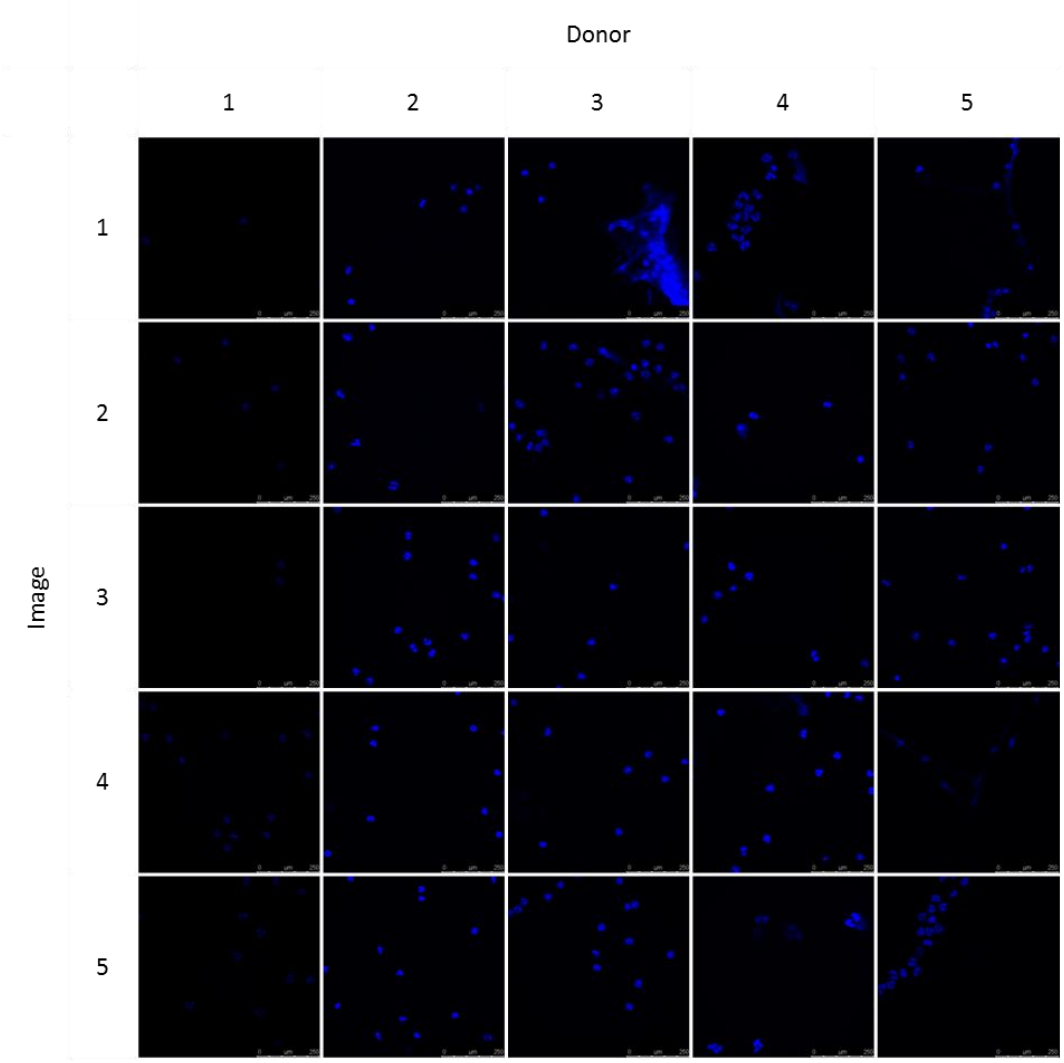
Appendix E, Figure 1. Unstimulated neutrophils stained with DAPI, and NETosis was visualised. Neutrophils were isolated from healthy adult donors (n=5) and were left to settle on cover slips for 4hrs. Neutrophils were subsequently fixed and stained with DAPI within mounting media. 5 images were taken per donor.



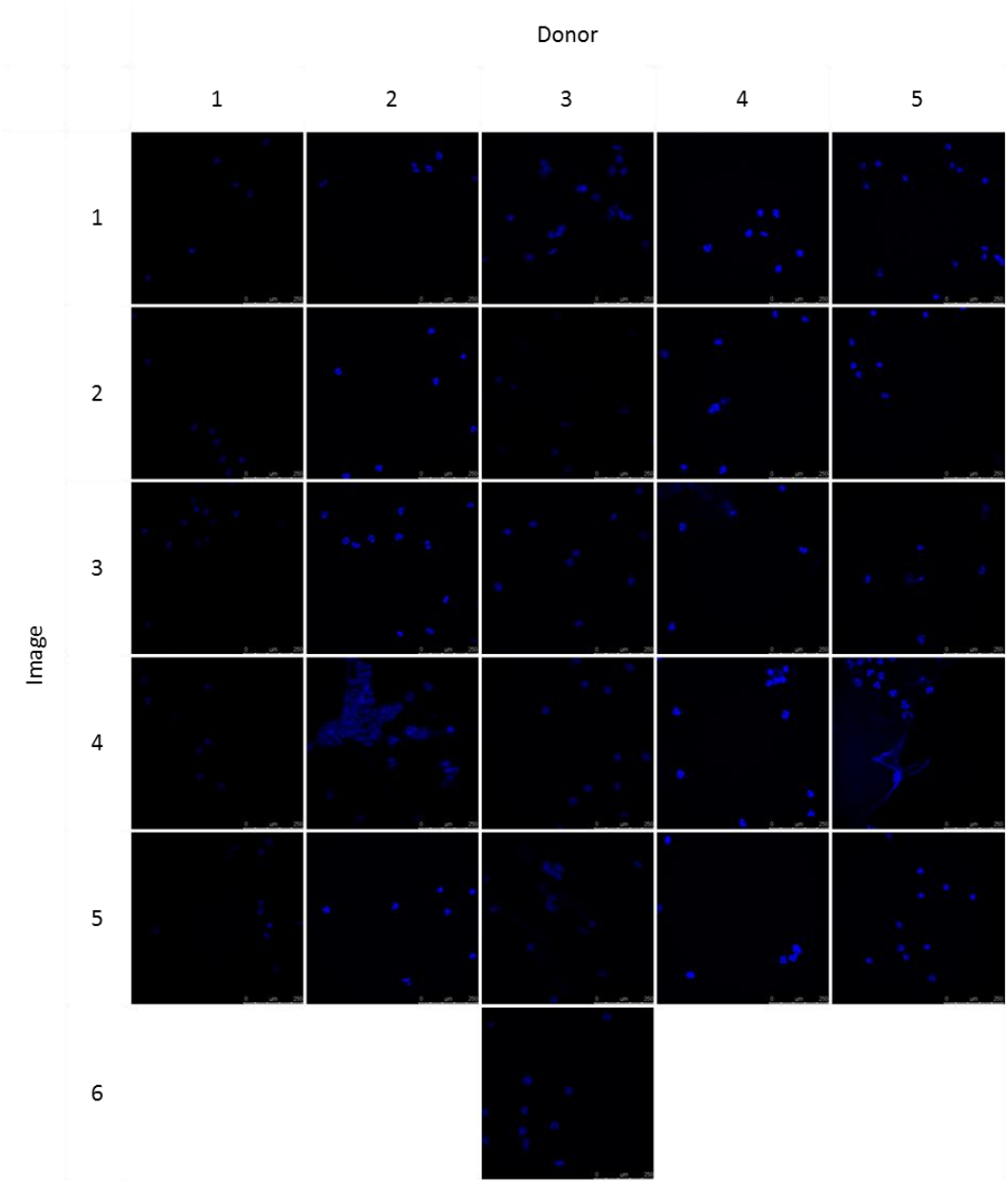
Appendix E, Figure 2. Neutrophils were stimulated with PMA stained with DAPI, and NETosis was visualised. Neutrophils were isolated from healthy adult donors (n=5) and were left to settle on cover slips for 1hr. Neutrophils were treated with 320nM PMA for 3hrs, and subsequently fixed and stained with DAPI within mounting media. 5 images were taken per donor.



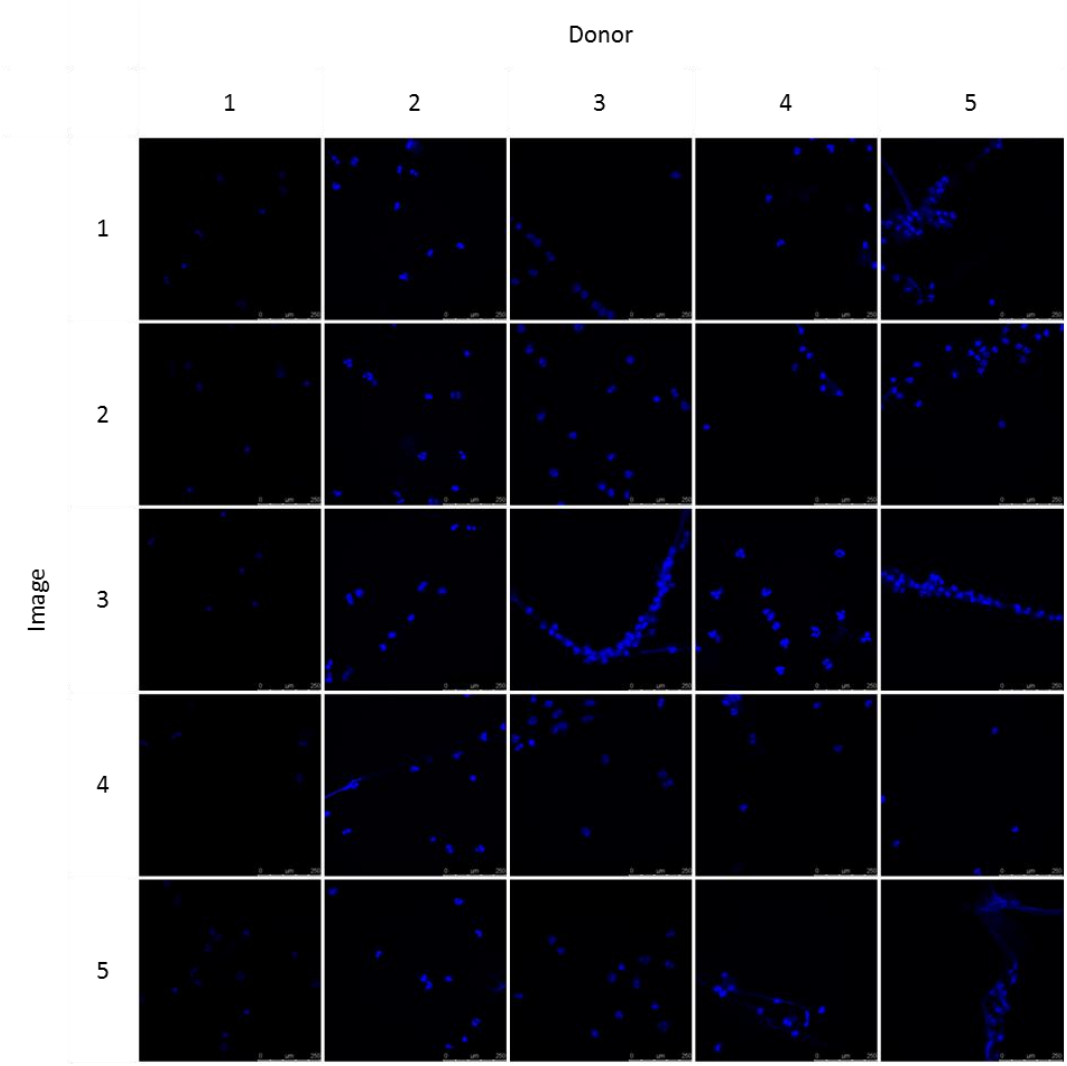
Appendix E, Figure 3. Neutrophils were stimulated with IFNα stained with DAPI, and NETosis was visualised. Neutrophils were isolated from healthy adult donors (n=5) and were left to settle on cover slips for 1hr. Neutrophils were treated with 10ng/ml IFNα for 3hrs, and subsequently fixed and stained with DAPI within mounting media. 5-6 images were taken per donor.



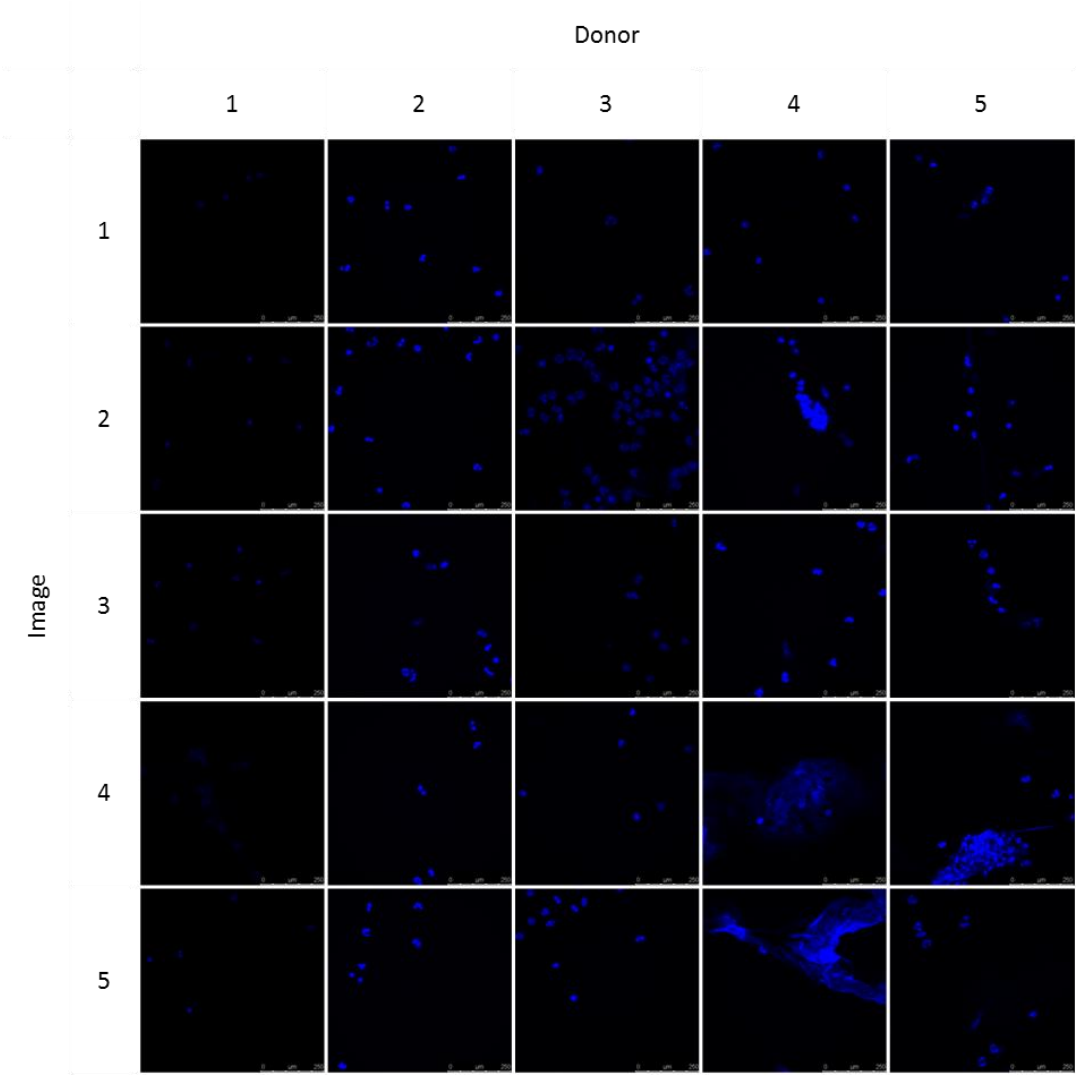
Appendix E, Figure 4. Neutrophils were stimulated with IFNγ and stained with DAPI, and NETosis was visualised. Neutrophils were isolated from healthy adult donors (n=5) and were left to settle on cover slips for 1hr. Neutrophils were treated with 10ng/ml IFNγ for 3hrs, and subsequently fixed and stained with DAPI within mounting media. 5 images were taken per donor.



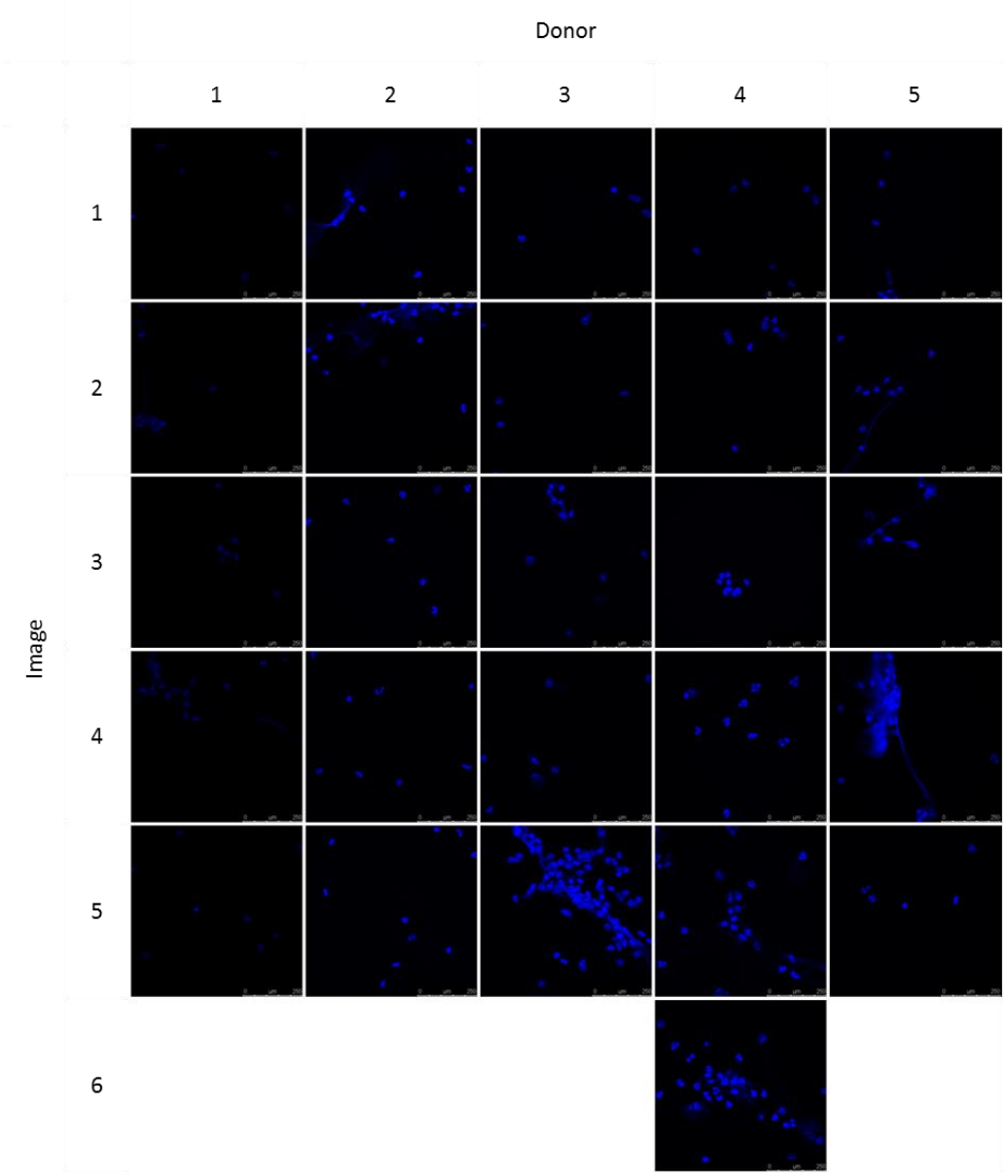
Appendix E, Figure 5. Neutrophils were stimulated with IFNβ and stained with DAPI, and NETosis was visualised. Neutrophils were isolated from healthy adult donors (n=5) and were left to settle on cover slips for 1hr. Neutrophils were treated with 10ng/ml IFNβ for 3hrs, and subsequently fixed and stained with DAPI within mounting media. 5-6 images were taken per donor.



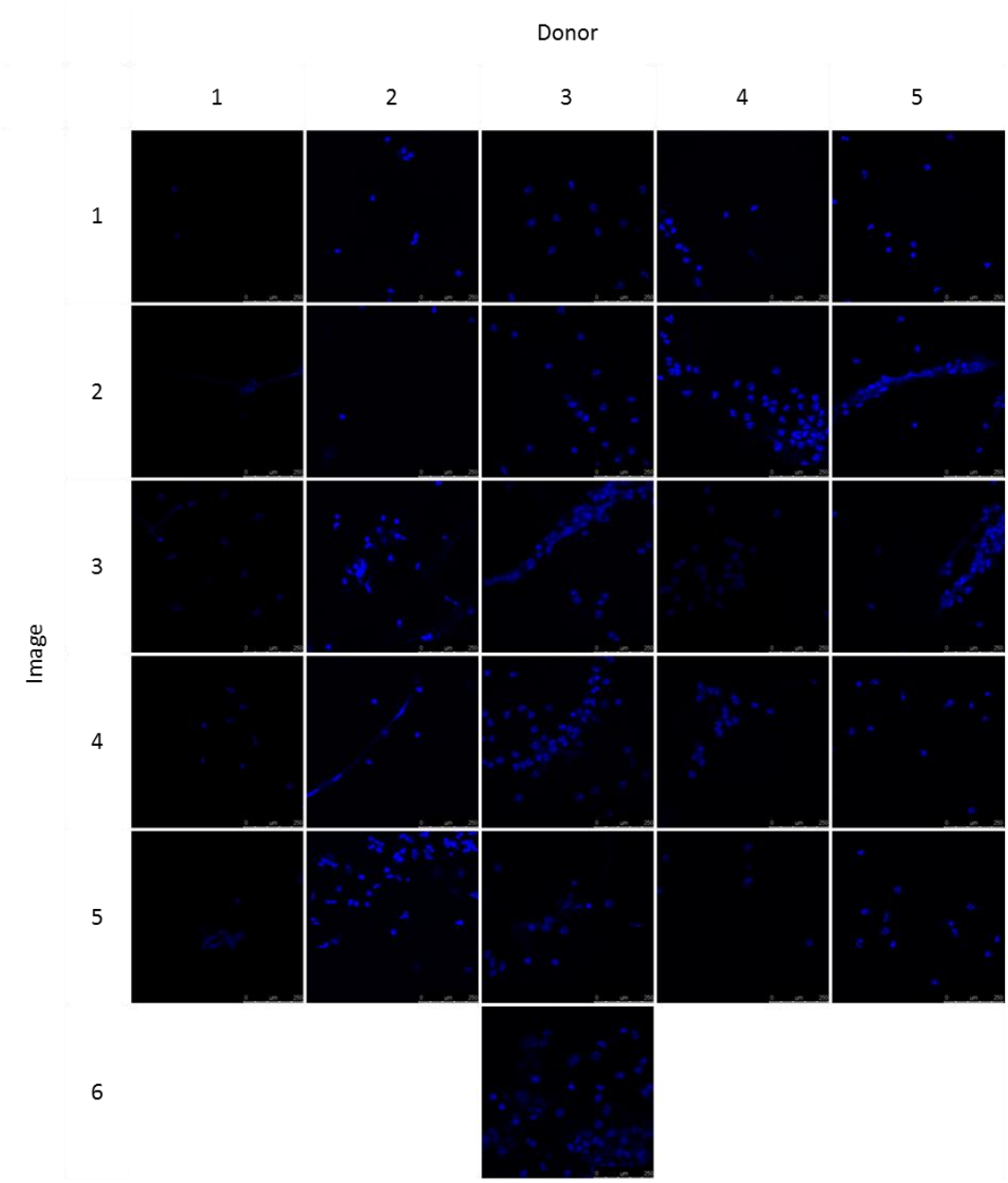
Appendix E, Figure 6. Neutrophils were primed with TNFα, stained with DAPI, and NETosis was visualised. Neutrophils were isolated from healthy adult donors (n=5) and were left to settle on cover slips for 30mins. Neutrophils were primed with 1µg/ml TNFα for 30mins and left unstimulated for 3hrs. Neutrophils were subsequently fixed and stained with DAPI within mounting media. 5 images were taken per donor.



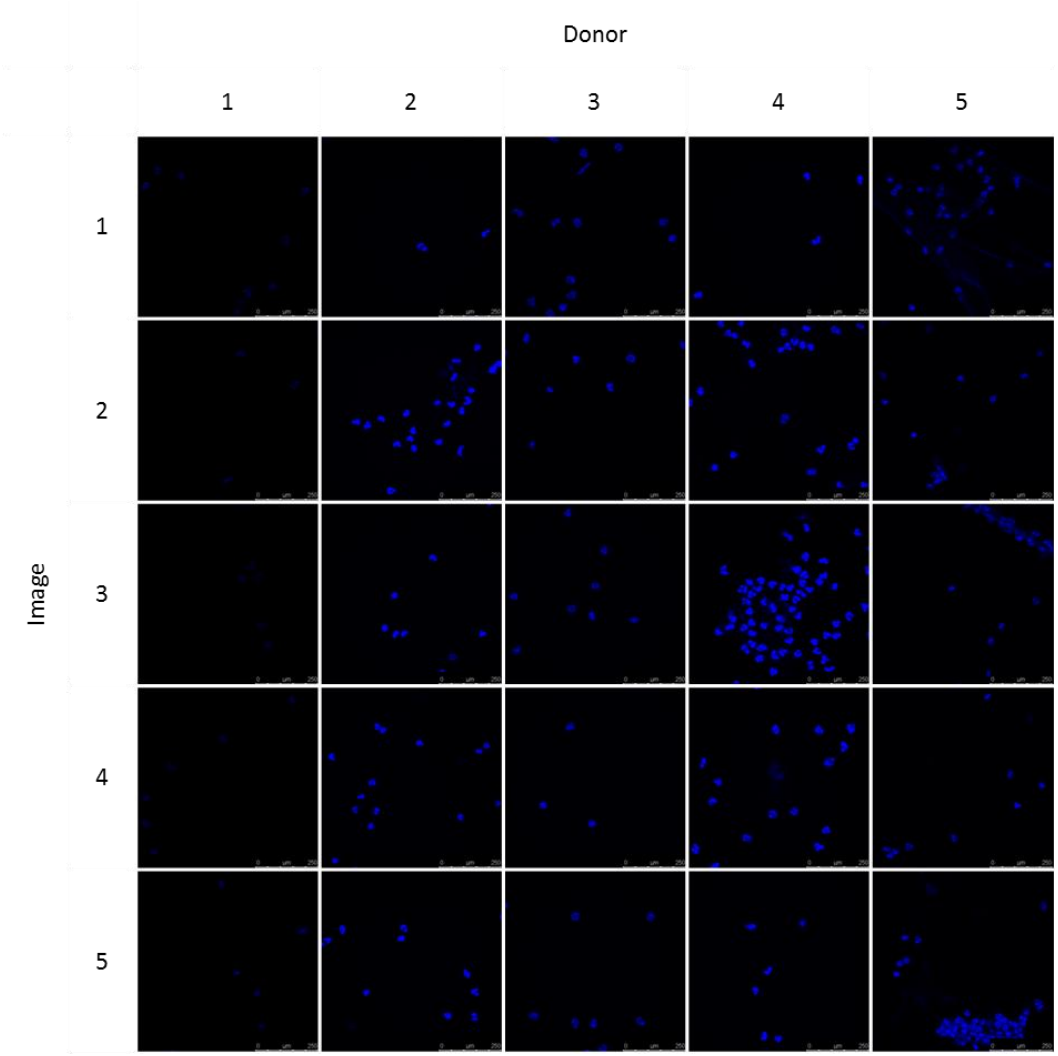
Appendix E, Figure 7. Neutrophils were primed with TNFα, stimulated with IFNα and stained with DAPI, and NETosis was visualised. Neutrophils were isolated from healthy adult donors (n=5) and were left to settle on cover slips for 30mins. Neutrophils were primed with 1µg/ml TNFα for 30mins, and stimulated with 10ng/ml IFNα for 3hrs. Neutrophils were subsequently fixed and stained with DAPI within mounting media. 5 images were taken per donor.



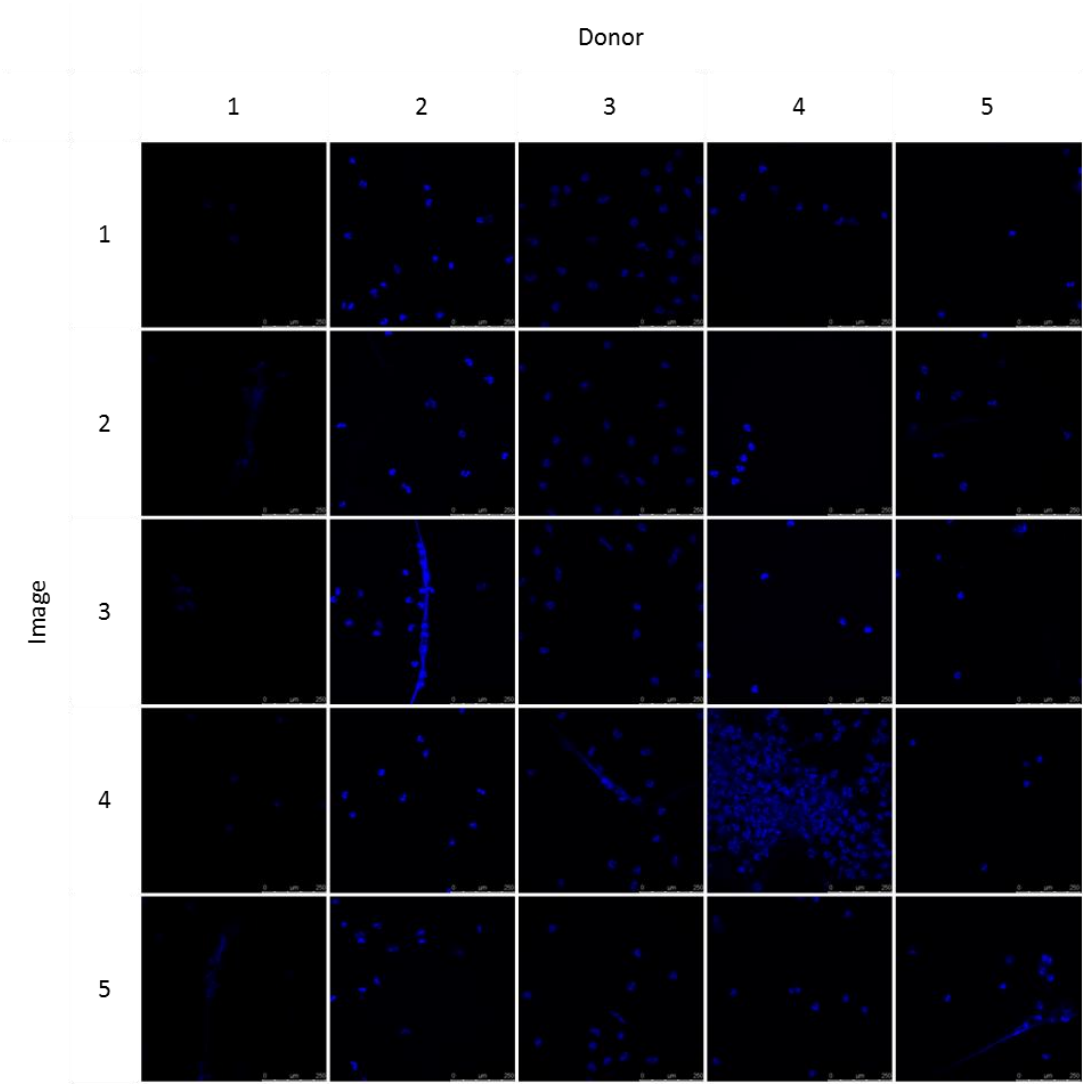
Appendix E, Figure 8. Neutrophils were primed with TNFα-primed, stimulated with IFNγ, stained with DAPI and NETosis was visualised. Neutrophils were isolated from healthy adult donors (n=5) and were left to settle on cover slips for 30mins. Neutrophils were primed with 1µg/ml TNFα for 30mins, and stimulated with 10ng/ml IFNγ for 3hrs. Neutrophils were subsequently fixed and stained with DAPI within mounting media. 5-6 images were taken per donor.



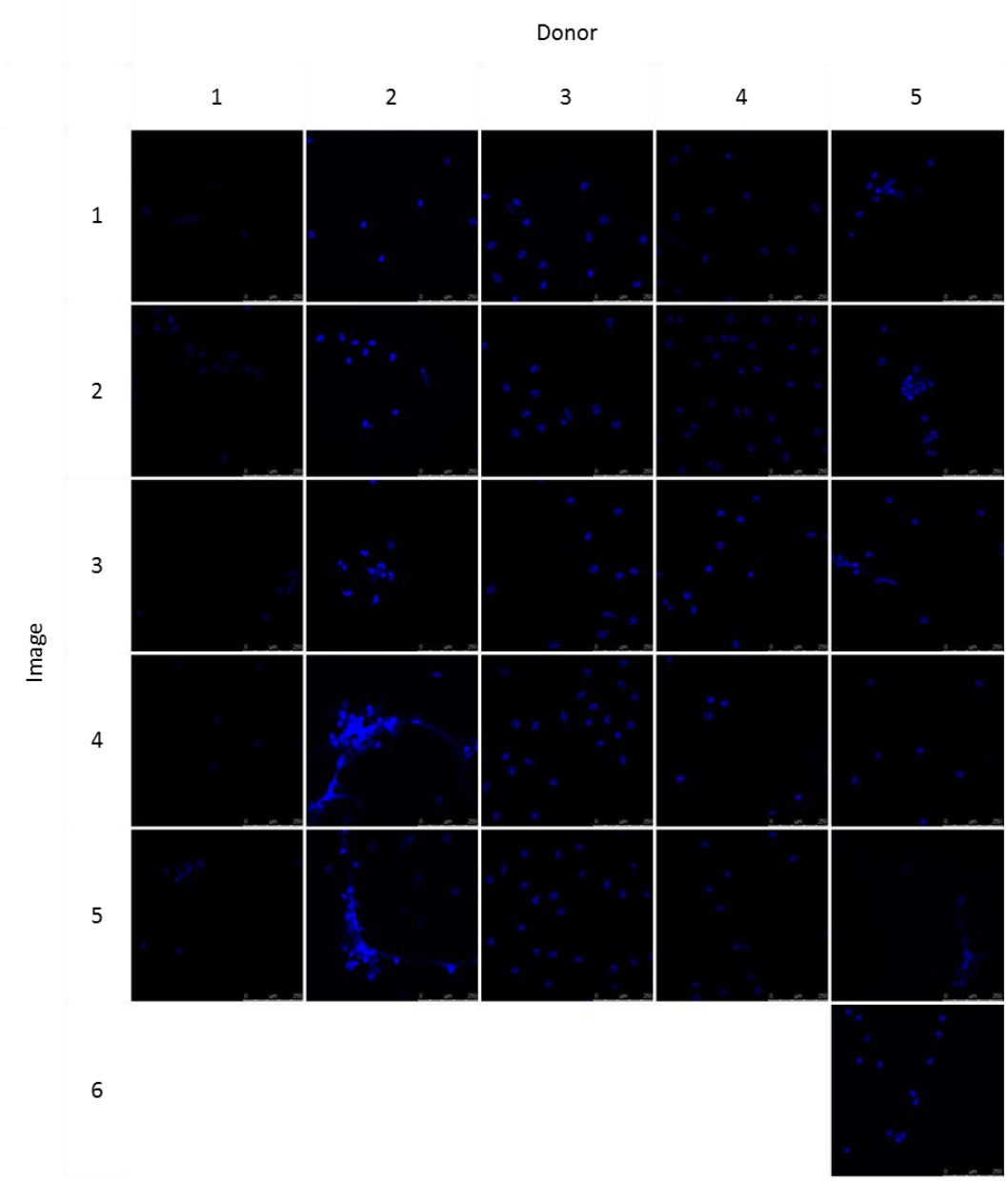
Appendix E, Figure 9. Neutrophils were primed with TNFα, stimulated with IFNβ and stained with DAPI and NETosis was visualised. Neutrophils were isolated from healthy adult donors (n=5) and were left to settle on cover slips for 30mins. Neutrophils were primed with 1µg/ml TNFα for 30mins, and stimulated with 10ng/ml IFNβ for 3hrs. They were subsequently fixed and stained with DAPI within mounting media. 5-6 images were taken per donor.



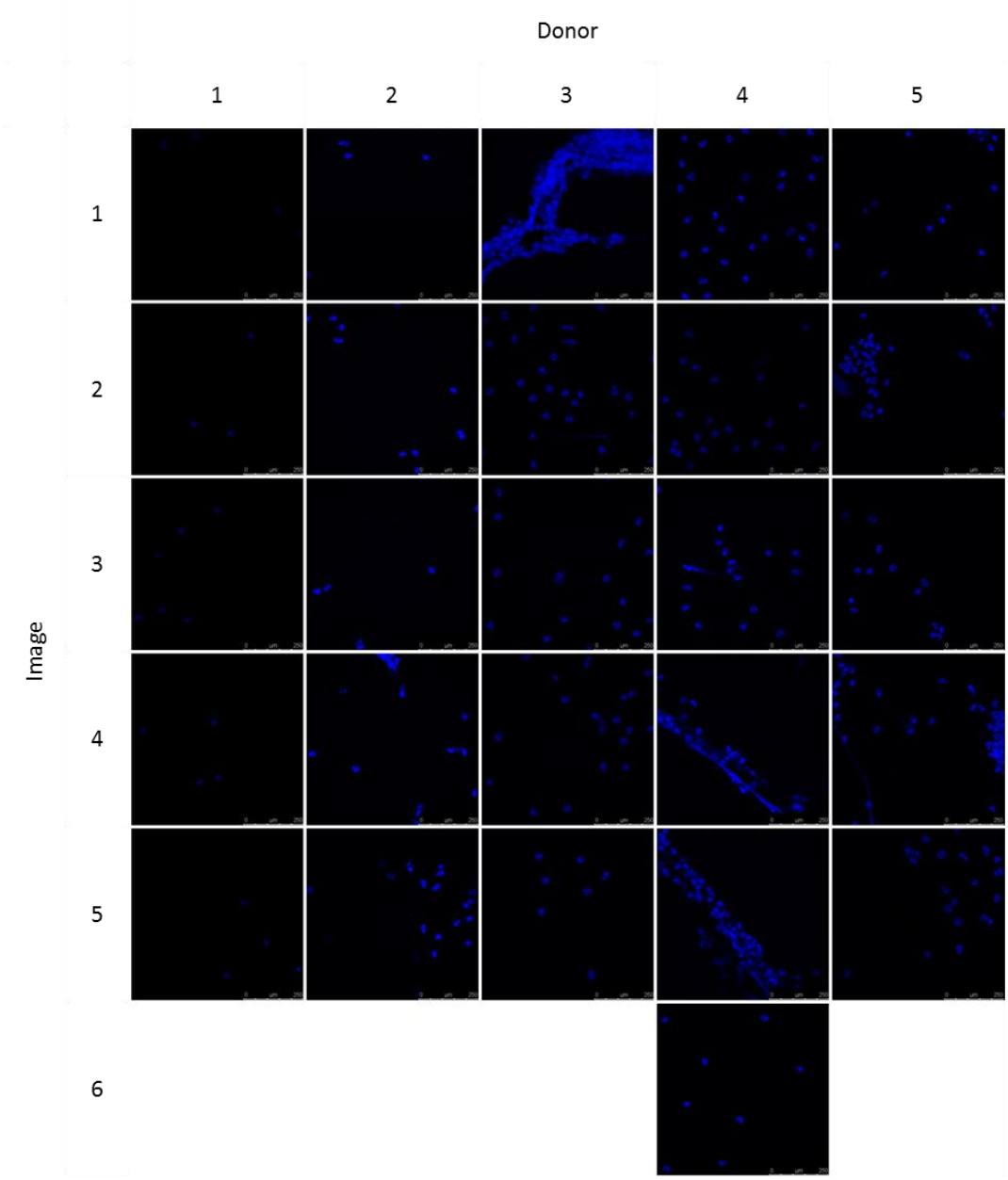
Appendix E, Figure 10. Naïve neutrophils were stimulated with LPS stained with DAPI staining and NETosis was visualised. Neutrophils were isolated from healthy adult donors (n=5) and were left to settle on cover slips for 1hr. Neutrophils were stimulated with 1µg/ml LPS for 3hrs. They were subsequently fixed and stained with DAPI within mounting media. 5 images were taken per donor.



Appendix E, Figure 11. Neutrophils were primed with IFNα, stimulated with LPS, stained with DAPI and NETosis was visualised. Neutrophils were isolated from healthy adult donors (n=5) and were left to settle on cover slips for 30mins. Neutrophils were primed with 10ng/ml IFNα for 30mins and stimulated with 1µg/ml LPS for 3hrs. They were subsequently fixed and stained with DAPI within mounting media. 5 images were taken per donor.



Appendix E. Figure 12. Neutrophils were primed with IFNγ stimulated with LPS, stained with DAPI and NETosis was visualised. Neutrophils were isolated from healthy adult donors (n=5) and were left to settle on cover slips for 30 mins. Neutrophils were primed with 10ng/ml IFNγ for 30mins and stimulated with 1µg/ml LPS for 3hrs. They were subsequently fixed and stained with DAPI within mounting media. 5-6 images were taken per donor.



Appendix E, Figure 13. Neutrophils were primed with IFNβ, stimulated with LPS, stained with DAPI and NETosis was visualised. Neutrophils were isolated from healthy adult donors (n=5) and were left to settle on cover slips for 30 mins. Neutrophils were primed with 10ng/ml IFNβ for 30mins and stimulated with 1µg/ml LPS for 3hrs. They were subsequently fixed and stained with DAPI within mounting media. 5-6 images were taken per donor (n=5).

References

1. Cojocaru M, Cojocaru IM, Silosi I, Vrabie CD. Manifestations of systemic lupus erythematosus. Maedica (Buchar). 2011;6(4):330-6.

2. Yurasov S, Wardemann H, Hammersen J, Tsuiji M, Meffre E, Pascual V, et al. Defective B cell tolerance checkpoints in systemic lupus erythematosus. J Exp Med. 2005;201(5):703-11.

3. Mortensen ES, Fenton KA, Rekvig OP. Lupus nephritis: the central role of nucleosomes revealed. Am J Pathol. 2008;172(2):275-83.

4. Armstrong DL, Zidovetzki R, Alarcón-Riquelme ME, Tsao BP, Criswell LA, Kimberly RP, et al. GWAS identifies novel SLE susceptibility genes and explains the association of the HLA region. Genes Immun. 2014;15(6):347-54.

5. Rood MJ, Van Der Velde EA, Ten Cate R, Breedveld FC, Huizinga TW. Female sex hormones at the onset of systemic lupus erythematosus affect survival. Br J Rheumatol. 1998;37(9):1008-10.

6. James JA, Neas BR, Moser KL, Hall T, Bruner GR, Sestak AL, et al. Systemic lupus erythematosus in adults is associated with previous Epstein-Barr virus exposure. Arthritis Rheum. 2001;44(5):1122-6.

7. Brunner HI, Gladman DD, Ibañez D, Urowitz MD, Silverman ED. Difference in disease features between childhood-onset and adult-onset systemic lupus erythematosus. Arthritis Rheum. 2008;58(2):556-62.

8. Klein-Gitelman M, Reiff A, Silverman ED. Systemic lupus erythematosus in childhood. Rheum Dis Clin North Am. 2002;28(3):561-77, vi-vii.

9. Watson L, Leone V, Pilkington C, Tullus K, Rangaraj S, McDonagh JE, et al. Disease activity, severity, and damage in the UK Juvenile-Onset Systemic Lupus Erythematosus Cohort. Arthritis Rheum. 2012;64(7):2356-65.

10. Ambrose N, Morgan TA, Galloway J, Ionnoau Y, Beresford MW, Isenberg DA, et al. Differences in disease phenotype and severity in SLE across age groups. Lupus. 2016.

11. Riboldi P, Gerosa M, Moroni G, Radice A, Allegri F, Sinico A, et al. Anti-DNA antibodies: a diagnostic and prognostic tool for systemic lupus erythematosus? Autoimmunity. 2005;38(1):39-45.

12. Rees F, Doherty M, Grainge MJ, Lanyon P, Zhang W. The worldwide incidence and prevalence of systemic lupus erythematosus: a systematic review of epidemiological studies. Rheumatology (Oxford). 2017;56(11):1945-61.

13. Tan EM, Cohen AS, Fries JF, Masi AT, McShane DJ, Rothfield NF, et al. The 1982 revised criteria for the classification of systemic lupus erythematosus. Arthritis Rheum. 1982;25(11):1271-7.

14. Hochberg MC. Updating the American College of Rheumatology revised criteria for the classification of systemic lupus erythematosus. Arthritis Rheum. 1997;40(9):1725.

15. Isenberg DA, Rahman A, Allen E, Farewell V, Akil M, Bruce IN, et al. BILAG 2004. Development and initial validation of an updated version of the British Isles Lupus Assessment Group's disease activity index for patients with systemic lupus erythematosus. Rheumatology (Oxford). 2005;44(7):902-6.

16. Smith EMD, Jorgensen AL, Beresford MW, Group UJS. Do classic blood biomarkers of JSLE identify active lupus nephritis? Evidence from the UK JSLE Cohort Study. Lupus. 2017;26(11):1212-7.

17. Petri M, Orbai AM, Alarcón GS, Gordon C, Merrill JT, Fortin PR, et al. Derivation and validation of the Systemic Lupus International Collaborating Clinics classification criteria for systemic lupus erythematosus. Arthritis Rheum. 2012;64(8):2677-86.

18. Adhami E. Calculating the etiology of systemic lupus erythematosus. Med Hypotheses. 2004;62(2):237-46.

19. Haddon DJ, Diep VK, Price JV, Limb C, Utz PJ, Balboni I. Autoantigen microarrays reveal autoantibodies associated with proliferative nephritis and active disease in pediatric systemic lupus erythematosus. Arthritis Res Ther. 2015;17:162.

20. Schneider P, MacKay F, Steiner V, Hofmann K, Bodmer JL, Holler N, et al. BAFF, a novel ligand of the tumor necrosis factor family, stimulates B cell growth. J Exp Med. 1999;189(11):1747-56.

21. Kisiel BM, Kosinska J, Wierzbowska M, Rutkowska-Sak L, Musiej-Nowakowska E, Wudarski M, et al. Differential association of juvenile and adult systemic lupus erythematosus with genetic variants of oestrogen receptors alpha and beta. Lupus. 2011;20(1):85-9.

22. Pontillo A, Reis EC, Liphaus BL, Silva CA, Carneiro-Sampaio M. Inflammasome polymorphisms in juvenile systemic lupus erythematosus. Autoimmunity. 2015;48(7):434-7.

23. Ziaee V, Tahghighi F, Moradinejad MH, Harsini S, Mahmoudi M, Rezaei A, et al. Interleukin-6, interleukin-1 gene cluster and interleukin-1 receptor polymorphisms in Iranian patients with juvenile systemic lupus erythematosus. Eur Cytokine Netw. 2014;25(2):35-40.

24. Mahmoudi M, Tahghighi F, Ziaee V, Harsini S, Rezaei A, Soltani S, et al. Interleukin-4 single nucleotide polymorphisms in juvenile systemic lupus erythematosus. Int J Immunogenet. 2014;41(6):512-7.

25. Mahmoudi M, Rezaiemanesh A, Salmaninejad A, Harsini S, Poursani S, Bahrami T, et al. PDCD1 single nucleotide genes polymorphisms confer susceptibility to juvenile-onset systemic lupus erythematosus. Autoimmunity. 2015;48(7):488-93.

26. Liao CH, Yao TC, Chung HT, See LC, Kuo ML, Huang JL. Polymorphisms in the promoter region of RANTES and the regulatory region of monocyte chemoattractant protein-1 among Chinese children with systemic lupus erythematosus. J Rheumatol. 2004;31(10):2062-7.

27. Baca V, Velázquez-Cruz R, Salas-Martínez G, Espinosa-Rosales F, Saldaña-Alvarez Y, Orozco L. Association analysis of the PTPN22 gene in childhood-onset systemic lupus erythematosus in Mexican population. Genes Immun. 2006;7(8):693-5.

28. Rönnblom L, Pascual V. The innate immune system in SLE: type I interferons and dendritic cells. Lupus. 2008;17(5):394-9.

29. Kahlenberg JM, Kaplan MJ. The inflammasome and lupus: another innate immune mechanism contributing to disease pathogenesis? Curr Opin Rheumatol. 2014;26(5):475-81.

30. Brocker C, Thompson D, Matsumoto A, Nebert DW, Vasiliou V. Evolutionary divergence and functions of the human interleukin (IL) gene family. Hum Genomics. 2010;5(1):30-55.

31. Kufareva I, Salanga CL, Handel TM. Chemokine and chemokine receptor structure and interactions: implications for therapeutic strategies. Immunol Cell Biol. 2015;93(4):372-83.

32. Keir ME, Butte MJ, Freeman GJ, Sharpe AH. PD-1 and its ligands in tolerance and immunity. Annu Rev Immunol. 2008;26:677-704.

33. Lydyard P, Whelan A, Fanger MW. Immunology. Hames BD, editor: Garland Scuence/BIOS Scientific Publishers Limited; 2004. 332 p.

34. Abbas AKa, Lichtman AHa, Pillai Sa. Cellular and molecular immunology. Eighth edition. ed. Philadelphia: Elsevier; 2015.

35. Kruger P, Saffarzadeh M, Weber AN, Rieber N, Radsak M, von Bernuth H, et al. Neutrophils: Between host defence, immune modulation, and tissue injury. PLoS Pathog. 2015;11(3):e1004651.

36. Summers C, Rankin SM, Condliffe AM, Singh N, Peters AM, Chilvers ER. Neutrophil kinetics in health and disease. Trends Immunol. 2010;31(8):318-24.

37. Brinkmann V, Reichard U, Goosmann C, Fauler B, Uhlemann Y, Weiss DS, et al. Neutrophil extracellular traps kill bacteria. Science. 2004;303(5663):1532-5.

38. Lee WL, Harrison RE, Grinstein S. Phagocytosis by neutrophils. Microbes Infect. 2003;5(14):1299-306.

39. Hacbarth E, Kajdacsy-Balla A. Low density neutrophils in patients with systemic lupus erythematosus, rheumatoid arthritis, and acute rheumatic fever. Arthritis Rheum. 1986;29(11):1334-42.

40. Carmona-Rivera C, Kaplan MJ. Low-density granulocytes: a distinct class of neutrophils in systemic autoimmunity. Semin Immunopathol. 2013;35(4):455-63.

41. Bennett L, Palucka AK, Arce E, Cantrell V, Borvak J, Banchereau J, et al. Interferon and granulopoiesis signatures in systemic lupus erythematosus blood. J Exp Med. 2003;197(6):711-23.

42. Denny MF, Yalavarthi S, Zhao W, Thacker SG, Anderson M, Sandy AR, et al. A distinct subset of proinflammatory neutrophils isolated from patients with systemic lupus erythematosus induces vascular damage and synthesizes type I IFNs (vol 184, pg 3284, 2010). Journal of Immunology. 2010;185(6):3779-.

43. Saenz SA, Taylor BC, Artis D. Welcome to the neighborhood: epithelial cell-derived cytokines license innate and adaptive immune responses at mucosal sites. Immunol Rev. 2008;226:172-90.

44. Vernier A, Diab M, Soell M, Haan-Archipoff G, Beretz A, Wachsmann D, et al. Cytokine production by human epithelial and endothelial cells following exposure to oral viridans streptococci involves lectin interactions between bacteria and cell surface receptors. Infect Immun. 1996;64(8):3016-22.

45. Bruewer M, Luegering A, Kucharzik T, Parkos CA, Madara JL, Hopkins AM, et al. Proinflammatory cytokines disrupt epithelial barrier function by apoptosis-independent mechanisms. J Immunol. 2003;171(11):6164-72.

46. Sarma JV, Ward PA. The complement system. Cell Tissue Res. 2011;343(1):227-35.

47. Heit B, Tavener S, Raharjo E, Kubes P. An intracellular signaling hierarchy determines direction of migration in opposing chemotactic gradients. J Cell Biol. 2002;159(1):91-102.

48. Becker S, Quay J, Soukup J. Cytokine (tumor necrosis factor, IL-6, and IL-8) production by respiratory syncytial virus-infected human alveolar macrophages. J Immunol. 1991;147(12):4307-12.

49. Noah TL, Becker S. Respiratory syncytial virus-induced cytokine production by a human bronchial epithelial cell line. Am J Physiol. 1993;265(5 Pt 1):L472-8.

50. Chen LY, Pan WW, Chen M, Li JD, Liu W, Chen G, et al. Synergistic induction of inflammation by bacterial products lipopolysaccharide and fMLP: an important microbial pathogenic mechanism. J Immunol. 2009;182(4):2518-24.

51. Bertolotto M, Contini P, Ottonello L, Pende A, Dallegri F, Montecucco F. Neutrophil migration towards C5a and CXCL8 is prevented by non-steroidal anti-inflammatory drugs via inhibition of different pathways. Br J Pharmacol. 2014;171(14):3376-93.

52. Haynes DR, Harkin DG, Bignold LP, Hutchens MJ, Taylor SM, Fairlie DP. Inhibition of C5a-induced neutrophil chemotaxis and macrophage cytokine production in vitro by a new C5a receptor antagonist. Biochem Pharmacol. 2000;60(5):729-33.

53. Geiser T, Dewald B, Ehrengruber MU, Clark-Lewis I, Baggiolini M. The interleukin-8-related chemotactic cytokines GRO alpha, GRO beta, and GRO gamma activate human neutrophil and basophil leukocytes. J Biol Chem. 1993;268(21):15419-24.

54. Glynn PC, Henney EM, Hall IP. Peripheral blood neutrophils are hyperresponsive to IL-8 and Gro-alpha in cryptogenic fibrosing alveolitis. Eur Respir J. 2001;18(3):522-9.

55. Brazil DP, Hemmings BA. Ten years of protein kinase B signalling: a hard Akt to follow. Trends Biochem Sci. 2001;26(11):657-64.

56. Crooks SW, Stockley RA. Leukotriene B4. Int J Biochem Cell Biol. 1998;30(2):173-8.

57. Nam YH, Min A, Kim SH, Lee YA, Kim KA, Song KJ, et al. Leukotriene B(4) receptors BLT1 and BLT2 are involved in interleukin-8 production in human neutrophils induced by Trichomonas vaginalis-derived secretory products. Inflamm Res. 2012;61(2):97-102.

58. Shin EH, Lee HY, Bae YS. Leukotriene B4 stimulates human monocyte-derived dendritic cell chemotaxis. Biochem Biophys Res Commun. 2006;348(2):606-11.

59. Ribeiro RA, Cunha FQ, Ferreira SH. Recombinant gamma interferon causes neutrophil migration mediated by the release of a macrophage neutrophil chemotactic factor. Int J Exp Pathol. 1990;71(5):717-25.

60. Rosner D, Stoneman V, Littlewood T, McCarthy N, Figg N, Wang Y, et al. Interferon-gamma induces Fas trafficking and sensitization to apoptosis in vascular smooth muscle cells via a PI3K- and Akt-dependent mechanism. Am J Pathol. 2006;168(6):2054-63.

61. van Kessel KP, Bestebroer J, van Strijp JA. Neutrophil-Mediated Phagocytosis of Staphylococcus aureus. Front Immunol. 2014;5:467.

62. Fossati G, Moots RJ, Bucknall RC, Edwards SW. Differential role of neutrophil Fcgamma receptor IIIB (CD16) in phagocytosis, bacterial killing, and responses to immune complexes. Arthritis Rheum. 2002;46(5):1351-61.

63. Fuchs TA, Abed U, Goosmann C, Hurwitz R, Schulze I, Wahn V, et al. Novel cell death program leads to neutrophil extracellular traps. J Cell Biol. 2007;176(2):231-41.

64. Yipp BG, Petri B, Salina D, Jenne CN, Scott BN, Zbytnuik LD, et al. Infection-induced NETosis is a dynamic process involving neutrophil multitasking in vivo. Nat Med. 2012;18(9):1386-93.

65. Röhm M, Grimm MJ, D'Auria AC, Almyroudis NG, Segal BH, Urban CF. NADPH oxidase promotes neutrophil extracellular trap formation in pulmonary aspergillosis. Infect Immun. 2014;82(5):1766-77.

66. Tadie JM, Bae HB, Jiang S, Park DW, Bell CP, Yang H, et al. HMGB1 promotes neutrophil extracellular trap formation through interactions with Toll-like receptor 4. Am J Physiol Lung Cell Mol Physiol. 2013;304(5):L342-9.

67. Ma CY, Jiao YL, Zhang J, Yang QR, Zhang ZF, Shen YJ, et al. Elevated plasma level of HMGB1 is associated with disease activity and combined alterations with IFN-α and TNF-α in systemic lupus erythematosus. Rheumatol Int. 2012;32(2):395-402.

68. Kanakoudi-Tsakalidou F, Farmaki E, Tzimouli V, Taparkou A, Paterakis G, Trachana M, et al. Simultaneous changes in serum HMGB1 and IFN-α levels and in LAIR-1 expression on plasmatoid dendritic cells of patients with juvenile SLE. New therapeutic options? Lupus. 2014;23(3):305-12.

69. Kerr JF, Wyllie AH, Currie AR. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. Br J Cancer. 1972;26(4):239-57.

70. Renehan AG, Booth C, Potten CS. What is apoptosis, and why is it important? BMJ. 2001;322(7301):1536-8.

71. Courtney PA, Crockard AD, Williamson K, Irvine AE, Kennedy RJ, Bell AL. Increased apoptotic peripheral blood neutrophils in systemic lupus erythematosus: relations with disease activity, antibodies to double stranded DNA, and neutropenia. Ann Rheum Dis. 1999;58(5):309-14.

72. Midgley A, McLaren Z, Moots RJ, Edwards SW, Beresford MW. The role of neutrophil apoptosis in juvenile-onset systemic lupus erythematosus. Arthritis Rheum. 2009;60(8):2390-401.

73. Elmore S. Apoptosis: a review of programmed cell death. Toxicol Pathol. 2007;35(4):495-516.

74. Portt L, Norman G, Clapp C, Greenwood M, Greenwood MT. Anti-apoptosis and cell survival: a review. Biochim Biophys Acta. 2011;1813(1):238-59.

75. Zhang M, Zheng J, Nussinov R, Ma B. Release of Cytochrome C from Bax Pores at the Mitochondrial Membrane. Sci Rep. 2017;7(1):2635.

76. Boatright KM, Salvesen GS. Mechanisms of caspase activation. Curr Opin Cell Biol. 2003;15(6):725-31.

77. Derouet M, Thomas L, Cross A, Moots RJ, Edwards SW. Granulocyte macrophage colony-stimulating factor signaling and proteasome inhibition delay neutrophil apoptosis by increasing the stability of Mcl-1. J Biol Chem. 2004;279(26):26915-21.

78. Hasegawa T, Suzuki K, Sakamoto C, Ohta K, Nishiki S, Hino M, et al. Expression of the inhibitor of apoptosis (IAP) family members in human neutrophils: up-regulation of cIAP2 by granulocyte colony-stimulating factor and overexpression of cIAP2 in chronic neutrophilic leukemia. Blood. 2003;101(3):1164-71.

79. Deveraux QL, Reed JC. IAP family proteins--suppressors of apoptosis. Genes Dev. 1999;13(3):239-52.

80. Swain SD, Rohn TT, Quinn MT. Neutrophil priming in host defense: role of oxidants as priming agents. Antioxid Redox Signal. 2002;4(1):69-83.

81. McColl SR, Beauseigle D, Gilbert C, Naccache PH. Priming of the human neutrophil respiratory burst by granulocyte-macrophage colony-stimulating factor and tumor necrosis factor-alpha involves regulation at a post-cell surface receptor level. Enhancement of the effect of agents which directly activate G proteins. J Immunol. 1990;145(9):3047-53.

82. Buyon JP, Shadick N, Berkman R, Hopkins P, Dalton J, Weissmann G, et al. Surface expression of Gp 165/95, the complement receptor CR3, as a marker of disease activity in systemic Lupus erythematosus. Clin Immunol Immunopathol. 1988;46(1):141-9.

83. Molad Y, Buyon J, Anderson DC, Abramson SB, Cronstein BN. Intravascular neutrophil activation in systemic lupus erythematosus (SLE): dissociation between increased expression of CD11b/CD18 and diminished expression of L-selectin on neutrophils from patients with active SLE. Clin Immunol Immunopathol. 1994;71(3):281-6.

84. Langereis JD, Franciosi L, Ulfman LH, Koenderman L. GM-CSF and TNFα modulate protein expression of human neutrophils visualized by fluorescence two-dimensional difference gel electrophoresis. Cytokine. 2011;56(2):422-9.

85. McLeish KR, Knall C, Ward RA, Gerwins P, Coxon PY, Klein JB, et al. Activation of mitogen-activated protein kinase cascades during priming of human neutrophils by TNF-alpha and GM-CSF. J Leukoc Biol. 1998;64(4):537-45.

86. Dang PM, Dewas C, Gaudry M, Fay M, Pedruzzi E, Gougerot-Pocidalo MA, et al. Priming of human neutrophil respiratory burst by granulocyte/macrophage colony-stimulating factor (GM-CSF) involves partial phosphorylation of p47(phox). J Biol Chem. 1999;274(29):20704-8.

87. el Benna J, Ruedi JM, Babior BM. Cytosolic guanine nucleotide-binding protein Rac2 operates in vivo as a component of the neutrophil respiratory burst oxidase. Transfer of Rac2 and the cytosolic oxidase components p47phox and p67phox to the submembranous actin cytoskeleton during oxidase activation. J Biol Chem. 1994;269(9):6729-34.

88. Benna JE, Dang PM, Gaudry M, Fay M, Morel F, Hakim J, et al. Phosphorylation of the respiratory burst oxidase subunit p67(phox) during human neutrophil activation. Regulation by protein kinase C-dependent and independent pathways. J Biol Chem. 1997;272(27):17204-8.

89. Kurt-Jones EA, Mandell L, Whitney C, Padgett A, Gosselin K, Newburger PE, et al. Role of toll-like receptor 2 (TLR2) in neutrophil activation: GM-CSF enhances TLR2 expression and TLR2-mediated interleukin 8 responses in neutrophils. Blood. 2002;100(5):1860-8.

90. Jabłońska E, Kiluk M, Markiewicz W, Jabłoński J. Priming effects of GM-CSF, IFN-gamma and TNF-alpha on human neutrophil inflammatory cytokine production. Melanoma Res. 2002;12(2):123-8.

91. Liu Y, Liao J, Zhao M, Wu H, Yung S, Chan TM, et al. Increased expression of TLR2 in CD4(+) T cells from SLE patients enhances immune reactivity and promotes IL-17 expression through histone modifications. Eur J Immunol. 2015;45(9):2683-93.

92. Urbonaviciute V, Starke C, Pirschel W, Pohle S, Frey S, Daniel C, et al. Toll-like receptor 2 is required for autoantibody production and development of renal disease in pristane-induced lupus. Arthritis Rheum. 2013;65(6):1612-23.

93. Postal M, Peliçari KO, Sinicato NA, Marini R, Costallat LT, Appenzeller S. Th1/Th2 cytokine profile in childhood-onset systemic lupus erythematosus. Cytokine. 2013;61(3):785-91.

94. McCarthy EM, Smith S, Lee RZ, Cunnane G, Doran MF, Donnelly S, et al. The association of cytokines with disease activity and damage scores in systemic lupus erythematosus patients. Rheumatology (Oxford). 2014;53(9):1586-94.

95. Wittmann S, Rothe G, Schmitz G, Fröhlich D. Cytokine upregulation of surface antigens correlates to the priming of the neutrophil oxidative burst response. Cytometry A. 2004;57(1):53-62.

96. Todd RF. The continuing saga of complement receptor type 3 (CR3). J Clin Invest. 1996;98(1):1-2.

97. Sánchez-Madrid F, Corbí AL. Leukocyte integrins: structure, function and regulation of their activity. Semin Cell Biol. 1992;3(3):199-210.

98. Zhou H, Liao J, Aloor J, Nie H, Wilson BC, Fessler MB, et al. CD11b/CD18 (Mac-1) is a novel surface receptor for extracellular double-stranded RNA to mediate cellular inflammatory responses. J Immunol. 2013;190(1):115-25.

99. Beller DI, Springer TA, Schreiber RD. Anti-Mac-1 selectively inhibits the mouse and human type three complement receptor. J Exp Med. 1982;156(4):1000-9.

100. Springer T, Galfré G, Secher DS, Milstein C. Mac-1: a macrophage differentiation antigen identified by monoclonal antibody. Eur J Immunol. 1979;9(4):301-6.

101. Tedder TF, Steeber DA, Chen A, Engel P. The selectins: vascular adhesion molecules. FASEB J. 1995;9(10):866-73.

102. Waldner H. The role of innate immune responses in autoimmune disease development. Autoimmun Rev. 2009;8(5):400-4.

103. Sakaguchi S, Sakaguchi N, Asano M, Itoh M, Toda M. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. J Immunol. 1995;155(3):1151-64.

104. Navegantes KC, de Souza Gomes R, Pereira PAT, Czaikoski PG, Azevedo CHM, Monteiro MC. Immune modulation of some autoimmune diseases: the critical role of macrophages and neutrophils in the innate and adaptive immunity. J Transl Med. 2017;15(1):36.

105. Hoffman WH, Burek CL, Waller JL, Fisher LE, Khichi M, Mellick LB. Cytokine response to diabetic ketoacidosis and its treatment. Clin Immunol. 2003;108(3):175-81.

106. Valle A, Giamporcaro GM, Scavini M, Stabilini A, Grogan P, Bianconi E, et al. Reduction of circulating neutrophils precedes and accompanies type 1 diabetes. Diabetes. 2013;62(6):2072-7.

107. Diana J, Lehuen A. Macrophages and β-cells are responsible for CXCR2-mediated neutrophil infiltration of the pancreas during autoimmune diabetes. EMBO Mol Med. 2014;6(8):1090-104.

108. Khandpur R, Carmona-Rivera C, Vivekanandan-Giri A, Gizinski A, Yalavarthi S, Knight JS, et al. NETs are a source of citrullinated autoantigens and stimulate inflammatory responses in rheumatoid arthritis. Sci Transl Med. 2013;5(178):178ra40.

109. Ramanujam M, Davidson A. Targeting of the immune system in systemic lupus erythematosus. Expert Rev Mol Med. 2008;10:e2.

110. Chiewchengchol D, Midgley A, Sodsai P, Deekajorndech T, Hirankarn N, Beresford MW, et al. The protective effect of GM-CSF on serum-induced neutrophil apoptosis in juvenile systemic lupus erythematosus patients. Clin Rheumatol. 2015;34(1):85-91.

111. Midgley A, Beresford MW. Cellular localization of nuclear antigen during neutrophil apoptosis: mechanism for autoantigen exposure? Lupus. 2011;20(6):641-6.

112. Wu SA, Yeh KW, Lee WI, Yao TC, Kuo ML, Huang B, et al. Impaired phagocytosis and susceptibility to infection in pediatric-onset systemic lupus erythematosus. Lupus. 2013;22(3):279-88.

113. Ballantine L, Midgley A, Harris D, Richards E, Burgess S, Beresford MW. Increased soluble phagocytic receptors sMer, sTyro3 and sAxl and reduced phagocytosis in juvenile-onset systemic lupus erythematosus. Pediatr Rheumatol Online J. 2015;13:10.

114. Ren Y, Tang J, Mok MY, Chan AW, Wu A, Lau CS. Increased apoptotic neutrophils and macrophages and impaired macrophage phagocytic clearance of apoptotic neutrophils in systemic lupus erythematosus. Arthritis Rheum. 2003;48(10):2888-97.

115. Biswas D, Mathias A, Dayal R, Aggarwal A, Misra R, Naik S. Presence of antibodies to SSB/La is associated with decreased phagocytic efficiency of neutrophils in patients with systemic lupus erythematosus. Clin Rheumatol. 2008;27(6):717-22.

116. Goetzl EJ. Defective responsiveness to ascorbic acid of neutrophil random and chemotactic migration in Felty's syndrome and systemic lupus erythematosus. Ann Rheum Dis. 1976;35(6):510-5.

117. Clark RA, Kimball HR, Decker JL. Neutrophil chemotaxis in systemic lupus erythematosus. Ann Rheum Dis. 1974;33(2):167-72.

118. Lit LC, Wong CK, Tam LS, Li EK, Lam CW. Raised plasma concentration and ex vivo production of inflammatory chemokines in patients with systemic lupus erythematosus. Ann Rheum Dis. 2006;65(2):209-15.

119. Midgley A, Beresford MW. Increased expression of low density granulocytes in juvenile-onset systemic lupus erythematosus patients correlates with disease activity. Lupus. 2016;25(4):407-11.

120. Kotenko SV, Gallagher G, Baurin VV, Lewis-Antes A, Shen M, Shah NK, et al. IFN-lambdas mediate antiviral protection through a distinct class II cytokine receptor complex. Nat Immunol. 2003;4(1):69-77.

121. Donnelly RP, Kotenko SV. Interferon-lambda: a new addition to an old family. J Interferon Cytokine Res. 2010;30(8):555-64.

122. Jordan WJ, Eskdale J, Srinivas S, Pekarek V, Kelner D, Rodia M, et al. Human interferon lambda-1 (IFN-lambda1/IL-29) modulates the Th1/Th2 response. Genes Immun. 2007;8(3):254-61.

123. Wang H, Li T, Chen S, Gu Y, Ye S. Neutrophil Extracellular Trap Mitochondrial DNA and Its Autoantibody in Systemic Lupus Erythematosus and a Proof-of-Concept Trial of Metformin. Arthritis Rheumatol. 2015;67(12):3190-200.

124. Bengtsson AA, Sturfelt G, Gullstrand B, Truedsson L. Induction of apoptosis in monocytes and lymphocytes by serum from patients with systemic lupus erythematosus - an additional mechanism to increased autoantigen load? Clin Exp Immunol. 2004;135(3):535-43.

125. Braun D, Caramalho I, Demengeot J. IFN-alpha/beta enhances BCR-dependent B cell responses. Int Immunol. 2002;14(4):411-9.

126. Ruuth K, Carlsson L, Hallberg B, Lundgren E. Interferon-alpha promotes survival of human primary B-lymphocytes via phosphatidylinositol 3-kinase. Biochem Biophys Res Commun. 2001;284(3):583-6.

127. Samuelsson CV, Lienenklaus S, Müller PP, Zawatzky R, Hauser H, Weiss S. Transformation of mouse fibroblasts alters the induction pattern of type I IFNs after virus infection. Biochem Biophys Res Commun. 2005;335(2):584-9.

128. Coccia EM. IFN regulation and functions in myeloid dendritic cells. Cytokine Growth Factor Rev. 2008;19(1):21-32.

129. Schroder K, Hertzog PJ, Ravasi T, Hume DA. Interferon-gamma: an overview of signals, mechanisms and functions. J Leukoc Biol. 2004;75(2):163-89.

130. Rawlings JS, Rosler KM, Harrison DA. The JAK/STAT signaling pathway. J Cell Sci. 2004;117(Pt 8):1281-3.

131. Liu J, Kern JA. Neuregulin-1 activates the JAK-STAT pathway and regulates lung epithelial cell proliferation. Am J Respir Cell Mol Biol. 2002;27(3):306-13.

132. Kieslinger M, Woldman I, Moriggl R, Hofmann J, Marine JC, Ihle JN, et al. Antiapoptotic activity of Stat5 required during terminal stages of myeloid differentiation. Genes Dev. 2000;14(2):232-44.

133. Sahni M, Raz R, Coffin JD, Levy D, Basilico C. STAT1 mediates the increased apoptosis and reduced chondrocyte proliferation in mice overexpressing FGF2. Development. 2001;128(11):2119-29.

134. Schindler C, Levy DE, Decker T. JAK-STAT signaling: from interferons to cytokines. J Biol Chem. 2007;282(28):20059-63.

135. Ihle JN. The Stat family in cytokine signaling. Curr Opin Cell Biol. 2001;13(2):211-7.

136. Lim CP, Cao X. Structure, function, and regulation of STAT proteins. Mol Biosyst. 2006;2(11):536-50.

137. Begitt A, Droescher M, Meyer T, Schmid CD, Baker M, Antunes F, et al. STAT1-cooperative DNA binding distinguishes type 1 from type 2 interferon signaling. Nat Immunol. 2014;15(2):168-76.

138. Chin YE, Kitagawa M, Kuida K, Flavell RA, Fu XY. Activation of the STAT signaling pathway can cause expression of caspase 1 and apoptosis. Mol Cell Biol. 1997;17(9):5328-37.

139. Xu X, Fu XY, Plate J, Chong AS. IFN-gamma induces cell growth inhibition by Fas-mediated apoptosis: requirement of STAT1 protein for up-regulation of Fas and FasL expression. Cancer Res. 1998;58(13):2832-7.

140. Yue C, Xu J, Tan Estioko MD, Kotredes KP, Lopez-Otalora Y, Hilliard BA, et al. Host STAT2/type I interferon axis controls tumor growth. Int J Cancer. 2015;136(1):117-26.

141. Sakamoto E, Hato F, Kato T, Sakamoto C, Akahori M, Hino M, et al. Type I and type II interferons delay human neutrophil apoptosis via activation of STAT3 and up-regulation of cellular inhibitor of apoptosis 2. J Leukoc Biol. 2005;78(1):301-9.

142. Nishikomori R, Usui T, Wu CY, Morinobu A, O'Shea JJ, Strober W. Activated STAT4 has an essential role in Th1 differentiation and proliferation that is independent of its role in the maintenance of IL-12R beta 2 chain expression and signaling. J Immunol. 2002;169(8):4388-98.

143. Ren Z, Aerts JL, Vandenplas H, Wang JA, Gorbenko O, Chen JP, et al. Phosphorylated STAT5 regulates p53 expression via BRCA1/BARD1-NPM1 and MDM2. Cell Death Dis. 2016;7(12):e2560.

144. Kälin S, Becker M, Ott VB, Serr I, Hosp F, Mollah MMH, et al. A Stat6/Pten Axis Links Regulatory T Cells with Adipose Tissue Function. Cell Metab. 2017;26(3):475-92.e7.

145. Kaplan MH, Schindler U, Smiley ST, Grusby MJ. Stat6 is required for mediating responses to IL-4 and for development of Th2 cells. Immunity. 1996;4(3):313-9.

146. Greenlund AC, Schreiber RD, Goeddel DV, Pennica D. Interferon-gamma induces receptor dimerization in solution and on cells. J Biol Chem. 1993;268(24):18103-10.

147. Wilmes S, Beutel O, Li Z, Francois-Newton V, Richter CP, Janning D, et al. Receptor dimerization dynamics as a regulatory valve for plasticity of type I interferon signaling. J Cell Biol. 2015;209(4):579-93.

148. Shuai K, Ziemiecki A, Wilks AF, Harpur AG, Sadowski HB, Gilman MZ, et al. Polypeptide signalling to the nucleus through tyrosine phosphorylation of Jak and Stat proteins. Nature. 1993;366(6455):580-3.

149. Uddin S, Chamdin A, Platanias LC. Interaction of the transcriptional activator Stat-2 with the type I interferon receptor. J Biol Chem. 1995;270(42):24627-30.

150. Colamonici OR, Uyttendaele H, Domanski P, Yan H, Krolewski JJ. p135tyk2, an interferon-alpha-activated tyrosine kinase, is physically associated with an interferon-alpha receptor. J Biol Chem. 1994;269(5):3518-22.

151. Gupta S, Yan H, Wong LH, Ralph S, Krolewski J, Schindler C. The SH2 domains of Stat1 and Stat2 mediate multiple interactions in the transduction of IFN-alpha signals. EMBO J. 1996;15(5):1075-84.

152. McBride KM, Banninger G, McDonald C, Reich NC. Regulated nuclear import of the STAT1 transcription factor by direct binding of importin-alpha. EMBO J. 2002;21(7):1754-63.

153. Ma J, Cao X. Regulation of Stat3 nuclear import by importin alpha5 and importin alpha7 via two different functional sequence elements. Cell Signal. 2006;18(8):1117-26.

154. Epling-Burnette PK, Zhong B, Bai F, Jiang K, Bailey RD, Garcia R, et al. Cooperative regulation of Mcl-1 by Janus kinase/stat and phosphatidylinositol 3-kinase contribute to granulocyte-macrophage colony-stimulating factor-delayed apoptosis in human neutrophils. J Immunol. 2001;166(12):7486-95.

155. Kim HS, Kim DC, Kim HM, Kwon HJ, Kwon SJ, Kang SJ, et al. STAT1 deficiency redirects IFN signalling toward suppression of TLR response through a feedback activation of STAT3. Sci Rep. 2015;5:13414.

156. Qing Y, Stark GR. Alternative activation of STAT1 and STAT3 in response to interferon-gamma. J Biol Chem. 2004;279(40):41679-85.

157. Ho J, Pelzel C, Begitt A, Mee M, Elsheikha HM, Scott DJ, et al. STAT2 Is a Pervasive Cytokine Regulator due to Its Inhibition of STAT1 in Multiple Signaling Pathways. PLoS Biol. 2016;14(10):e2000117.

158. Croker BA, Kiu H, Nicholson SE. SOCS regulation of the JAK/STAT signalling pathway. Semin Cell Dev Biol. 2008;19(4):414-22.

159. Marijanovic Z, Ragimbeau J, van der Heyden J, Uzé G, Pellegrini S. Comparable potency of IFNalpha2 and IFNbeta on immediate JAK/STAT activation but differential down-regulation of IFNAR2. Biochem J. 2007;407(1):141-51.

160. Pogue SL, Preston BT, Stalder J, Bebbington CR, Cardarelli PM. The receptor for type I IFNs is highly expressed on peripheral blood B cells and monocytes and mediates a distinct profile of differentiation and activation of these cells. J Interferon Cytokine Res. 2004;24(2):131-9.

161. Bernabei P, Coccia EM, Rigamonti L, Bosticardo M, Forni G, Pestka S, et al. Interferon-gamma receptor 2 expression as the deciding factor in human T, B, and myeloid cell proliferation or death. J Leukoc Biol. 2001;70(6):950-60.

162. Novelli F, Bernabei P, Ozmen L, Rigamonti L, Allione A, Pestka S, et al. Switching on of the proliferation or apoptosis of activated human T lymphocytes by IFN-gamma is correlated with the differential expression of the alpha- and beta-chains of its receptor. J Immunol. 1996;157(5):1935-43.

163. Garcia-Romo GS, Caielli S, Vega B, Connolly J, Allantaz F, Xu Z, et al. Netting neutrophils are major inducers of type I IFN production in pediatric systemic lupus erythematosus. Sci Transl Med. 2011;3(73):73ra20.

164. Yen JH, Ganea D. Interferon beta induces mature dendritic cell apoptosis through caspase-11/caspase-3 activation. Blood. 2009;114(7):1344-54.

165. Choi I, Muta K, Wickrema A, Krantz SB, Nishimura J, Nawata H. Interferon gamma delays apoptosis of mature erythroid progenitor cells in the absence of erythropoietin. Blood. 2000;95(12):3742-9.

166. Wang K, Scheel-Toellner D, Wong SH, Craddock R, Caamano J, Akbar AN, et al. Inhibition of neutrophil apoptosis by type 1 IFN depends on cross-talk between phosphoinositol 3-kinase, protein kinase C-delta, and NF-kappa B signaling pathways. J Immunol. 2003;171(2):1035-41.

167. Burt HM, Jackson JK. The priming action of tumour necrosis factor-alpha (TNF-alpha) and granulocyte-macrophage colony-stimulating factor (GM-CSF) on neutrophils activated by inflammatory microcrystals. Clin Exp Immunol. 1997;108(3):432-7.

168. Choi JC, Jung JW, Kwak HW, Song JH, Jeon EJ, Shin JW, et al. Granulocyte macrophage-colony stimulating factor (GM-CSF) augments acute lung injury via its neutrophil priming effects. J Korean Med Sci. 2008;23(2):288-95.

169. Condliffe AM, Chilvers ER, Haslett C, Dransfield I. Priming differentially regulates neutrophil adhesion molecule expression/function. Immunology. 1996;89(1):105-11.

170. Midgley A, Mayer K, Edwards SW, Beresford MW. Differential expression of factors involved in the intrinsic and extrinsic apoptotic pathways in juvenile systemic lupus erythematosus. Lupus. 2011;20(1):71-9.

171. Kim T, Kanayama Y, Negoro N, Okamura M, Takeda T, Inoue T. Serum levels of interferons in patients with systemic lupus erythematosus. Clin Exp Immunol. 1987;70(3):562-9.

172. Postal M, Sinicato NA, Peliçari KO, Marini R, Lavras Costallat LT, Appenzeller S. Clinical and serological manifestations associated with interferon-α levels in childhood-onset systemic lupus erythematosus. Clinics (Sao Paulo). 2012;67(2):157-62.

173. Baechler EC, Batliwalla FM, Karypis G, Gaffney PM, Ortmann WA, Espe KJ, et al. Interferon-inducible gene expression signature in peripheral blood cells of patients with

severe lupus. Proceedings of the National Academy of Sciences of the United States of America. 2003;100(5):2610-5.

174. Chiche L, Jourde-Chiche N, Whalen E, Presnell S, Gersuk V, Dang K, et al. Modular transcriptional repertoire analyses of adults with systemic lupus erythematosus reveal distinct type I and type II interferon signatures. Arthritis Rheumatol. 2014;66(6):1583-95.

175. Martinelli S, Urosevic M, Daryadel A, Oberholzer PA, Baumann C, Fey MF, et al. Induction of genes mediating interferon-dependent extracellular trap formation during neutrophil differentiation. J Biol Chem. 2004;279(42):44123-32.

176. Garcia-Romo GS, Caielli S, Vega B, Connolly J, Allantaz F, Xu ZH, et al. Netting Neutrophils Are Major Inducers of Type I IFN Production in Pediatric Systemic Lupus Erythematosus. Science Translational Medicine. 2011;3(73).

177. Stuve O, Chabot S, Jung SS, Williams G, Yong VW. Chemokine-enhanced migration of human peripheral blood mononuclear cells is antagonized by interferon beta-1b through an effect on matrix metalloproteinase-9. J Neuroimmunol. 1997;80(1-2):38-46.

178. Stüve O, Dooley NP, Uhm JH, Antel JP, Francis GS, Williams G, et al. Interferon beta-1b decreases the migration of T lymphocytes in vitro: effects on matrix metalloproteinase-9. Ann Neurol. 1996;40(6):853-63.

179. Bonecchi R, Polentarutti N, Luini W, Borsatti A, Bernasconi S, Locati M, et al. Up-regulation of CCR1 and CCR3 and induction of chemotaxis to CC chemokines by IFN-gamma in human neutrophils. J Immunol. 1999;162(1):474-9.

180. Pakianathan DR, Kuta EG, Artis DR, Skelton NJ, Hébert CA. Distinct but overlapping epitopes for the interaction of a CC-chemokine with CCR1, CCR3 and CCR5. Biochemistry. 1997;36(32):9642-8.

181. Combadiere C, Ahuja SK, Van Damme J, Tiffany HL, Gao JL, Murphy PM. Monocyte chemoattractant protein-3 is a functional ligand for CC chemokine receptors 1 and 2B. J Biol Chem. 1995;270(50):29671-5.

182. Daugherty BL, Siciliano SJ, DeMartino JA, Malkowitz L, Sirotina A, Springer MS. Cloning, expression, and characterization of the human eosinophil eotaxin receptor. J Exp Med. 1996;183(5):2349-54.

183. Rollag H, Degré M, Sonnenfeld G. Effects of interferon-alpha/beta and interferon-gamma preparations on phagocytosis by mouse peritoneal macrophages. Scand J Immunol. 1984;20(2):149-55.

184. Pechkovsky DV, Potapnev MP, Zalutskaya OM. Different patterns of cytokine regulation of phagocytosis and bacterial killing by human neutrophils. Int J Antimicrob Agents. 1996;7(1):33-40.

185. Schiff DE, Rae J, Martin TR, Davis BH, Curnutte JT. Increased phagocyte Fc gammaRI expression and improved Fc gamma-receptor-mediated phagocytosis after in vivo recombinant human interferon-gamma treatment of normal human subjects. Blood. 1997;90(8):3187-94.

186. Wang D, Feng X, Lu L, Konkel JE, Zhang H, Chen Z, et al. A CD8 T cell/indoleamine 2,3-dioxygenase axis is required for mesenchymal stem cell suppression of human systemic lupus erythematosus. Arthritis Rheumatol. 2014;66(8):2234-45.

187. Nacionales DC, Kelly-Scumpia KM, Lee PY, Weinstein JS, Lyons R, Sobel E, et al. Deficiency of the type I interferon receptor protects mice from experimental lupus. Arthritis Rheum. 2007;56(11):3770-83.

188. Bauer JW, Baechler EC, Petri M, Batliwalla FM, Crawford D, Ortmann WA, et al. Elevated serum levels of interferon-regulated chemokines are biomarkers for active human systemic lupus erythematosus. PLoS Med. 2006;3(12):e491.

189. Hsieh SC, Yu HS, Lin WW, Sun KH, Tsai CY, Huang DF, et al. Anti-SSB/La is one of the antineutrophil autoantibodies responsible for neutropenia and functional impairment of polymorphonuclear neutrophils in patients with systemic lupus erythematosus. Clin Exp Immunol. 2003;131(3):506-16.

190. Munroe ME, Lu R, Zhao YD, Fife DA, Robertson JM, Guthridge JM, et al. Altered type II interferon precedes autoantibody accrual and elevated type I interferon activity prior to systemic lupus erythematosus classification. Ann Rheum Dis. 2016;75(11):2014-21.

191. Chen J, Zhao J, Chen L, Dong N, Ying Z, Cai Z, et al. STAT1 modification improves therapeutic effects of interferons on lung cancer cells. J Transl Med. 2015;13:293.

192. Baskić D, Popović S, Ristić P, Arsenijević NN. Analysis of cycloheximide-induced apoptosis in human leukocytes: fluorescence microscopy using annexin V/propidium iodide versus acridin orange/ethidium bromide. Cell Biol Int. 2006;30(11):924-32.

193. Lehtonen A, Matikainen S, Julkunen I. Interferons up-regulate STAT1, STAT2, and IRF family transcription factor gene expression in human peripheral blood mononuclear cells and macrophages. J Immunol. 1997;159(2):794-803.

194. Karonitsch T, Feierl E, Steiner CW, Dalwigk K, Korb A, Binder N, et al. Activation of the interferon-gamma signaling pathway in systemic lupus erythematosus peripheral blood mononuclear cells. Arthritis Rheum. 2009;60(5):1463-71.

195. Karonitsch T, von Dalwigk K, Steiner CW, Blüml S, Steiner G, Kiener HP, et al. Interferon signals and monocytic sensitization of the interferon-γ signaling pathway in the peripheral blood of patients with rheumatoid arthritis. Arthritis Rheum. 2012;64(2):400-8.

196. Dong J, Wang QX, Zhou CY, Ma XF, Zhang YC. Activation of the STAT1 signalling pathway in lupus nephritis in MRL/lpr mice. Lupus. 2007;16(2):101-9.

197. Ikeda K, Hayakawa K, Fujishiro M, Kawasaki M, Hirai T, Tsushima H, et al. JAK inhibitor has the amelioration effect in lupus-prone mice: the involvement of IFN signature gene downregulation. BMC Immunol. 2017;18(1):41.

198. Ripoll È, de Ramon L, Draibe Bordignon J, Merino A, Bolaños N, Goma M, et al. JAK3-STAT pathway blocking benefits in experimental lupus nephritis. Arthritis Res Ther. 2016;18(1):134.

199. Mitchell TS, Moots RJ, Wright HL. Janus kinase inhibitors prevent migration of rheumatoid arthritis neutrophils towards interleukin-8, but do not inhibit priming of the respiratory burst or reactive oxygen species production. Clin Exp Immunol. 2017;189(2):250-8.

200. Frank DA, Mahajan S, Ritz J. Fludarabine-induced immunosuppression is associated with inhibition of STAT1 signaling. Nat Med. 1999;5(4):444-7.

201. Yao Y, Hausding M, Erkel G, Anke T, Förstermann U, Kleinert H. Sporogen, S14-95, and S-curvularin, three inhibitors of human inducible nitric-oxide synthase expression isolated from fungi. Mol Pharmacol. 2003;63(2):383-91.

202. Erkel G, Rether J, Anke T, Sterner O. S14-95, a novel inhibitor of the JAK/STAT pathway from a Penicillium species. J Antibiot (Tokyo). 2003;56(4):337-43.

203. Association GAotWM. World Medical Association Declaration of Helsinki: ethical principles for medical research involving human subjects. J Am Coll Dent. 2014;81(3):14-8.

204. Marks SD, Pilkington C, Woo P, Dillon MJ. The use of the British Isles Lupus Assessment Group (BILAG) index as a valid tool in assessing disease activity in childhood-onset systemic lupus erythematosus. Rheumatology (Oxford). 2004;43(9):1186-9.

205. Shen L, Smith JM, Shen Z, Hussey SB, Wira CR, Fanger MW. Differential regulation of neutrophil chemotaxis to IL-8 and fMLP by GM-CSF: lack of direct effect of oestradiol. Immunology. 2006;117(2):205-12.

206. Lokuta MA, Huttenlocher A. TNF-alpha promotes a stop signal that inhibits neutrophil polarization and migration via a p38 MAPK pathway. J Leukoc Biol. 2005;78(1):210-9.

207. Hammond ME, Lapointe GR, Feucht PH, Hilt S, Gallegos CA, Gordon CA, et al. IL-8 induces neutrophil chemotaxis predominantly via type I IL-8 receptors. J Immunol. 1995;155(3):1428-33.

208. Hakkim A, Fürnrohr BG, Amann K, Laube B, Abed UA, Brinkmann V, et al. Impairment of neutrophil extracellular trap degradation is associated with lupus nephritis. Proc Natl Acad Sci U S A. 2010;107(21):9813-8.

209. Dürr UH, Sudheendra US, Ramamoorthy A. LL-37, the only human member of the cathelicidin family of antimicrobial peptides. Biochim Biophys Acta. 2006;1758(9):1408-25.

210. Reinholz M, Ruzicka T, Schauber J. Cathelicidin LL-37: an antimicrobial peptide with a role in inflammatory skin disease. Ann Dermatol. 2012;24(2):126-35.

211. Lee SA, Kwak MS, Kim S, Shin JS. The role of high mobility group box 1 in innate immunity. Yonsei Med J. 2014;55(5):1165-76.

212. Villanueva E, Yalavarthi S, Berthier CC, Hodgin JB, Khandpur R, Lin AM, et al. Netting neutrophils induce endothelial damage, infiltrate tissues, and expose immunostimulatory molecules in systemic lupus erythematosus. J Immunol. 2011;187(1):538-52.

213. Li RHL, Ng G, Tablin F. Lipopolysaccharide-induced neutrophil extracellular trap formation in canine neutrophils is dependent on histone H3 citrullination by peptidylarginine deiminase. Vet Immunol Immunopathol. 2017;193-194:29-37.

214. Putker F, Bos MP, Tommassen J. Transport of lipopolysaccharide to the Gram-negative bacterial cell surface. FEMS Microbiol Rev. 2015;39(6):985-1002.

215. Guyre PM, Morganelli PM, Miller R. Recombinant immune interferon increases immunoglobulin G Fc receptors on cultured human mononuclear phagocytes. J Clin Invest. 1983;72(1):393-7.

216. Kraaij T, Tengström FC, Kamerling SW, Pusey CD, Scherer HU, Toes RE, et al. A novel method for high-throughput detection and quantification of neutrophil extracellular traps reveals ROS-independent NET release with immune complexes. Autoimmun Rev. 2016;15(6):577-84.

217. Coelho LP, Pato C, Friães A, Neumann A, von Köckritz-Blickwede M, Ramirez M, et al. Automatic determination of NET (neutrophil extracellular traps) coverage in fluorescent microscopy images. Bioinformatics. 2015;31(14):2364-70.

218. Kahlenberg JM, Carmona-Rivera C, Smith CK, Kaplan MJ. Neutrophil extracellular trap-associated protein activation of the NLRP3 inflammasome is enhanced in lupus macrophages. J Immunol. 2013;190(3):1217-26.

219. Wang KQ, Scheel-Toellner D, Wong SH, Craddock R, Caamano J, Akbar AN, et al. Inhibition of neutrophil apoptosis by type 1 IFN depends on cross-talk between phosphoinositol 3-kinase, protein kinase C-delta, and NF-kappa B signaling pathways. Journal of Immunology. 2003;171(2):1035-41.

220. Willis SN, Chen L, Dewson G, Wei A, Naik E, Fletcher JI, et al. Proapoptotic Bak is sequestered by Mcl-1 and Bcl-xL, but not Bcl-2, until displaced by BH3-only proteins. Genes Dev. 2005;19(11):1294-305.

221. Cross A, Moots RJ, Edwards SW. The dual effects of TNFalpha on neutrophil apoptosis are mediated via differential effects on expression of Mcl-1 and Bfl-1. Blood. 2008;111(2):878-84.

222. Niewold TB, Hua J, Lehman TJ, Harley JB, Crow MK. High serum IFN-alpha activity is a heritable risk factor for systemic lupus erythematosus. Genes Immun. 2007;8(6):492-502.

223. Moulding DA, Akgul C, Derouet M, White MR, Edwards SW. BCL-2 family expression in human neutrophils during delayed and accelerated apoptosis. J Leukoc Biol. 2001;70(5):783-92.

224. Koenig KF, Groeschl I, Pesickova SS, Tesar V, Eisenberger U, Trendelenburg M. Serum cytokine profile in patients with active lupus nephritis. Cytokine. 2012;60(2):410-6.

225. Ballantine LE, Ong J, Midgley A, Watson L, Flanagan BF, Beresford MW. The pro-inflammatory potential of T cells in juvenile-onset systemic lupus erythematosus. Pediatr Rheumatol Online J. 2014;12:4.

226. Reeves WH, Lee PY, Weinstein JS, Satoh M, Lu L. Induction of autoimmunity by pristane and other naturally occurring hydrocarbons. Trends Immunol. 2009;30(9):455-64.

227. Nacionales DC, Kelly KM, Lee PY, Zhuang H, Li Y, Weinstein JS, et al. Type I interferon production by tertiary lymphoid tissue developing in response to 2,6,10,14-tetramethyl-pentadecane (pristane). Am J Pathol. 2006;168(4):1227-40.

228. Socinski MA, Cannistra SA, Sullivan R, Elias A, Antman K, Schnipper L, et al. Granulocyte-macrophage colony-stimulating factor induces the expression of the CD11b surface adhesion molecule on human granulocytes in vivo. Blood. 1988;72(2):691-7.

229. Ricci F, Tedeschi A, Morra E, Montillo M. Fludarabine in the treatment of chronic lymphocytic leukemia: a review. Ther Clin Risk Manag. 2009;5(1):187-207.

230. Courtney PA, Crockard AD, Williamson K, Irvine AE, Kennedy RJ, Bell AL. Increased apoptotic peripheral blood neutrophils in systemic lupus erythematosus: relations with disease activity, antibodies to double stranded DNA, and neutropenia. Annals of the Rheumatic Diseases. 1999;58(5):309-14.

231. Bengtsson AA, Sturfelt G, Truedsson L, Blomberg J, Alm G, Vallin H, et al. Activation of type I interferon system in systemic lupus erythematosus correlates with disease activity but not with antiretroviral antibodies. Lupus. 2000;9(9):664-71.

232. Agramonte-Hevia J, González-Arenas A, Barrera D, Velasco-Velázquez M. Gram-negative bacteria and phagocytic cell interaction mediated by complement receptor 3. FEMS Immunol Med Microbiol. 2002;34(4):255-66.

233. Sakamoto E, Hato F, Kato T, Sakamoto C, Akahori M, Hino M, et al. Type I and type II interferons delay human neutrophil apoptosis via activation of STAT3 and up-regulation of cellular inhibitor of apoptosis 2. Journal of Leukocyte Biology. 2005;78(1):301-9.

234. Malide D, Russo P, Bendayan M. Presence of tumor necrosis factor alpha and interleukin-6 in renal mesangial cells of lupus nephritis patients. Hum Pathol. 1995;26(5):558-64.

235. de Jager W, Hoppenreijs EP, Wulffraat NM, Wedderburn LR, Kuis W, Prakken BJ. Blood and synovial fluid cytokine signatures in patients with juvenile idiopathic arthritis: a cross-sectional study. Ann Rheum Dis. 2007;66(5):589-98.

236. Greten FR, Weber CK, Greten TF, Schneider G, Wagner M, Adler G, et al. Stat3 and NF-kappaB activation prevents apoptosis in pancreatic carcinogenesis. Gastroenterology. 2002;123(6):2052-63.

237. Bhattacharya S, Ray RM, Johnson LR. STAT3-mediated transcription of Bcl-2, Mcl-1 and c-IAP2 prevents apoptosis in polyamine-depleted cells. Biochem J. 2005;392(Pt 2):335-44.

238. Sato VA, Marques ID, Goldenstein PT, Carmo LP, Jorge LB, Titan SM, et al. Lupus nephritis is more severe in children and adolescents than in older adults. Lupus. 2012;21(9):978-83.

239. Smith EM, Jorgensen AL, Midgley A, Oni L, Goilav B, Putterman C, et al. International validation of a urinary biomarker panel for identification of active lupus nephritis in children. Pediatr Nephrol. 2017;32(2):283-95.

240. Watson L, Midgley A, Pilkington C, Tullus K, Marks S, Holt R, et al. Urinary monocyte chemoattractant protein 1 and alpha 1 acid glycoprotein as biomarkers of renal disease activity in juvenile-onset systemic lupus erythematosus. Lupus. 2012;21(5):496-501.

241. Dransfield I, Buckle AM, Savill JS, McDowall A, Haslett C, Hogg N. Neutrophil apoptosis is associated with a reduction in CD16 (Fc gamma RIII) expression. J Immunol. 1994;153(3):1254-63.

242. Homburg CH, de Haas M, von dem Borne AE, Verhoeven AJ, Reutelingsperger CP, Roos D. Human neutrophils lose their surface Fc gamma RIII and acquire Annexin V binding sites during apoptosis in vitro. Blood. 1995;85(2):532-40.

243. Jönsen A, Gunnarsson I, Gullstrand B, Svenungsson E, Bengtsson AA, Nived O, et al. Association between SLE nephritis and polymorphic variants of the CRP and FcgammaRIIIa genes. Rheumatology (Oxford). 2007;46(9):1417-21.

244. Park-Min KH, Serbina NV, Yang W, Ma X, Krystal G, Neel BG, et al. FcgammaRIII-dependent inhibition of interferon-gamma responses mediates suppressive effects of intravenous immune globulin. Immunity. 2007;26(1):67-78.

245. Vermes I, Haanen C, Steffens-Nakken H, Reutelingsperger C. A novel assay for apoptosis. Flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labelled Annexin V. J Immunol Methods. 1995;184(1):39-51.

246. Furie R, Khamashta M, Merrill JT, Werth VP, Kalunian K, Brohawn P, et al. Anifrolumab, an Anti-Interferon-α Receptor Monoclonal Antibody, in Moderate-to-Severe Systemic Lupus Erythematosus. Arthritis Rheumatol. 2017;69(2):376-86.

247. Lloyds D, Brindle NP, Hallett MB. Priming of human neutrophils by tumour necrosis factor-alpha and substance P is associated with tyrosine phosphorylation. Immunology. 1995;84(2):220-6.

248. Salamone G, Giordano M, Trevani AS, Gamberale R, Vermeulen M, Schettinni J, et al. Promotion of neutrophil apoptosis by TNF-alpha. J Immunol. 2001;166(5):3476-83.

249. Sikorski K, Czerwoniec A, Bujnicki JM, Wesoly J, Bluyssen HA. STAT1 as a novel therapeutical target in pro-atherogenic signal integration of IFNγ, TLR4 and IL-6 in vascular disease. Cytokine Growth Factor Rev. 2011;22(4):211-9.

250. Martinez-Lostao L, Briones J, Forné I, Martinez-Gallo M, Ferrer B, Sierra J, et al. Role of the STAT1 pathway in apoptosis induced by fludarabine and JAK kinase inhibitors in B-cell chronic lymphocytic leukemia. Leuk Lymphoma. 2005;46(3):435-42.

251. Kumar A, Commane M, Flickinger TW, Horvath CM, Stark GR. Defective TNF-alpha-induced apoptosis in STAT1-null cells due to low constitutive levels of caspases. Science. 1997;278(5343):1630-2.

252. Wang Y, Wu TR, Cai S, Welte T, Chin YE. Stat1 as a component of tumor necrosis factor alpha receptor 1-TRADD signaling complex to inhibit NF-kappaB activation. Mol Cell Biol. 2000;20(13):4505-12.

253. Dhodapkar KM, Banerjee D, Connolly J, Kukreja A, Matayeva E, Veri MC, et al. Selective blockade of the inhibitory Fcgamma receptor (FcgammaRIIB) in human dendritic cells and monocytes induces a type I interferon response program. J Exp Med. 2007;204(6):1359-69.

254. McCracken JM, Allen LA. Regulation of human neutrophil apoptosis and lifespan in health and disease. J Cell Death. 2014;7:15-23.

255. Liu Y, Hu Y, Gu F, Liang J, Zeng Y, Hong X, et al. Phenotypic and clinical characterization of low density neutrophils in patients with advanced lung adenocarcinoma. Oncotarget. 2017;8(53):90969-78.

256. Denny MF, Yalavarthi S, Zhao W, Thacker SG, Anderson M, Sandy AR, et al. A distinct subset of proinflammatory neutrophils isolated from patients with systemic lupus erythematosus induces vascular damage and synthesizes type I IFNs. J Immunol. 2010;184(6):3284-97.

257. Niewold TB, Swedler WI. Systemic lupus erythematosus arising during interferon-alpha therapy for cryoglobulinemic vasculitis associated with hepatitis C. Clin Rheumatol. 2005;24(2):178-81.

258. Pouthier D, Theissen F, Humbel RL. Lupus syndrome, hypothyroidism and bullous skin lesions after interferon alfa therapy for hepatitis C in a haemodialysis patient. Nephrol Dial Transplant. 2002;17(1):174.

259. Ho V, Mclean A, Terry S. Severe systemic lupus erythematosus induced by antiviral treatment for hepatitis C. J Clin Rheumatol. 2008;14(3):166-8.

260. Fukuyama S, Kajiwara E, Suzuki N, Miyazaki N, Sadoshima S, Onoyama K. Systemic lupus erythematosus after alpha-interferon therapy for chronic hepatitis C: a case report and review of the literature. Am J Gastroenterol. 2000;95(1):310-2.

261. Schilling PJ, Kurzrock R, Kantarjian H, Gutterman JU, Talpaz M. Development of systemic lupus erythematosus after interferon therapy for chronic myelogenous leukemia. Cancer. 1991;68(7):1536-7.

262. Rönnblom LE, Alm GV, Oberg KE. Possible induction of systemic lupus erythematosus by interferon-alpha treatment in a patient with a malignant carcinoid tumour. J Intern Med. 1990;227(3):207-10.

263. Yao Y, Richman L, Higgs BW, Morehouse CA, de los Reyes M, Brohawn P, et al. Neutralization of interferon-alpha/beta-inducible genes and downstream effect in a phase I trial of an anti-interferon-alpha monoclonal antibody in systemic lupus erythematosus. Arthritis Rheum. 2009;60(6):1785-96.

264. Merrill JT, Wallace DJ, Petri M, Kirou KA, Yao Y, White WI, et al. Safety profile and clinical activity of sifalimumab, a fully human anti-interferon α monoclonal antibody, in systemic lupus erythematosus: a phase I, multicentre, double-blind randomised study. Ann Rheum Dis. 2011;70(11):1905-13.

265. Petri M, Wallace DJ, Spindler A, Chindalore V, Kalunian K, Mysler E, et al. Sifalimumab, a human anti-interferon-alpha monoclonal antibody, in systemic lupus erythematosus: a phase I randomized, controlled, dose-escalation study. Arthritis Rheum. 2013;65(4):1011-21.

266. McBride JM, Jiang J, Abbas AR, Morimoto A, Li J, Maciuca R, et al. Safety and pharmacodynamics of rontalizumab in patients with systemic lupus erythematosus: results of a phase I, placebo-controlled, double-blind, dose-escalation study. Arthritis Rheum. 2012;64(11):3666-76.

267. Kalunian KC, Merrill JT, Maciuca R, McBride JM, Townsend MJ, Wei X, et al. A Phase II study of the efficacy and safety of rontalizumab (rhuMAb interferon-α) in patients with systemic lupus erythematosus (ROSE). Ann Rheum Dis. 2016;75(1):196-202.

268. Peng L, Oganesyan V, Wu H, Dall'Acqua WF, Damschroder MM. Molecular basis for antagonistic activity of anifrolumab, an anti-interferon-α receptor 1 antibody. MAbs. 2015;7(2):428-39.

269. Zhou Z, Hamming OJ, Ank N, Paludan SR, Nielsen AL, Hartmann R. Type III interferon (IFN) induces a type I IFN-like response in a restricted subset of cells through signaling pathways involving both the Jak-STAT pathway and the mitogen-activated protein kinases. J Virol. 2007;81(14):7749-58.

270. Wu Q, Yang QR, Lourenco E, Sun HS, Zhang YC. Interferon-lambda1 induces peripheral blood mononuclear cell-derived chemokines secretion in patients with systemic lupus erythematosus: its correlation with disease activity. Arthritis Research & Therapy. 2011;13(3).

271. Zickert A, Oke V, Parodis I, Svenungsson E, Sundström Y, Gunnarsson I. Interferon (IFN)-λ is a potential mediator in lupus nephritis. Lupus Sci Med. 2016;3(1):e000170.

272. Amezcua-Guerra LM, Márquez-Velasco R, Chávez-Rueda AK, Castillo-Martínez D, Massó F, Páez A, et al. Type III Interferons in Systemic Lupus Erythematosus: Association Between Interferon λ3, Disease Activity, and Anti-Ro/SSA Antibodies. J Clin Rheumatol. 2017.

273. Sekiya S, Gotoh S, Yamashita T, Watanabe T, Saitoh S, Sendo F. Selective depletion of rat neutrophils by in vivo administration of a monoclonal antibody. J Leukoc Biol. 1989;46(2):96-102.

274. Santos LL, Morand EF, Hutchinson P, Boyce NW, Holdsworth SR. Anti-neutrophil monoclonal antibody therapy inhibits the development of adjuvant arthritis. Clin Exp Immunol. 1997;107(2):248-53.

275. Quint JK, Wedzicha JA. The neutrophil in chronic obstructive pulmonary disease. J Allergy Clin Immunol. 2007;119(5):1065-71.

276. Vij N, Min T, Bodas M, Gorde A, Roy I. Neutrophil targeted nano-drug delivery system for chronic obstructive lung diseases. Nanomedicine. 2016;12(8):2415-27.

277. Hamming OJ, Gad HH, Paludan S, Hartmann R. Lambda Interferons: New Cytokines with Old Functions. Pharmaceuticals (Basel). 2010;3(4):795-809.

278. Jewell NA, Cline T, Mertz SE, Smirnov SV, Flaño E, Schindler C, et al. Lambda interferon is the predominant interferon induced by influenza A virus infection in vivo. J Virol. 2010;84(21):11515-22.

279. Li W, Lewis-Antes A, Huang J, Balan M, Kotenko SV. Regulation of apoptosis by type III interferons. Cell Prolif. 2008;41(6):960-79.