Neutrophil function in Juvenile Systemic Lupus Erythematosus (JSLE)

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor in Philosophy by

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I declare that this thesis entitled:

"Neutrophil Function in

Juvenile Systemic Lupus Erythematosus (JSLE)"

is entirely my own work

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ABSTRACT

Objectives This project aimed to investigate the abnormal neutrophil functions in terms of cell survival, phagocytosis, ROS production and chemotaxis in JSLE patients. Furthermore, factors that could affect normal neutrophil functions (such as patient sera, recombinant human cytokines and commonly-used medications) were investigated.

Methods Neutrophils and sera were isolated from whole blood of JSLE patients, healthy juvenile controls and healthy adult controls. Neutrophils from healthy adult controls were incubated with 10% serum from either JSLE patients or juvenile controls. Different recombinant cytokines or hydroxychloroquine were added in 10% human AB serum to neutrophils from healthy adult controls. Neutrophil apoptosis was measured using flow cytometry using annexin V-FITC and propidium iodide. The expression of proteins (Mcl-1, caspases 3,7,8 and 9) was measured by Western blotting. Phagocytosis and ROS production from neutrophils incubated with *S. aureus* opsonised with JSLE serum were quantified. Levels of cytokines in JSLE serum were measured using a Luminex assay and effects of GM-CSF and TNF-α on neutrophil apoptosis induced by JSLE serum were measured. JSLE neutrophil apoptosis was measured and Mcl-1 mRNA expression from JSLE neutrophils was quantified using real-time PCR. Phagocytosis, ROS production and neutrophil chemotaxis by JSLE neutrophils were investigated. Lastly, the effects of hydroxychloroquine on neutrophil functions were explored.

Results The results showed that neutrophils incubated with inactive and active JSLE sera had significantly increased apoptosis at 6 h compared to control sera. Cleaved (active) forms of caspases 3,7,8 were identified in neutrophils incubated with inactive and active JSLE sera (that showed high rates of apoptosis) compared to control sera. Decreased bacterial opsonisation leading to defective phagocytosis and ROS production was observed in neutrophils incubated with *S. aureus* opsonised with JSLE serum. Serum analysis showed IL-8 levels in active JSLE patients were significantly increased. GM-CSF was the most potent cytokine in delaying apoptosis and significantly saved neutrophil apoptosis induced by JSLE serum. Low concentrations of TNF-α significantly protected neutrophils against apoptosis by down-regulating several genes and proteins involved in death receptor signaling pathway (e.g. TNFR, FADD, TRADD, caspases 8 and 10). No significant differences were detected in apoptosis, phagocytosis, ROS production and chemotaxis of neutrophils isolated from JSLE patients compared to healthy juvenile controls, and healthy adult neutrophils treated with hydroxychloroquine compared to untreated cells.

Conclusions This study demonstrated that JSLE serum played an important role in regulating the functions of JSLE neutrophils. Factor(s) in JSLE serum induced neutrophil apoptosis and caused decreased bacterial opsonisation, leading to defective neutrophil phagocytosis and ROS production. Increased IL-8 levels could be used as an indicator of disease activity. GM-CSF was the most protective cytokine and overcame the pro-apoptotic effects of JSLE serum; thus, GM-CSF could potentially be used as an alternative treatment in JSLE patients. Effects of TNF-α are probably tissue-specific and the clinical application of TNF blockers in JSLE patients needs to be carefully considered. The functions of JSLE neutrophils were unimpaired and hydroxychloroquine showed no effects on neutrophil functions.

PUBLICATIONS AND PRESENTATIONS

Publications

1. **Chiewchengchol, D**., Murphy, R., Edwards, S.W., Beresford M.W.

"Mucocutaneous manifestations in Juvenile-onset Systemic lupus erythematosus: A Review of literature" (*In preparation)*

2. **Chiewchengchol, D.,** Wright, H.L., Thomas, H.B., Lam, C., Roberts, K.J., Beresford M.W., Moots, R.J., Edwards, S.W.

"Down Regulation of Death Receptor Signaling in Human Neutrophils following TNF-α Stimulation" *(In preparation)*

3. **Chiewchengchol, D**., Murphy, R., Morgan, T., Edwards, S.W., Leone, V., Friswell, M., Pilkington, C., Tullus, K., Rangaraj, S., McDonagh, J.E., Gardner-Medwin, J., Wilkinson, N., Riley, P., Tizard, J., Armon, K., Sinha, M.D., Ioannou, Y., Mann, R., Bailey, K., Davidson, J., Baildam, E.M., Pain, C.E., Cleary, G., McCann, L.J., and Beresford, M.W., On behalf of the UK Juvenile-onset SLE study group

"Mucocutaneous manifestations in a National Cohort of Juvenile-onset Systemic Lupus Erythematosus Patients" *(Submitted to Rheumatology Oxford Journal)*

Poster Presentations

1. British Society for Paediatric and Adolescent Rheumatology 2011 Annual Conference, Nottingham, United Kingdom, September 22-23 2011: The expression of Myeloid Cell Leukemia-1 (Mcl-1) in Juvenile Systemic Lupus Erythematosus serum induced-neutrophil apoptosis.

Poster Presentations (continued)

2. The neutrophil in Immunity 2012 Annual Conference, Quebec, Canada, June 9-12 2012: Defective Neutrophil Phagocytosis in Juvenile Systemic Lupus Erythematosus.

3. The neutrophil in Immunity 2012 Annual Conference, Quebec, Canada, June 9-12 2012: Down Regulation of Death Receptor Signaling in Neutrophils following TNF-α stimulation.

4. Musculoskeletal Biology Science Day, Liverpool, United Kingdom, February 25 2013: Defective Neutrophil Function in Juvenile Systemic Lupus Erythematosus.

ABBREVIATIONS

ACR	American College of Rheumatology
ACTB	β-actin
Akt	Protein kinase B
ANA	Anti-nuclear antigen
AP-1	Activator protein-1
Apaf-1	Apoptotic protease-activating factor-1
APS	Ammonium persulphate
ARDS	Acute respiratory distress syndrome
ATP	adenosine triphosphate
AZA	Azathioprine
B_2M	β_2 microglobulin
Bad	B-cell leukaemia-2 associated death promoter protein
Bak	B-cell leukaemia-2 homologous antagonist/killer protein
Bax	B-cell leukaemia-2 associated protein-X
Bcl-2	B-cell leukaemia-2 protein
BCL2A1	B-cell leukaemia-2 related protein A1
Bcl-B	B-cell leukaemia-B protein
Bcl-w	B-cell leukaemia-w protein
Bcl-X_L	B-cell lymphoma-extra large protein
Bfl-1/A1	B-cell leukaemia-2 related protein A1
BGI	Beijing Genomics Institute
BH	B-cell leukaemia-2 homology domain

Bid	BH3 interacting domain death antagonist protein
Bik	BCL2-interacting killer (apoptosis-inducing)
Bim	B-cell leukaemia-2 like protein-11
BLys	B Lymphocyte stimulator
Bmf	BCL-2 modifying factor
Boo	BCL-2 homolog of ovary
BSA	Bovine serum albumin
BX471	CCR-1 specific antagonist
C3	Complement factor 3
C4	Complement factor 4
Caspase	Cysteine-aspartic acid protease
CCL	Chemokine (C-C motif) ligand
CCR	Chemokine (C-C motif) receptor
CD	Cluster of differentiation
C/EBP	CCAAT/enhancer-binding protein
CFLAR	Caspase 8 and FADD-like apoptosis regulator
Cfu	Colony-forming units
CGD	Chronic Granulomatous Disease
CHX	Cycloheximide
cIAP	Cellular inhibitor of apoptosis
CMV	Cytomegalovirus
CNS	Central nervous system
COPD	Chronic Obstructive Pulmonary Disease

CORE	Committee for research ethics
CR	Complement receptor
CREB	cAMP response element-binding protein
CREM	cAMP response element modulator
CRISP	Cysteine-rich secretory protein
CrmA	Cytokine response modifier A
CRP	C-reactive protein
CXCL	C-X-C motif ligand
CXCR	C-X-C motif receptor
DEPC	Dethylpyrocarbonate
DHR123	Dihydrorhodramine 123
DISC	Death-inducing signalling complex
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dsDNA	double stranded DNA
DTT	Dithiothreitol
EBV	Epstein-Barr virus
EBNA	Epstein-Barr nuclear antigen
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetracetic acid
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinase
ESL-1	E-selectin ligand 1

ESR	Erythrocyte sedimentation rate
FAD	Flavin adenine dinucleotide
FADD	Fas-associated death domain-containing protein
FasL	Fas ligand
FcγR	Fcγ receptor
FDA	Food and Drug Administration
FITC	Fluorescein isothiocyanate
FLIP	FLICE-like inhibitory protein
fMLP	N-formyl-methionyl-leucyl-phenylalanine
FPR	Formyl-peptide receptor
G6P	Glucose-6-phosphate
G6PDH	G6P dehydrogenase
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
G-CSF	Granulocyte colony stimulating factor
GM-CSF	Granulocyte macrophage colony stimulating factor
GPI	Glycosyl-phosphatidylinositol
GROβ	Growth-regulated oncogene β
GSK3	Glycogen synthase kinase-3
GTPase	Guanosine triphosphatase
H_2O_2	Hydrogen peroxide
HBSS	Hanks balanced salt solution
HDAC	Histone deacetylases
HIF1α	Hypoxia-inducible factor 1α

HMP	Hexose monophosphate
HOCl	Hypochlorous acid
HRP	Horseradish peroxidase
HSA	Human serum albumin
ICAM	Intercellular adhesion molecule
IFN-γ	Interferon-γ
IgG	Immunoglobulin G
IκB	nuclear factor of kappa light polypeptide gene in B cells inhibitor
ICAM	Intercellular adhesion molecule
IKK	Inhibitor of nuclear factor kappa B kinase
IL	Interleukin
IL-1Ra	IL-1 receptor antagonist
IPA	Ingenuity pathway analysis
IRAK	Interleukin 1 receptor associated kinase
IRF	Interferon regulatory factor
ITGAM	Integrin alpha M
JAK	Janus kinase
JAM	Junctional adhesion molecule
JAZF1	Juxtaposed with another zinc finger protein 1
JIA	Juvenile idiopathic arthritis
JNK	C-Jun N-terminal kinase
JSLE	Juvenile systemic lupus erythematosus

kDa	kilo Dalton
LDGs	Low-density granulocytes
LF	Lactoferrin
LFA-1	Lymphocyte function-associated antigen 1
LL-37	Human cathelicidin
LPS	Lipopolysaccharide
MAC-1	Macrophage-1 antigen
MAPK	Mitogen-activated protein kinase
Mcl-1	Myeloid cell leukaemia 1
MCP-1	Monocyte chemotactic protein 1
MEK1	Mitogen-activated protein kinase 1
MEKK1	Mitogen-activated protein kinase kinase 1
MHCII	Class II major histocompatibility complex
MIP1	Macrophage inflammatory protein 1
MMF	Mycophenolate mofetil
MMP	Matrix-metalloproteinase
MPO	Myeloperoxidase
mRNA	messenger ribonucleic acid
NADP	Nicotinate adenine dinucleotide phosphate
NAD	Nicotinamide adenine dinucleotide (oxidised)
NADH	Nicotinamide adenine dinucleotide (reduced)
NADK	Nicotinamide adenine dinucleotide kinase
NADP	Nicotinamide adenine dinucleotide phosphate (oxidised)

NADPH	Nicotinamide adenine dinucleotide phosphate (reduced)
NET	Neutrophil extracellular trap
NF-1	Nuclear factor-1
NF-κB	Nuclear factor κB
NFAT	Nuclear Factor of activated T cells
NGAL	Neutrophil gelatinase associated lipocalin
NIK	NFκB-inducing kinase
NK cells	Natural Killer cells
NMN	Nicotinamide mononucleotide
NMNAT	Nicotinamide mononucleotide adenylyl transferase
NSAID	Non-steroidal anti-inflammatory drug
PAMP	Pathogen-associated molecular pattern
PBS	Phosphate buffered saline
PECAM	Platelet endothelial cell adhesion molecule
PEST	Proline-glutamic acid-serine-threonine
PET	Polyethylene terephthalate
PFA	Paraformaldehyde
PHOX	Phagocyte oxidase
PI	Propidium iodide
PMA	Phorbol 12-myristate 13-acetate
PRPP	5-phosphoribosyl-pyrophosphate
PRT	Phosphoribosyltransferase
PSGL-1	P-selectin glycoprotein ligand 1

PU.1	Transcription factor PU.1
PUMA	p53 up-regulated modulator of apoptosis
PVDF	Polyvinylidene fluoride
RA	Rheumatoid arthritis
Rac1/2	Ras-related C3 botulinum toxin substrate 1/2
RANKL	Receptor activator of nuclear factor kappa-B ligand
REL	Proto-oncogene REL
RF	Rheumatoid factor
RhoGDI	Rho guanine nucleotide dissociation inhibitor
RIN	RNA integrity number
RNP	Ribonucleoprotein
ROR	RAR-related orphan receptor
ROS	Reactive oxygen species
RPKM	Reads per kilobase-pair per million mapped reads
SDS	Sodium dodecyl sulphate
siRNA	Small interfering ribonucleic acid
SLE	Systemic lupus erythematosus
SLEDAI	SLE disease activity index
Sm	Smooth muscle
SNP	Single nucleotide polymorphisms
SP-1	Specificity Protein-1
SSA/Ro	Sjögren’s syndrome A antigen
SSB/La	Sjögren’s syndrome B antigen

STAT	Signal transducer and activator of transcription
TCR	T cell receptor
TEMED	Tetramethylethylenediamine
Th cells	T helper cells
TLR	Toll-like receptor
TNFAIP	Tumour necrosis factor alpha-induced protein
TNFR	Tumour necrosis factor receptor
TNFRSF	Tumour necrosis factor receptor superfamily
TNF-α	Tumour necrosis factor-α
TRADD	TNFR-associated death domain-containing proteins
TRAIL	TNF-related apoptosis-inducing ligand
Treg cells	Regulatory T cells
UHRF1BP1	UHRF1 binding protein 1
XIAP	X-linked inhibitor of apoptosis protein

Chapter 1: Introduction

1.1 Neutrophils

Neutrophilic polymorphonuclear leukocytes (neutrophils) are the most abundant white blood cells in the circulation of human blood. They play a major role during acute inflammation, as they are the first white blood cells to migrate through the vasculature to the inflammatory site and are able to recognize and eliminate pathogens (1, 2). Neutrophils normally constitute about 40-65% of all human white blood cells and are found at concentrations around 3-5 x 10^6 cells/mL blood. Their shape in the circulation is approximately spherical, and they have a diameter of 7-10 μm. Their cytoplasm has many granules that contain essential enzymes, and they possess a multi-lobed nucleus. Neutrophils have a short life span in the circulation (estimated at 8-20 h), but infections may be prolong their life span up to several days, especially when the neutrophils migrate from the blood circulation into infected tissues (3).

1.1.1 Neutrophil production and maturation

Hematopoietic stem cells in the bone marrow continuously-generate white blood cells (leukocytes), including neutrophils, and release them into the blood circulation. The rate of neutrophil production is approximately 1-2 x 10^{11} cells per day in a normal adult human and this process is essentially regulated by G-CSF in response to IL-17A

synthesized by T cells. IL-17A production is itself regulated by IL-23 that is released from macrophages and dendritic cells (2). During inflammation, the number of neutrophils in the circulation is increased, but over time, the cells become apoptotic, and are removed by macrophages and dendritic cells, or other phagocytic cells. This safe removal process down-regulates IL-23 synthesis, and then decreases G-CSF production. For this reason, neutrophil production is generally controlled by the removal of apoptotic neutrophils in tissues (4).

Neutrophil maturation (terminal granulocytopoiesis) is regulated by the expression of transcription factors, especially PU.1 and CCAAT/enhancer-binding protein (C/EBP) α-ζ (4). The balance between PU.1 and C/EBPα activity determines the direction of differentiation towards either the granulocytic or monocytic lineages. For example, high expression of PU.1 results in monocytic differentiation, whilst the expression of C/EBPα is required for granulocytopoiesis. Other C/EBP transcription factors are also involved during neutrophil maturation e.g. C/EBPβ is essential for the production of mature neutrophils in response to fungal infections (4).

The formation of granules occurs during the process of neutrophil maturation. There are three different major types of granules, based on their protein content and the control of their mobilization. Primary (azurophilic or peroxidase-positive) granules contain myeloperoxidase

(MPO), defensins and azurocidin. Secondary (specific or peroxidase-negative) granules contain lactoferrin, ficolin 1, cysteine-rich secretory protein 3 (CRISP3) and gelatinase. Tertiary (gelatinase) granules contain matrix metalloproteinase 9 (MMP9) or gelatinase B. These granules, containing various proteins and enzymes, have different functions. For example, primary granules mainly store proteins and peptides for killing and digestion of ingested microorganism, whereas secondary granules enrich membranes for proteins, such as receptors and oxidase components while tertiary granules deliver proteases for digesting basement membranes, hence allowing neutrophil transmigration (5).

In addition, neutrophils also possess secretory vesicles, containing a number of proteins and receptors involved in neutrophil adhesion, migration and chemotaxis. These vesicles can be transported to the cell surface and they either release their proteins into phagosomes or incorporate their membranes into the surface membrane and so their proteins become expressed on the surface membrane, for example, in response to cell rolling and adhesion (e.g. β_2-integrins) (4, 6). However, the full characterization of the protein content of secretory vesicles is still unknown.

After the processes of neutrophil production and maturation, neutrophils are released into the circulation, but mature cells can also be found in the bone marrow, lung, spleen and liver where they may exist as

reservoirs of mature cells (7). It is believed that these reservoirs (marginal pool) can rapidly deploy mature neutrophils to the site of infection or inflammation.

1.1.2 Neutrophil response to infections or inflammation

Neutrophils are recruited to sites of bacterial, viral and fungal infections to rapidly attack and remove these pathogens. Their numbers can dramatically increase in cases of infection, and they may move effectively into tissues in order to respond to cytokines and chemo-attractive factors generated during infections or inflammation (8). The role of neutrophils during infection or inflammation is regulated at several steps (4). First, many chemotactic factors are produced at the site of infection, including those released by bacteria, injured host cells, microbes engulfed by macrophages or complement activation. These chemoattractants (e.g. TNF-α, GM-CSF, IL-8 and IFN-γ) can prime neutrophils (neutrophil priming) to express increased number and/or affinity of membrane receptors, to release cytokines, and trigger the release of secretory granules containing pre-formed receptors to the surface membrane, thereby preparing the cell for the process of cell migration (8). They also affect the endothelial cells lining the blood vessels, which themselves generate cytokines, such as TNF-α, IL-1β. These cytokines are recognized by membrane receptors on the surface of circulating neutrophils and promote neutrophils to roll onto and attach to

the endothelial cells (neutrophil rolling and adhesion). These processes are regulated by interactions between receptors and ligands on the surfaces of neutrophils and endothelial cells. For example, activated endothelial cells express P-selectin and E-selectin which bind to the Selectin ligands on circulating neutrophils, such as PSGL-1, L-selectin, ESL-1 or CD44 (9). These neutrophils then roll slowly until they stop and attach to the activated endothelial cells. The inflammed tissue produces and transports chemoattractant cytokines or chemokines, such as IL-8, to the luminal surface of endothelial cells. The rolling neutrophils express chemokine receptors that bind with these chemokines and the expression of leukocyte adhesion molecules (β_2-integrins, LFA-1 and Mac-1) is increased. These adhesion molecules interact with ICAM-1 and ICAM-2 on endothelial cells and mediate strong adhesion of neutrophils to endothelial cells (10). The endothelial tight junctions are opened by molecules expressed by neutrophils, such as PECAM-1, β_2-integrins LFA-1 and Mac-1 (4). Neutrophils then transmigrate through the gaps by diapedesis (neutrophil transmigration), a process that changes neutrophil morphology during movement into the inflamed tissues by the process of chemotaxis. There are two possible methods of neutrophil transmigration: paracellular (between endothelial cells) and transcellular (through an endothelial cell). Neutrophils usually migrate paracellularly because this way is more efficient and less time-consuming (Figure 1.1).

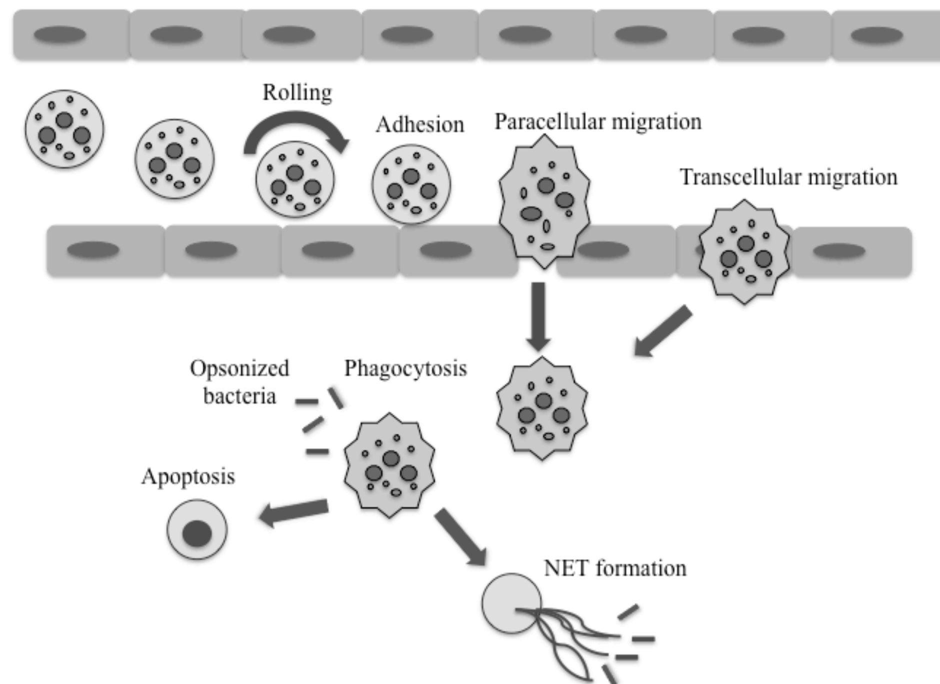


Figure 1.1 The sequential steps of neutrophil responses to infection or inflammation. After neutrophils are primed by chemoattractants, they start rolling on and then adhering to endothelial cells by the interaction between membrane receptors and corresponding ligands. Neutrophils then transmigrate through endothelial cells either paracellularly or transcellularly. These process result in changes to neutrophil morphology and mobilize neutrophils toward the site of infection or inflammation. The neutrophils phagocytose microorganisms and then undergo either apoptosis or NET formation (see sections 1.1.3 and 1.1.4).

By the time the neutrophils reach the site of infection, the microbes may be opsonised by complement fragments, immunoglobulins or acute-phase reactant proteins in serum. Neutrophils can detect the opsonised microbes and ingest them by opsono-phagocytosis (4).

1.1.3 Neutrophil phagocytosis and respiratory burst

Phagocytosis is an important process for neutrophils to eliminate invasive pathogens and also to activate other innate immune responses. Numerous molecules and cell surface receptors have been identified that are required for the process of phagocytosis and activation of microbicidal mechanisms e.g. CD16 (FcγRIII), CD32 (FcγRII), CD64 (FcγRI), ICAM-1, integrins, CD11b, CD62-L and complement receptors (11). Activation of these molecules and receptors can stimulate the neutrophil cell membrane to extend pseudopodia and engulf microorganisms. For example, opsonins, activated complement proteins (C3) and IgG antibodies in serum bind to invasive pathogens such as *Staphylococcus aureus*, *Escherichia coli*. The opsonised pathogens can then bind to neutrophil opsonic receptors (the number and affinity of these receptors on the neutrophil surface can be increased) and these membrane-bound glycoprotein complexes trigger neutrophil phagocytosis (12, 13).

One of the most important neutrophil receptors recognizing bacterial peptides is the N-formyl-methionyl-leucyl-phenylalanine

(fMLP) receptor. fMLP is a very potent bacterial-derived peptide and it binds to FRP receptors. Up-regulation of complement receptors CR1 and CR3 (CD11b/CD18 or Mac-1) additionally occurs during neutrophil priming and these complement receptors bind with the complement factors (C3bi-opsonised pathogens, C3a and C5a) to initiate neutrophil adhesion, chemotaxis and phagocytosis (14, 15). Furthermore, bacteria may be opsonised by immunoglobulins such that the exposed Fc domains are recognized by immunoglobulin receptors on neutrophils, namely Fcγ receptors. Neutrophils express several types of Fcγ receptors on their surface such as FcγRII (CD32) and FcγRIII (CD16) on both peripheral blood and inflammatory neutrophils, whilst FcγRI (CD64) is only expressed on neutrophils primed by cytokines such as GM-CSF and interferon-γ (IFN-γ) (16). FcγRI and FcγRII binds to monomeric IgG, FcγRI has a high affinity whereas FcγRII has a low affinity but its affinity is greater when IgG dimerization or aggregation occurs (17). There are 2 forms of FcγRIII (FcγRIII -a and -b), which bind to IgG with low affinity and merely FcγRIIIb is expressed on neutrophils (18). FcγRIIIb is expressed abundantly with 100,000-200,000 receptors per cell and it is shed rapidly by cytokine stimulation such as GM-CSF, and the internal stores of this receptor will maintain cell surface expression (19). These receptors facilitate phagocytosis (20).

During the process of phagocytosis, the neutrophil plasma membrane first flows around and engulfs the bacteria to form a phagosome. The neutrophil morphology usually changes from spherical to amoeboid shape following the formation of pseudopodia, in order to engulf those organisms. Next, neutrophil granules are released into the phagocytic vacuoles to form a phagolysosome. These granules contain anti-microbial peptides, proteins and enzymes such as myeloperoxidase, hydrolases, elastase and proteinases to kill and digest the invasive pathogens (4, 21). In addition, neutrophils can also produce cytotoxic molecules, such as reactive oxygen metabolites and can also release from their granules a range of proteins with cytotoxic properties. They may also generate neutrophil extracellular traps (NETs) which entrap and kill extracellular pathogens that cannot be phagocytosed (2).

Reactive oxygen species are generated by the membrane-bound reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (22). Once the oxidase is activated, it accepts electrons from NADPH to reduce O_2 and generate the superoxide anion (O_2^-) as a primary oxidant, during a process known as the respiratory burst. NADPH is generated from $NADP^+$ via the hexose monophosphate (HMP) shunt. O_2^- is relatively unstable and rapidly dismutates into secondary oxidants; hydrogen peroxide (H_2O_2) and hypochlorous acid (HOCl). Myeloperoxidase utilizes H_2O_2 to generate HOCl, which is highly

bacteriocidal (Figure 1.2). Degranulation of cytotoxic enzymes and activation of the NADPH oxidase enzyme occur concurrently and these combine to kill and digest the microbes. Finally, when the infection is cleared, neutrophils will enter apoptosis (programmed cell death), which eventually leads to their clearance by phagocytosis via tissue macrophages.

NETs (neutrophil extracellular traps) are web-like structures, that have recently been shown to trap invasive pathogens such as bacteria, viruses, fungi and protozoa to aid the killing and clearance of extracellular microorganisms (2, 4, 11). NET structures are composed of nuclear components (e.g. large strands of de-condensed DNA, nuclear histones), proteins from neutrophil granules and several anti-microbial enzymes (23). The efficiency of NETs as a killing mechanism has not been clearly documented and their role *in vivo* is still controversial (24).

1.1.4 Neutrophil apoptosis (programmed cell death)

Human neutrophils can release a range of toxic molecules to kill invading microorganisms, but many of these molecules can also indiscriminately attack host tissues. Therefore, there must be a safe mechanism to remove neutrophils when they have completed their role in infections. When neutrophils complete their lifespan or their role in infection, they become apoptotic. This allows for the safe removal of cells and limits the release of destructive neutrophil products into

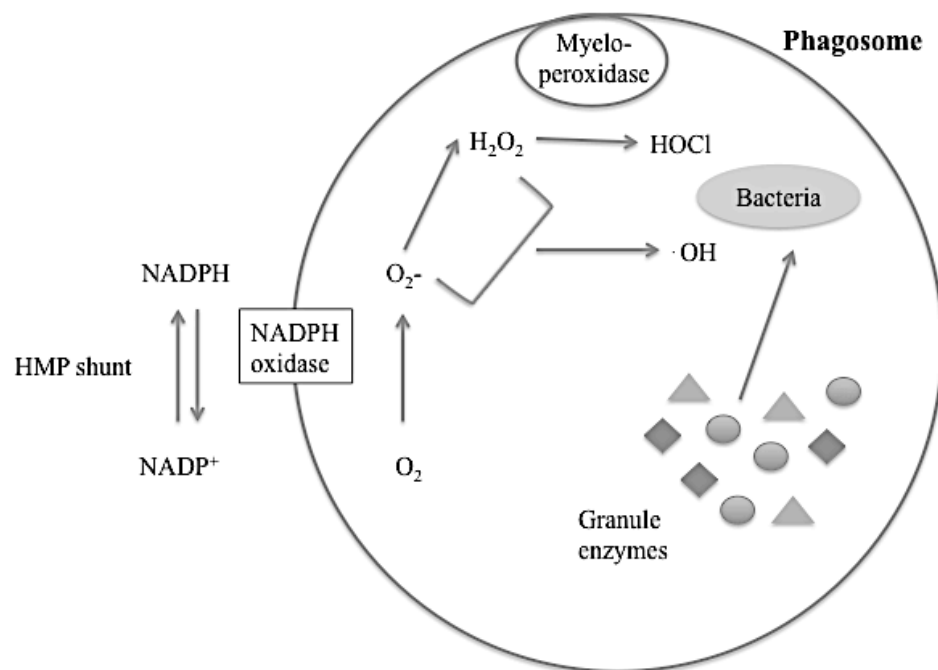


Figure 1.2 Reactive oxygen species production in neutrophils: the NADPH oxidase receives electrons from NADPH to reduce O_2 to the superoxide anion (O_2^-), which then dismutates into hydrogen peroxide (H_2O_2): $^{\cdot}OH$ formation may also occur. Myeloperoxidase reacts with H_2O_2 and Cl^- to produce hypochlorous acid (HOCl). The combination of HOCl, $^{\cdot}OH$ and granule enzymes provides an environment within the phagosome that is able to destroy the invasive pathogens (e.g. bacteria).

surrounding tissues that would occur via if neutrophils died by the process of necrosis (25). Apoptotic cells undergo characteristic morphological changes: they round up, start blebbing, their mitochondrial membrane potential is dissipated, expression of many cell surface markers is altered (e.g. phosphatidylserine residues are expressed on the cell surface) (Figure 1.3) (26), the nucleus breaks down and, if not phagocytosed by macrophages, the cell will undergo secondary necrosis.

Two major pathways control neutrophil apoptosis (26–28). The *extrinsic pathway* is initiated by extracellular proteins, which bind to membrane receptors, such as death receptors, to activate proteins and proteolytic cascades inside the cell. The *intrinsic pathway* is controlled by the mitochondria and the Bcl-2 family members (Figure 1.4) (29, 30).

1.1.5 The extrinsic pathway and death receptors

The extrinsic apoptotic pathway occurs after engagement of cell membrane receptors or death receptors. This activates the caspase cascade but can be linked to the intrinsic apoptotic pathway that is regulated by pro-apoptotic Bcl-2 family members, via Bid, suggesting a level of crosstalk between these two pathways. There are two main factors regulating apoptosis: pro-apoptotic factors that induce cell death (e.g. FasL, TRAIL and high dose TNF-α) and anti-apoptotic factors that prevent cell death (e.g. interferons, G-CSF, GM-CSF and low dose TNF-α) (31).

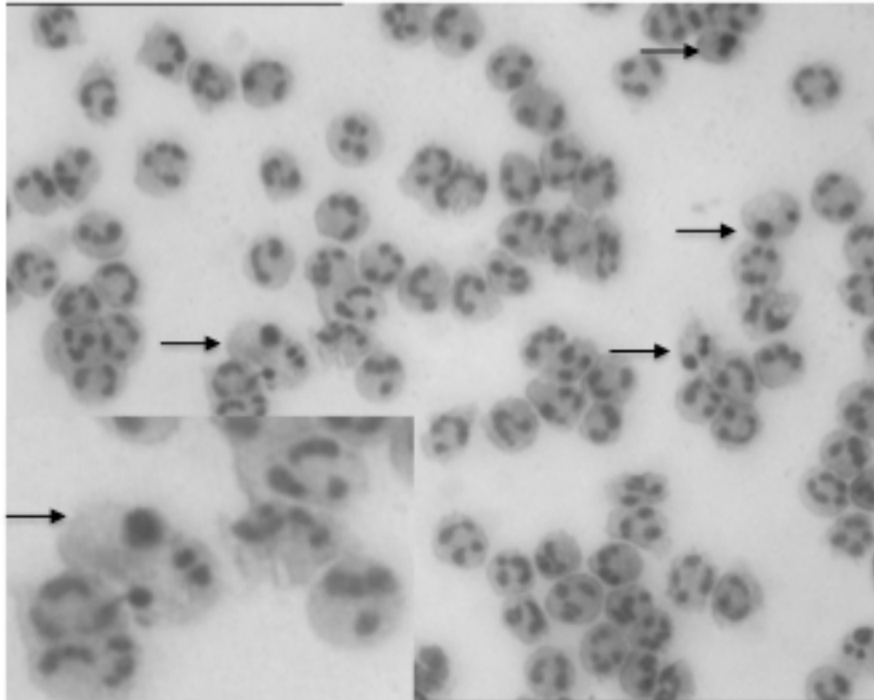


Figure 1.3 Morphological differences between healthy neutrophils and apoptotic neutrophils. Healthy neutrophils possess a multi-lobed nucleus and granular cytoplasm, whilst apoptotic neutrophils (arrowed) have a condensed nucleus and cytoplasm, and are smaller and rounder.

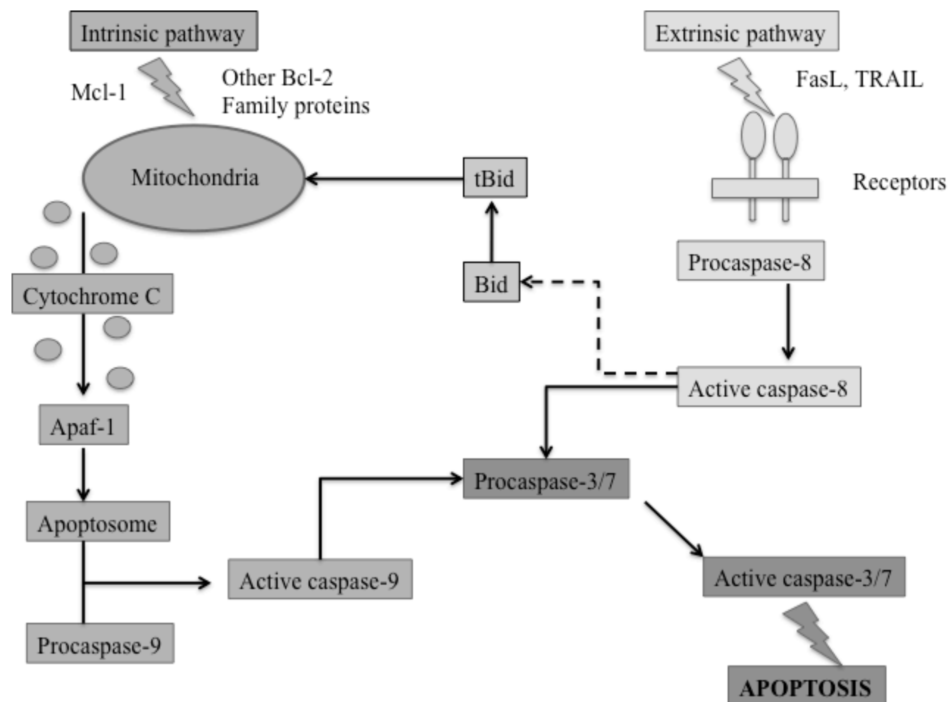


Figure 1.4 Summary of the pathways that control neutrophil apoptosis: The *extrinsic* pathway involves receptors e.g. death receptors (such as Fas, TRAIL) and is activated via caspase-8. The extrinsic pathway usually may be linked to the intrinsic pathway via Bid. The *intrinsic* pathway involves mitochondria, Bcl-2 family proteins and Mcl-1. When this pathway is activated, cytochrome c is released which then combines with Apaf-1, ATP and pro-caspase-9 to form an apoptosome, and activates caspase-9. Both pathways finally activate caspases -3, -7 and induce neutrophil apoptosis (26).

The two most characterized neutrophil death receptors are TNFR1 and Fas (32). TNFR1 binds TNF-α, whilst Fas receptor binds Fas ligand (FasL), and both receptors sequentially activate the caspase cascade. A group of adaptor proteins are recruited during the engagement of death receptors, such as TNF-associated death domain-containing proteins (TRADD) and Fas-associated death domain-containing protein (FADD). These adaptor proteins facilitate interaction with other proteins, including pro-apoptotic proteins. For example, the clustering of Fas, FADD and pro-caspase-8 (the death-inducing signaling complex; DISC) can cleave and activate pro-caspase-8, and initiate the caspase cascade (33). The clustering of TRADD with TNFR1 usually includes FADD, pro-caspase-8 and several adaptor proteins to induce and trigger apoptosis (34).

1.1.6 The intrinsic pathway, mitochondria and Bcl-2 family members

The intrinsic apoptotic pathway is initiated by mitochondria and regulated by Bcl-2 family members. The proteins of the Bcl-2 family comprise both pro-apoptotic (e.g. Bid, Bax, Bim, Bak, Bik) and anti-apoptotic proteins (e.g. Mcl-1, XIAP, Bcl-X_L) (35). They are classified into 3 groups; the anti-apoptotic (prosurvival), pro-apoptotic and the BH3-only proteins (36). The anti-apoptotic Bcl-2 family members are crucial regulators of mitochondrial membrane integrity (mainly of the intrinsic apoptotic pathway) and include Bcl-2, Bcl-X_L, Bcl-w, A1, Mcl-1, Boo/DIVA/Bcl2-L-10 and Bcl-B. The proteins contain three or four

regions of Bcl-2 homology (BH regions) (Figure 1.5). Their function is mainly to protect against the release of cytochrome c from mitochondria into the cytoplasm, and to keep the mitochondrial membranes intact. Another group of proteins is the pro-apoptotic proteins, such as Bax and Bak, which have three BH domains (BH1, 2 and 3). These proteins move to the outer mitochondrial membrane and promote the release of cytochrome c from mitochondria, which then induces caspase activation via the apoptosome. The last group in the family is the BH3-only proteins, which are essential initiators of the mitochondrial apoptotic pathway and are able to bind to Bcl-2-like prosurvival proteins and initiate cell apoptosis. There are at least 10 BH3-only proteins in most cells, such as Bid, Bim, Bmf, Puma, etc. which are required for programmed cell death.

One of the most important Bcl-2 family members regulating intrinsic neutrophil apoptosis is Myeloid Cell Leukemia-1 (Mcl-1). Mcl-1 is an anti-apoptotic that was first described as an early induction gene expressed during the differentiation of the ML-1 myeloid cell line along the monocytic lineage (37). Up-regulation of expression of this protein prevents intrinsic neutrophil apoptosis (38). It can be expressed as short and long forms after the processes of transcription and alternative splicing, and it shares some homology with other members of the Bcl-2 family (Figure 1.5). Differences to the other family members (Bcl-2, Bcl-

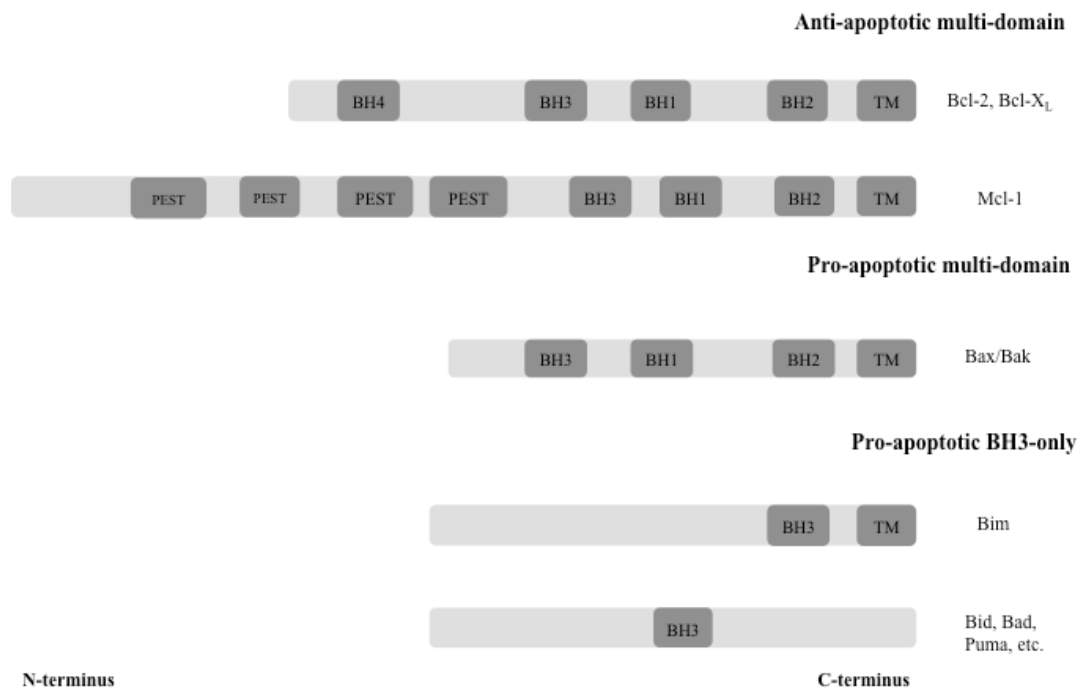


Figure 1.5 Bcl-2 family proteins. Most proteins have a transmembrane domain (TM) helping them to bind to intracellular membranes. BH=Bcl-2 homology domains (1-4), PEST=Proline/Glutamic acid/Serine/Threonine residues (39).

X), include: (a) it is a larger protein (42 kDa compared 26 kDa to for Bcl-2); (b) its N-terminal region contains PEST sequences that are targets for phosphorylation and ubiquitination; (c) Mcl-1 does not contain a BH4 domain, which suggests that it interacts with different proteins compared with Bcl-2 and Bcl-X, which both have this domain (39).

Neutrophils express the anti-apoptotic protein Mcl-1 and the level of expression of this protein correlates with neutrophil survival (38). Mcl-1 has a very short half-life of approximately 2 h in neutrophils because of the PEST domains (proline, glutamic acid, serine and threonine residues) that target it for rapid turnover by the proteasome, which is a very large protein complex that degrades unnecessary or damaged proteins by proteolysis (39). This is in contrast to the function of pro-apoptotic Bcl-2 homologues such as Bax, Bid and Bim that induce neutrophil apoptosis (35), which are more stable. Therefore, a balance between anti- and pro-apoptotic proteins controls neutrophil fate. If there is an excess level of pro-apoptotic proteins or an attenuation of anti-apoptotic expression, including Mcl-1 protein, neutrophils lose their outer mitochondrial membrane protection and become apoptotic (40).

Mcl-1 expression can be enhanced by some factors (e.g. GM-CSF, IL-1β, sodium butyrate and lipopolysaccharide), that induce neutrophil survival (41). For example, GM-CSF is a cytokine that functions as a growth factor for white blood cells, especially granulocytes

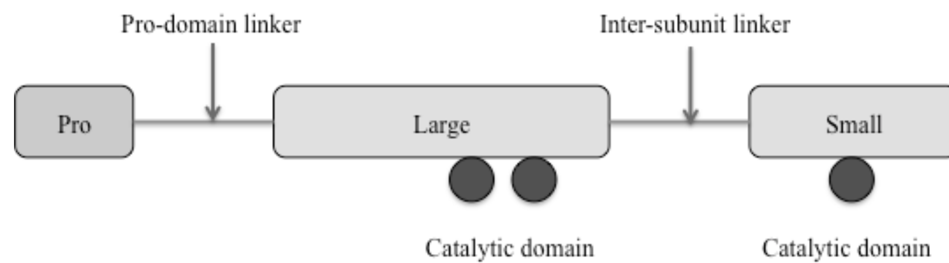
and monocytes. GM-CSF enhances neutrophil survival by changing Mcl-1 turnover rate, enhancing Mcl-1 stability and also up-regulating mRNA levels of Mcl-1.

1.1.7 Caspase cascade

Caspases are an important family of intracellular enzymes involved in the regulation of programmed cell death (apoptosis). Their name comes from their description: cysteine-dependent aspartate-specific protease. The pro-forms of caspases are single-chain proteins and comprise an N-terminal pro-domain preceding the conserved catalytic domains, which are then cleaved into large and small portions during activation to form active dimeric molecules (Figure 1.6) (42).

The classification of human caspases is based on their function and location within signaling pathways, and they are categorized into two groups; apoptotic and pro-inflammatory. This classification is useful, although there are some overlaps between the groups. The apoptotic subgroup contains two subtypes of caspases that are (a) the initiator (apical) and (b) the executioner (effector, downstream) caspases. The initiators (caspase -8, -9 and -10) activate the executioners (caspase -3, -6 and -7) to induce the apoptotic cascade. The initiator caspases are activated by homodimerization, which is facilitated by adaptor proteins, and are divided into 2 sub-types: caspases involved in the extrinsic (caspase -8, -10) or intrinsic (caspase -9) apoptotic pathways (Figure 1.4).

A.



B.

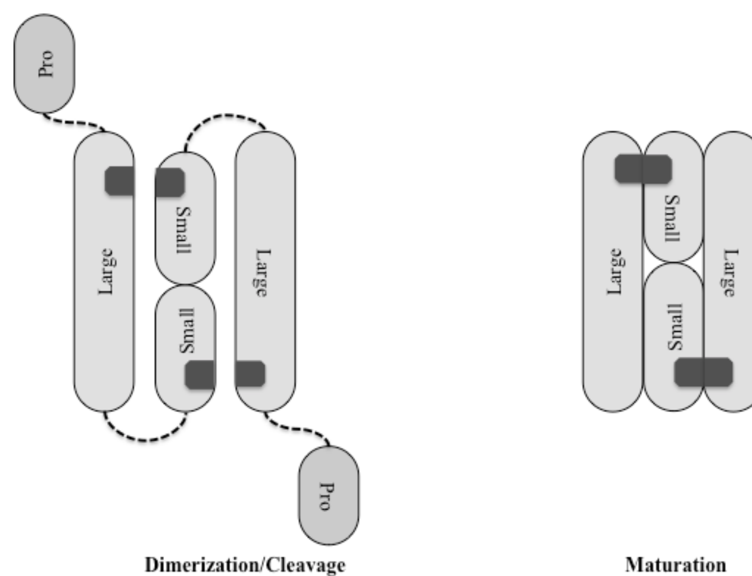


Figure 1.6 (A) The N-terminal pro-domain precedes the catalytic domain, while the large and small portions are covalently-linked subunits. **(B)** Caspase activation occurs by dimerization or cleavage of inter-subunit and pro-domain linkers of pro-caspases, followed by maturation as an active caspase tetramer with constituted active sites.

Caspase activation occurs by proteolytic maturation or auto-proteolytic cleavage. This activation mechanism is associated with trimming/removal of the pro-domain and the linkers of caspases, and the formation of dimers (particularly in initiator caspases), which stabilizes the caspase catalytic domain and activates downstream signaling (43).

There are three feedback strategies to prevent caspase over-activity; caspase inhibition, caspase degradation and decoy inhibitors. Caspase inhibitors in human cells, such as XIAP (X-linked inhibitor of apoptotic protein) can effectively and selectively inhibit caspases-9, -3 and -7. Many viruses encode proteins that circumvent the host apoptotic response, and so viruses can survive and proliferate in the host, by generating proteins that are caspase inhibitors; for example, CrmA (cytokine response modifier A) in cowpox virus or p35/p49 proteins in baculovirus (44, 45).

1.1.8 Neutrophils and diseases

The primary role of neutrophils is in the control of infections or inflammation. They are major players in the elimination of invasive pathogens, especially intracellular and extracellular bacteria (e.g. *Staphylococcus spp.*, group A *Streptococcus* and *Mycobacterium tuberculosis*) and can use several mechanisms to achieve this. Neutrophil phagocytosis, release of granule enzymes containing anti-microbial peptides, reactive oxygen species production and NETs formation

essentially occur during the process of bacterial elimination, as mentioned previously (2, 4). It has been reported that impairment of these functions and/or a decreased number of neutrophils can cause severe immunodeficiency in humans (46). Thus, neutrophil recruitment and appropriate activation of their killing mechanisms at the site of infection are crucial.

However, neutrophils also play damaging roles in the pathophysiology of many types of human disease, including chronic inflammation and autoimmune diseases (11). Examples of the role of neutrophils contributing to the exacerbation of chronic inflammation are rheumatoid arthritis (RA), chronic obstructive pulmonary disease (COPD) and Behçet's Disease. Neutrophils are inappropriately activated in these conditions to release cytotoxic granules, cytokines and chemokines, leading to persistent inflammation in the joints in RA, the lungs in COPD, and many organ systems in Behçet's disease (8, 47, 48). In autoimmune diseases, one example of neutrophil contribution to disease pathology is systemic lupus erythematosus (SLE). Abnormalities in neutrophil functions have been reported in SLE patients, such as increased neutrophil aggregation and apoptosis, ineffective neutrophil phagocytosis, a decreased cytokine and chemokine response and the presence of neutrophil autoantibodies (49). Interestingly, one subgroup of SLE, patients of which often present with severe clinical manifestations,

is juvenile SLE (JSLE). JSLE patients usually have multi-organ involvement at presentation, especially renal, hematological and neurological manifestations, and these clinical features usually indicate the worst prognosis. There are increasing number of reports of a correlation between abnormal neutrophil function and disease pathogenesis, including disease activity and severity in JSLE patients (50–52).

1.2 Juvenile Systemic Lupus Erythematosus (JSLE)

Systemic Lupus Erythematosus (SLE) is one of the most important autoimmune diseases and it has a varied clinical spectrum and abnormal immune regulation (53). Although there are numerous groups working on SLE and JSLE worldwide, the etiology remains unknown. Some of the current studies are associated with the search for particular genetic backgrounds, but environmental factors also appear to contribute to immune-pathogenesis. These genetic and environmental factors may lead to altered innate and adaptive immune responses in patients (54).

1.2.1 Epidemiology

Most SLE patients are adults, with a prevalence 40 to 50 per 100,000 people globally, particularly in childbearing aged females (16-55 years old); however, 15-20% of patients present in childhood (55). The

incidence of childhood SLE or JSLE across the world varies between 0.3-0.9 per 100,000 cases per year, and its prevalence rate is approximately 4-250 per 100,000. Asian, Hispanic and African-American children have a higher incidence of disease (56, 57). JSLE is commonly-diagnosed up to 16 years of age (age range from 14 to 20 years in different studies). The female to male ratio changes from 4:3 during the first decade of life to 4:1 during the second decade (58).

1.2.2 Diagnosis

Patients diagnosed with JSLE have a broad range of organ system involvement and the clinical symptoms, including immunological findings, are similar in both adults and children. The diagnosis of JSLE is based upon any 4 out of 11 criteria from The American College of Rheumatology (1997 revision) and the criteria are used for both adults and children (Table 1.1) (59).

1.2.3 Clinical Manifestations

The clinical signs and symptoms of JSLE range from mild, chronic and slowly progressive disease with exacerbations and remissions, to a severe, acute and rapidly-aggressive disease (60). Most JSLE patients initially present with symptoms such as low-grade fever, malaise, poor appetite and weight loss (55). Notably, JSLE is clinically more severe than adult SLE and most patients often present with major

Table 1.1 Classification criteria for JSLE or SLE from the American College of Rheumatology

- Malar (butterfly) rash
- Discoid rash
- Photosensitivity
- Oral or nasal mucocutaneous ulceration
- Nonerosive arthritis
- Nephritis
    - Proteinuria >0.5 g/day
    - Cellular casts
- Encephalopathy
    - Seizures
    - Psychosis
- Pleuritis or pericarditis
- Cytopenia
- Positive immunoserology
    - Antibodies to dsDNA
    - Antibodies to Sm nuclear antigen
    - Positive finding of antiphospholipid antibodies based on:
    - (a) IgG or IgM anticardiolipin antibodies, or
    - (b) Lupus anti-coagulant, or
    - (c) False-positive serologic test for syphilis for at least 6 months, confirmed by *Treponema pallidum* immobilization or fluorescent treponemal antibody absorption test
- Positive antinuclear antibody test

(*Hochberg M. Arthritis Rheum 1997; 40:1725)*

organ or systemic involvement, particularly renal, neuropsychiatric and hematological symptoms (61). Table 1.2 demonstrates differences in the frequency of clinical manifestations in JSLE and adult SLE.

Table 1.2 Differences in the frequency of clinical characteristics in JSLE and adult SLE according to the ACR classification criteria (55, 61–64).

Clinical features	JSLE	Adult SLE
Malar rash	44-85 %	40-52%
Discoid rash	< 10%	20%
Photosensitivity	35-50%	35-63%
Oral ulcers	20-30%	20-30%
Non-erosive arthritis	60-70%	80-100%
Renal disease	75-80%	35-67%
CNS involvement	20-45%	10-25%
Pulmonary involvement	15-40%	20-40%
Cardiac involvement	10-15%	25-30%
Hematological involvement	50-100%	42-72%

Renal disease is more common in JSLE compared to adult SLE (55, 61). It has been estimated that approximately 75-80% JSLE patients exhibit nephritis either at presentation, or some time over the course of the follow-up period. Nephritis in JSLE is usually more severe than in adults, and it represents the most dominant feature of prognosis and

mortality in children (65). The most common manifestations of renal disease in JSLE are microscopic hematuria (79%), proteinuria (60-70%) and hypertension (25-40%) (66). Many reports have shown that most JSLE patients with severe and aggressive renal disease required higher doses of corticosteroids or immunosuppressive drugs, compared with adult SLE. Moreover, studies also reported that these JSLE patients showed a higher risk of acute renal failure and a requirement for renal dialysis (67).

Neuropsychiatric involvement is not uncommon in JSLE, with approximately 20-45% incidence, and up to 65% of patients has been reported in some case series (68, 69). These clinical manifestations vary from mild cognitive dysfunction, such as memory or attention deficit, to severe neurological and psychiatric symptoms, including seizures and psychosis. Neuropsychiatric disorder usually occurs within the first year after disease onset, and psychosis with visual hallucination is a hallmark of this disorder in JSLE patients. Severe neuropsychiatric disorder is a high risk factor of poor outcome in JSLE and the patients usually require the most aggressive therapies and immunosuppressive drugs in order to suppress symptoms (63).

Hematologic features are more common in JSLE compared with adult SLE (50-100% VS 42-72%). More than 50% of JSLE patients present with cytopenia, particularly leukopenia as the most common hematological involvement (60). Anemia is also common and can be

presented as Coombs-positive hemolytic anemia, anemia of chronic disease or iron-deficiency anemia (61). Thrombocytopenia caused by autoimmune-mediated mechanisms is also observed in JSLE. The symptoms range from minor bleeding to life-threatening blood loss (60). Hematological involvement in JSLE usually predicts a high mortality rate and strongly correlates to disease activity and severity.

Other clinical signs and symptoms, which are non-specific for JSLE and do not form part of the ACR classification criteria, can sometimes be detected at presentation (55, 60, 61), and these are summarised in Table 1.3.

1.2.4 Pathophysiology

In spite of the fact that there have been many studies designed to understand the pathogenesis of SLE, the actual cause of the disease is still unknown. The pathophysiology of SLE is characterized by the presence of autoantibodies directed against nuclear antigens and abnormal immune regulation, and these clinical markers are similar in both adult SLE and JSLE (70). Autoantigen and autoantibody complexes are capable of damaging human tissues and organs via immune cell activation. It has been proposed that the triggers for the formation of these immune complexes are multifactorial, including genetic, environmental and immunological factors (54).

Table 1.3 Other clinical findings in JSLE

Constitutional	Low grade fever, poor appetite, weight loss
Cutaneous	Non-scaring alopecia, maculopapular lupus rash, cutaneous vasculitis, Raynaud phenomenon, livedo reticularis
Musculoskeletal	Arthralgia, myalgia, myositis, osteopenia
Cardiopulmonary	Myocarditis, non-infective (Libmann-Sack) endocarditis, accelerated atherosclerosis, interstitial pneumonitis, pulmonary hemorrhage, pulmonary hypertension
Gastrointestinal	Abdominal vessel vasculitis, pancreatitis, hepatomegaly, splenomegaly
Neuropsychiatric	Headache, cognitive dysfunction, depression, anxiety, movement disorder (chorea), neuropathy
Renal	Nephrotic syndrome, tubulointerstitial nephritis, hypertension

A genetic predisposition associated with the development of SLE has been shown a number of ways. The combination effects of numerous genes belonging to different pathways that may contribute to autoimmunity include IRF5, STAT4, IRAK1 and TLR8 (54). Interestingly, a recent large-scale replication study has found that single-nucleotide polymorphisms (SNPs) of some candidate genes e.g. TNIP1,

PRDM1, JAZF1, UHRF1BP1 and IL10, significantly increase the risk of SLE (71). However, the identified loci are found only 15% of SLE patients and their relatives, and the importance of each gene in the development of the disease and their contribution to phenotype and disease severity, is unclear. Therefore, the relationship between candidate genes and the pathophysiology needs to be further explored in future studies.

Abnormal immune regulation has been widely described in many studies and T and B cell lymphocytes play important roles in the abnormal immune response in patient with SLE (54), although most patients present with lymphopenia. It has been demonstrated that T cells from SLE patients activate B cells to produce autoantibodies directed against a variety of autoantigens (72).

Enhanced activation of T cell signaling pathways occurs when T cell receptors (TCRs) are engaged by factors in SLE sera, such as cytokines, autoantibodies and immune complexes (54, 72). Consequently, activation of transcription factors and gene expression is dysregulated, particularly in CD4 T cells; this results in up-regulation of nuclear factor of activated T cells (NFAT) and CRE-modulator (CREM), and down-regulation of CRE-binding (CREB) and activator protein 1 (AP-1). Dysregulation of the function of these genes results in an increased number of SLE CD4 T cells and increased levels of pro-inflammatory cytokines, such as IL-17 (73–75). The abnormally high levels of IL-17

have been reported to contribute to T cell recruitment to damaged organs, and B cell activation and proliferation (76). Moreover, regulatory T cells, which maintain tolerance to self-antigens and suppress the activation of abnormal responses such as self-reactivity, are low and functionally abnormal in SLE patients (77). Therefore, these lupus T cells are capable of activating B cells to become hyperactive.

Hyperactivity of B cells is a prominent finding in SLE patients as they produce an array of autoantibodies against autoantigens, such as antinuclear antibodies (ANA) (54, 70). Analysis of the B cell subpopulation demonstrated an increased number of $CD27^{high}$ plasma cells that was significantly correlated with levels of serum autoantibodies, autoantibody production and disease activity in lupus patients (78). Furthermore, it has been reported that the expression of genes involved in inhibitory intracellular signaling of B cells such as Lyn, complement receptor 2 (CR2), surface receptor CD22 and FcγRIIB, is decreased, suggesting defects in inhibitory processes, leading to lupus B cell hyperactivity (79–81).

Apart from T and B lymphocyte over-activity, over-stimulation and abnormal functions of dendritic cells have been reported in lupus patients (82). Dendritic cell over-stimulation can be induced by autoantigens released from deregulated apoptotic cells that may accumulate either via defective clearance by macrophages or other phagocytic cells, or by increases in the rate of cell apoptosis. Following

autoantigen engulfment and processing, these dendritic cells activate T and B cells to produce a range of autoantibodies and form immune complexes. Moreover, dendritic cells also secrete large amounts of type I interferon particularly IFN-α, which has a major role in the pathogenesis of SLE, increasing the expression of autoantigens (e.g. SSA/Ro) and promoting dendritic cell maturation (83). Figure 1.7 summaries one hypothesis for the pathogenesis of SLE based on abnormal phagocytosis.

Environmental factors have been reported as another contributing factor in the pathogenesis of SLE. Exposure to UV and smoking are thought to be triggering factors in lupus patients, and they are associated with disease activity. Viral infections, such as parvovirus B19, cytomegalovirus (CMV) and Epstein-Barr virus (EBV) are also common in lupus patients, and it has been found that some viral infections, particularly EBV, may precede the development of autoantibody production (e.g. anti-SSA/Ro) via cross-reactivity of self-antigens and EBV protein (EBNA-1) (84, 85). Other environmental factors may include sex hormones (e.g. estrogen and prolactin), and drug-induced lupus has been reported (e.g. hydralazine and procainamide) (86, 87).

1.2.5 Laboratory findings

Routine laboratory tests during active disease episodes often show anemia, leukopenia (lymphopenia and thrombocytopenia), and elevated inflammatory markers, such as erythrocyte sedimentation rate (ESR) and

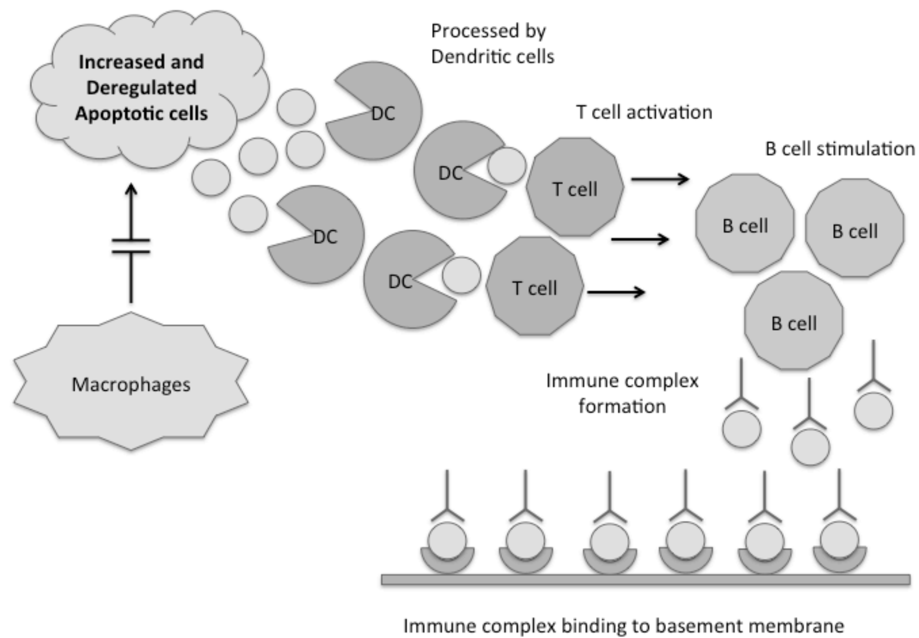


Figure 1.7 A hypothesis to explain the pathogenesis of SLE. Increased and deregulated apoptotic cells may arise from defective clearance by macrophages, leading to the release of autoantigens. These autoantigens are phagocytosed and processed by dendritic cells (DC), and then presented to T and B cells, which then induces autoantibody production. Consequently, immune complexes are formed and the complexes eventually bind to basement membrane to initiate abnormal autoimmune responses leading to tissue and organ damage (72).

C-reactive protein (CRP). Urinary analysis also reveals hematuria and proteinuria with cellular casts. These laboratory results together with the clinical signs and symptoms, are usually sufficient to make a diagnosis of SLE (55, 60). However, other laboratory tests are usually required to confirm the diagnosis, particularly autoantibody profiles which are a hallmark of SLE; for example, ANA, anti-dsDNA and anti-Sm autoantibodies.

Antinuclear antibody (ANA) is present in more than 95% of JSLE patients (55, 60, 88). The serum titer of this autoantibody is often higher than 1:80 in children, but there is no difference in ANA levels between JSLE and adult SLE patients (89). A positive ANA titer may also be present in other non-specific conditions, such as infections, following certain medications, or other autoimmune diseases (e.g. Juvenile Rheumatoid Arthritis) (55). Thus, the presence of ANA alone is not appropriate to make a diagnosis and other specific autoantibodies, such as anti-dsDNA and anti-Sm autoantibodies, must be tested for to confirm a diagnosis.

Anti-double stranded DNA (dsDNA) and anti-smooth muscle (Sm) autoantibodies are usually measured alongside the ANA profile, as they have high specificity for JSLE. They are found in approximately 61-93% and 50% of children, respectively. Both autoantibodies are correlated with renal involvement and disease severity (60). Other

autoantibodies observed in JSLE include anti SSA/Ro, anti-SSB/La, anti-ribonuclear protein (anti-RNP), anti-histone and Rheumatoid Factor (55).

Another prominent laboratory finding is low levels of serum complement proteins, particularly C3 and C4. They are very helpful for disease monitoring and present in up to 90% of JSLE patients. Low serum complement levels, cytopenias (anemia, leukopenia, lymphopenia or thrombocytopenia) and elevated ESR or CRP can be indicators that the disease has become active (89).

1.2.6 Treatment

As the clinical signs and symptoms, such as lupus nephritis, seizure, psychosis, autoimmune hemolytic anemia, leukopenia and thrombocytopenia can lead to long-term morbidity and mortality, appropriate and early treatment should be started as soon as possible. However, current treatments can only suppress the disease activity and prevent organ or system damage, rather than eliminate the disease. Commonly-used treatments include corticosteroids, anti-malarial drugs, immunosuppressive drugs and new biologics. All medications have side effects, and so the relative risk and benefit needs to be considered.

Corticosteroids is the mainstay of treatment as they are the most effective and rapidly-acting drugs for JSLE patients (55, 60). The dose and duration of corticosteroid therapy depends on clinical presentation, types of involved organs or systems, and disease activity and severity. It

has been observed that more than 90% of children diagnosed with JSLE will receive corticosteroids at some point during the course of treatment and follow up. However, these benefits must be counter-balanced by serious side effects, such as growth retardation, delayed puberty, osteoporosis and osteonecrosis, opportunistic infections and Cushing syndrome.

Anti-malarial drugs (hydroxychloroquine) have become an important component for the treatment in JSLE, especially in children presenting with cutaneous lesions and non-erosive arthritis (90). These drugs have shown many beneficial effects in lupus patients and long-term use is now recommended. Hydroxychloroquine can prevent disease flares and protect against irreversible organ damage. Furthermore, they can lower serum cholesterol levels and prevent against premature atherosclerosis in JSLE patients (64). The most serious side effect is an irreversible ocular toxicity (retinopathy), which is very rare and it can be prevented by annual eye examinations (91).

Immunosuppressive drugs are given to patients who are resistant to corticosteroids and hydroxychloroquine or who require steroid sparing: alternatively, they may be given to patients with persistent glomerulonephritis or protracted neuropsychiatric disorders (55, 60). For example, Methotrexate is the first line immunosuppressive therapy for children with persistent arthritis who are first treated with corticosteroids or hydroxychloroquine, but who show no improvement.

Cyclophosphamide is given for severe and life-threatening conditions, such as proliferative lupus nephritis and acute psychosis. Azathioprine is effective for non-erosive arthritis, lupus nephritis, severe cytopenias and serositis. Mycophenolate mofetil is commonly-used in children with lupus nephritis for either induction of remission and maintenance therapy.

New therapies are currently under investigation and show beneficial effects in lupus patients (55, 60). Rituximab, a monoclonal antibody against CD20 on B cells, has been reported to be effective in patients with cytopenia, refractory nephritis and pulmonary hemorrhage (92, 93). Belimumab is a B lymphocyte stimulator (BLyS) antibody, which has been recently received FDA approval for use in combination with other therapies, and is used for the treatment of active adult SLE (55). However, studies examining the effectiveness of Belimumab in JSLE are still ongoing.

1.2.7 Prognosis

As previously mentioned, JSLE patients have more severe clinical manifestations and higher morbidity and mortality rates than adult SLE. Nevertheless, the prognosis of JSLE has improved dramatically over the past ten years because of effective treatments and new therapies. 5-year survival rates of 90-95%, and 10-year survival rates of > 90% have been reported after short and long-term treatment (63).

In spite of these improvements in therapy, lupus nephritis, neuropsychiatric disorders and hematological involvement are still significant causes of morbidity and mortality. The major causes of death in JSLE include severe disease flares, severe lupus nephritis, and particularly infections, often due to the side effects of medication and immune-suppression (55, 60, 63).

1.3 Neutrophils and Juvenile Systemic Lupus Erythematosus

Many studies have been reported the involvement of neutrophils in the pathogenesis of SLE. An increase of neutrophil apoptosis and enhancement of neutrophil extracellular traps (NETs) has been observed in neutrophils from lupus patients, which probably are a key role in the induction of autoimmune response and the development of organ and system damage (49). Moreover, SLE-derived low-density granulocytes (LDGs), an abnormal subset of neutrophils isolated from lupus patients, are able to induce vascular damage and increase levels of type I interferon; an important cytokine involved in the pathogenesis of the disease (94).

Neutrophil functional defects have been described in patients with SLE. They are not only associated with the pathogenesis of SLE, but are also involved in the etiology of several types of infection in patients. One of the most common infections results from pathogenic bacteria (e.g. *S.*

aureus and *E. coli*) and they are responsible for more than half of all infected cases (95). Noticeably, these microorganisms are usually effectively engulfed and destroyed by neutrophils and many studies have reported defective neutrophil function in these patients, including impaired phagocytosis, ROS production and chemotaxis (96–98).

1.3.1 Neutrophil apoptosis

It is hypothesised that the pathogenesis of the disease is triggered by autoantigens that induce autoantibody production. It has been found that autoantigens may be clustered in blebs on the surface of apoptotic cells (99). It has also been found that there are increased numbers and impaired clearance of apoptotic white blood cells such as neutrophils, monocytes and lymphocytes, in patients with SLE (100). Moreover, a high rate of secondary necrosis in lupus neutrophils is observed *in vitro*. The increased levels of neutrophil apoptosis and defective clearance of apoptotic cells are associated with disease activity (101).

During apoptosis, many kinase- and proteolytic-pathways are activated and these may lead to the production of autoreactive lymphocytes, and result in expression of autoantigens (99). For example, Ro, La, dsDNA or RNP are presented on the cell membrane of apoptotic cells, which are increased in SLE patients compared to healthy controls and are significantly correlated with disease activity. In particular, neutrophils, the most abundant white blood cells in human blood, show

increased apoptosis and may also contribute to the expression of autoantigens, and probably are involved in the pathogenesis of the disease.

The actual causes of increased neutrophil apoptosis are not fully defined. Several studies have reported the development of apoptotic neutrophils in lupus patients is probably because of increased death receptor expression particularly Fas (100), an imbalance of neutrophil apoptosis regulatory cytokines (such as IL-1β, GM-CSF, which delay apoptosis and TNF-α, which induces apoptosis) (101), and the autoantibody production against dsDNA and ribonucleoprotein La (102). These possibilities need to be further investigated. Notably, much of the data on neutrophil dysfunction comes from studies of adult SLE; there have been only a few studies in JSLE. Recently, it has been found that an imbalance in pro-apoptotic and anti-apoptotic factors in JSLE results in increased neutrophil apoptosis, and nuclear components from apoptotic neutrophils are expressed on the cell surface, presumably acting as a major source of autoantigens leading to autoantibody production (103, 104). It has also been found that factors within JSLE serum induce neutrophil apoptosis via both the intrinsic and extrinsic pathways (105).

1.3.2 NETosis

NETosis is a process described as a specialized form of neutrophil death and results in the formation of neutrophil extracellular traps (NETs)

(49). It is induced by metabolites of reactive oxygen species, in addition to proteins from neutrophil granules, which generate chromatin decondensation and nuclear membrane breakdown. An accumulation of these nuclear components causes an expulsion of a mesh-like structure, namely a NET, into the extracellular compartment (106). As this web-like structure contains chromatin and DNA, therefore it can potentially be another source for extracellular DNA or autoantigen exposure, leading to the development of autoantibodies in lupus patients.

A number of studies have shown an important role for NETosis and the formation of extracellular DNA in the pathogenesis of SLE. Increased NET formation and impairment of NET degradation have been demonstrated in patients with SLE (51, 107, 108). NETs are usually degraded by the action of deoxyribonuclease 1 (DNase 1), but it has been found that this enzyme is inhibited in patients with SLE, because of an increased production of anti-NET antibodies and DNase 1 inhibitors (107). Moreover, autoantibodies to neutrophil anti-microbial peptides LL-37 detected in patient serum, could attach to NETs and promote NETosis (108). The process of NETosis was also identified in the skin and kidney of lupus patients, and it correlated with increased levels of anti-dsDNA autoantibodies in serum (109). A study in JSLE patients recently reported that mature neutrophils exposed to serum-derived anti-ribonucleoprotein antibodies from JSLE patients die by NETosis, and the

products from this form of cell death (NETs), are able to activate plasmacytoid dendritic cells to produce type I interferon (e.g. IFN-α) in a TLR9- and DNA- dependent manner (51).

1.3.3 Low-density granulocytes

Several studies have demonstrated an abnormal subset of neutrophils in preparations of peripheral blood mononuclear cells (PBMCs) from both JSLE and adult SLE patients (94, 110, 111). These cells have been shown to express type I interferon (94). More importantly, high numbers of low-density granulocytes are associated with skin involvement and vasculitis in patients with SLE.

Low-density granulocytes have an immature morphology and present a pro-inflammatory phenotype (94). They secrete type I and type II interferon and TNF-α after stimulation, which promote organ and tissue damage. Furthermore, they have an increased ability to destroy cell-cell interactions on endothelial cells and exhibit high rates of NET formation (109). Nevertheless, the origin and the full function of low-density granulocytes in patients with SLE need to be further investigated.

1.3.4 Defective neutrophil function

Defective phagocytic ability by neutrophils and decreased opsonisation of *S. aureus* by sera from adult SLE patients with active disease has been demonstrated (112). Interestingly, these conditions are

more common in patients who are not treated with corticosteroids or other immunosuppressive drugs (96, 112). It has been found that circulating autoantibodies in patient serum e.g. autoantibodies directed against CD11b/CD18 (Mac-1) block opsonin receptor binding on neutrophils, thereby decreasing their phagocytic ability (113). Moreover, factors in serum of SLE patients are able to induce neutrophil aggregation and interfere with granule enzyme release (114). Other studies have shown that neutrophils activated by serum from SLE patients overexpressed adhesion molecules on their cell surface, which promoted aggregation (115). These findings suggest that serum factors most likely cause defective neutrophil phagocytosis as they activate neutrophil aggregation, and also interrupt the process of opsonisation and neutrophil phagocytosis and killing.

The release of granule enzymes and anti-microbial peptides by neutrophils during phagocytosis in SLE patients is controversial. One study has demonstrated that serum levels of lysosomal enzyme released from neutrophils were decreased in SLE patients, compared to controls, but the decrease was unrelated to disease activity (98). Another recent study showed that the expression level of lactoferrin, a secondary granule of neutrophils, was lower in SLE neutrophils compared to normal control neutrophils (116). In contrast, some evidence has described increased levels of anti-microbial peptides such as defensins and lactoferrin in

serum from SLE patients (117–119). These protein levels, released by neutrophils, also correlated with disease activity and severity. While the presence of these autoantibodies against these proteins in patient serum has been shown, the actual role of these released neutrophilic enzymes and anti-microbial peptides in the pathogenesis of the disease remains unclear.

Some reports have shown that SLE neutrophils express high levels of FcγRI (CD64) and complement receptor CR3 (CD11b/ITGAM), inflammatory receptors leading to downstream inflammatory process (115, 120, 121). FcγRIIB (CD32), which is an inhibitory receptor in normal immune response, is expressed at lower levels on SLE neutrophils, compared with healthy controls (122). The decreased expression of FcγRIIB, may therefore result in a decreased ability to down-regulate inflammation after cell stimulation in SLE patients. Such studies have demonstrated that the expression of cell surface receptors and adhesion molecules on neutrophils is dysregulated and this could be another contributing factor of the abnormal inflammatory process in SLE.

Defective neutrophil chemotaxis has also been reported in patients with SLE (97, 123). The data demonstrated that serum chemotactic factors produced by SLE neutrophils after endotoxin exposure or activation by insoluble immune complexes was markedly decreased. Neutrophil chemotactic activity was significantly decreased and these

findings correlated with a high incidence of infection in SLE patients. In contrast, another study demonstrated that neutrophil chemotaxis was hyperactive (124). The expression of cell surface markers such as MCP-1c, MIP-1α, CCL-5, CXCR4 and CXCL-12, which are involved in neutrophil chemotaxis, and their receptors, were increased in SLE patients both *in vitro* and *ex vivo* (125–127). Moreover, other studies have shown that BX471, a CCR1 antagonist and CXCR4 and CXCL12 blockers were able to prevent the progression of lupus nephritis in mice (128, 129). These findings suggest that hyperactive neutrophil chemotaxis may be involved in inflammatory processes leading to tissue- or organ damage in SLE patients.

Several studies have demonstrated that ROS production is decreased in JSLE patients (130, 131). The data showed that superoxide production after phorbol myristate acetate (PMA) stimulation was decreased in patients compared to healthy controls, regardless of the immunosuppressive treatments. However, there was no significant difference in neutrophil ROS production between inactive and active cases of lupus patients (131). In contrast, increased ROS production in SLE neutrophils has been reported in several studies (124). For example, after stimulation with bacterial peptide, (N-formyl-methionyl-leucyl-phenylalanine; fMLP) or patient serum, lupus neutrophils showed increased superoxide production compared to normal control neutrophils

(132). The degree of oxidative metabolism from lupus neutrophils relates to the disease activity and tissue or organ damage observed in the patients (e.g. vascular damage, skin lesions and lupus nephritis) (49, 133). In addition, clinical administration of anti-oxidants, such as Vitamin E resulted in decreased autoantibody production in SLE patients (134). Taken together, these findings support the idea that an increase in ROS production is involved in the pathogenesis of SLE, particularly in terms of autoantigen and autoantibody production. Nevertheless, the regulation of ROS production in SLE is still unclear, and further investigations are required.

1.3.5 Neutrophils and tissue or organ damage

Current evidence suggests that neutrophils are key players in the development of tissue or organ damage in patients with SLE, particularly in patients presenting with cutaneous lupus lesions, cardiovascular complications and renal disorders (135). Neutrophil infiltration has been demonstrated in various types of cutaneous lupus lesions (135). The most common skin lesions infiltrated by neutrophils include bullous lupus erythematosus, cutaneous vasculitis, and urticarial vasculitis (136–138). The prominent findings in skin histopathology often have abundant neutrophil infiltration and neutrophil debris at the dermo-epidermal junction, deep dermis and skin appendages, including blood vessels (139). It has been found that autoantigens, such as dsDNA, anti-microbial

peptides (including LL-37) and pro-inflammatory cytokines, such as IL-17, are produced by neutrophils via the process of NETosis in the affected skin (109). Moreover, NETosis of affected skin lesions are associated with increased serum levels of autoantibodies in these patients.

JSLE patients have a higher risk of cardiovascular and cerebrovascular events, particularly premature atherosclerosis (64). The accelerated vascular damage in these patients results from an imbalance between endothelial damage and repair mechanisms. It has been demonstrated that type I interferon produced by low-density granulocytes, interferes endothelial progenitor cells and circulating myeloid angiogenic cell functions (140, 141). Dysfunction of these two types of cells leads to ineffective vascular repair. Moreover, low-density granulocytes are able to induce endothelial cytotoxicity (94). For this reason, this abnormal subset of neutrophils seems to be most involved in the induction of premature atherosclerosis in patients with JSLE.

Lupus nephritis is one of the worst prognostic factors in patients with JSLE and adult-onset SLE (55, 60, 61). Neutrophils are most likely to become involved in kidney inflammation during in disease flare. It has been demonstrated that neutrophils are mainly localized within the glomeruli in lupus nephritis and numerous enzymes produced by neutrophils, such as myeloperoxidase, elastase and cathepsin, are released into the affected area leading to glomerular inflammation and kidney

damage (142–144). This finding is supported by evidence of the presence of the neutrophilic enzyme, neutrophil gelatinase-associated lipocalin (NGAL), in urine and plasma of patients with JSLE (145). NGAL is released by neutrophils in certain circumstances, particularly in acute renal damage. It has been found that the levels of NGAL could predict the worsening of lupus nephritis and to correlate with disease activity. In addition, NET formation, associated with the development of glomerulonephritis in lupus patients, has been described (107, 109). Neutrophils producing NETs are identified in patient kidneys and correlated with the levels of autoantibodies in serum.

1.4 Role of Cytokines in Juvenile Systemic Lupus Erythematosus

A number of reports suggest that an imbalance of cytokine levels in serum is probably one of the major factors involved in the immune-pathogenesis of JSLE and adult-onset SLE (146). The serum levels of IL-2, IL-6, IL-10, IL-17, IL-18, IL-21, type I IFNs, TNF-α and B lymphocytes stimulators (BLys), are suggested as important factors to correlate with the degree of disease activity in SLE patients (146). It has been found that patients with active SLE have significantly increased serum IL-6, IL-17 and TNF-α, particularly IL-6 and TNF-α, which are also demonstrated in kidney biopsies of lupus nephritic patients (83).

1.4.1 Interleukin 6 (IL-6)

The association between IL-6 and the pathogenesis of SLE has been reported in many studies (83, 146–148). IL-6 is expressed by many cell types, such as monocytes, fibroblasts, endothelial cells and lymphocytes, including T- and B- cells, and it has a different biological effects on these target cells (149). One of its most important effects in the pathogenesis of SLE is to promote B cell differentiation into plasma cells and induce IgG production (150). IL-6 signaling requires a coupling of the heteromeric receptor complex, which is composed of IL-6 receptor (IL-6R) and a signal transducing chain (gp130). Binding of IL-6 to IL-6R induces gp130 dimerization followed by JAK1 activation and tyrosine phosphorylation of gp130. This signal subsequently triggers activation of the ERK/MAPK and p-STAT3 pathways and promotes differentiation and maturation of target cells (149).

The role of IL-6 in the development of SLE has been demonstrated in murine lupus mice (MRL/lpr) (151). Increased serum levels of IL-6 and IL-6R correlate with age in lupus mice (152). IL-6-deficient mice showed a decreased infiltration of T-lymphocytes and macrophages, and lowered deposition of IgG and C3 in kidneys. These lupus mice had a delayed onset of lupus nephritis and increased survival. In addition, exogenous administration of recombinant human IL-6 in NZB/W F1 mice accelerated glomerulonephritis, whilst IL-6 receptor

blockage decreased renal proteinuria. The serum levels of IgG antibodies, particularly anti-dsDNA autoantibodies, were decreased and this therapy significantly increased the survival rate of these animals (153, 154).

In active lupus patients, increased serum levels of IL-6 correlate with B-cell hyperactivity, anti-DNA levels and disease activity (155–157). Circulating IgG anti-DNA antibodies secreted by B-cells were decreased by IL-6 blockade and restored by exogenous IL-6 administration. Several studies showed increased levels of urinary IL-6 and high expression of IL-6 in the glomeruli and renal tubules of the kidneys from patients with lupus nephritis (158). Moreover, elevated IL-6 levels were also observed in cerebrospinal fluid of patients with neuropsychiatric disorders (159). These studies proposed that IL-6 and IL-6R could serve as markers for monitoring disease activity and disease flare (160). IL-6 shows a correlation with lupus nephritis; therefore, its receptor antagonist may be potentially be used for treatment.

Tocilizumab is an IL-6 receptor antagonist (a humanized monoclonal antibody), that has been tested in an open label-phase I trial in patients with SLE (161). The preliminary results showed an improvement of disease activity score, decreased levels of anti-dsDNA and acute phase reactants.

1.4.2 Interleukin 17 (IL-17)

IL-17 is a potent pro-inflammatory cytokine primarily produced by activated T-lymphocytes, including $CD4^+$ T cells, $CD8^+$ T cells, $CD4^-$ $CD8^-$ $CD3^+$ T cells and γδ-T cells, but it is also reported to be released by other cell types such as neutrophils, mast cells and natural killer (NK) cells (162). Several effects of IL-17 on different cell types via IL-17 receptors (IL-17R) have been reported (163, 164). There are six IL-17 family members (A to F) and five IL-17Rs (A to E). Signaling via these receptors induces inflammatory responses by increasing production of pro-inflammatory cytokines and chemokines, particularly IL-1β, IL-6 and IL-8 in SLE (165). Moreover, IL-17 signaling promotes up-regulation of adhesion molecules (e.g. ICAM1), B cell differentiation into plasma cells and autoantibody production (76, 166, 167).

One of the $CD4^+$ T cell subsets that specifically produces IL-17, is the Th17 cell. Th17 lymphocytes originate from naïve T cells and they are initially activated by certain cytokines (e.g. TGF-β, IL-1β, IL-6, IL-21 and IL-23) leading to an up-regulation of the retinoic acid receptor-related orphan receptor-γt (RORγt), expression and then selectively produce IL-17 (165, 168). IL-23 is one of the most important factors for Th17 differentiation and the association between the IL-23/IL-17 axis and the pathogenesis of SLE has been reported in both animal and human studies (147, 169).

In MRL/lpr mice, increased production of IL-17 by $CD4^- CD8^- CD3^+$ T cells and up-regulation of IL-17R and IL23R expression on lymphocytes have been described, and is associated with disease progression (170). Lymphoid cells from these mice after IL-23 treatment *in vitro* and then transferred to other non-autoimmune mice and were able to induce lupus nephritis (171). In addition, IL-23R deficiency in lupus-prone mice (C57BL/6-lpr/lpr) resulted in lowered numbers of $CD4^- CD8^- CD3^+$ T cells and IL-17 producing cells in the lymph nodes. These mice had decreased levels of anti-DNA antibodies and were devoid of pathological and clinical features of lupus nephritis (170).

Increased serum levels of IL-17 and IL-23 were observed in SLE patients and these levels correlated with disease activity (172). IL-17 can promote B lymphocyte proliferation and differentiation, leading to autoantibody production in SLE patients (76). IL-17 producing cells were also identified in skin, lungs and kidneys in the patients and were more likely to be involved in the inflammatory process of tissue or organ damage (173–175).

Despite IL-17 being an important cytokine in the pathogenesis of SLE, the therapeutic targeting of IL-17 or IL-17 blockade is still inconclusive (147). A relationship between Th17 cells and other T cell subsets has been in several studies. For example, one study showed that Th17 cells down-regulated Th1 differentiation, and improved acute graft-

versus-host-disease in recipient mice (176). Neutralizing IL-17 may induce exacerbation of the disease. Another study demonstrated that an increased number of Th17 cells were associated with a decrease in regulatory T (Treg) cells in active SLE patients (177). Notably, Treg cells and Th17 cells differentiate from the same precursor cell and they can inhibit each other's function (178). Therefore, IL-17 blockade and the manipulation of the balance between Th17 cells and other T cell subsets should be considered for the treatment of SLE.

1.4.3 Type I interferon (type I IFN)

All types of white blood cells, including plasmacytoid dendritic cells can synthesize type I IFN (179). Several mechanisms have been postulated to explain how type I IFN may play a role in the pathogenesis of SLE. One possibility is that autoantigen-autoantibody complexes activate plasmacytoid dendritic cells to secrete type I IFN and this cytokine then promotes dendritic cell maturation, and enhance autoantigen presentation (180, 181). Moreover, type I IFN promotes B cell hyperactivity and autoantibody production (182).

In lupus-prone mice (B6 lpr), a study has shown that a potent inducer of type I IFN, polyinosinic:polycytidylic acid, increased numbers of macrophages and activated lymphocytes (183). In this study, levels of autoantibody and immune complex deposition were measured, followed by progression of lupus nephritis. Another study demonstrated that mice

deficient in type I IFN receptors, had decreased titers of anti-DNA autoantibodies, and diminished kidney manifestations, including disease severity (184). Numbers of activated dendritic cells and lymphocytes were also decreased (185). These finding support the important role of type I IFN in the pathogenesis of SLE.

The association of Toll-like receptors (TLRs) and type I IFN production has been well documented in the lupus mouse model. TLR-7 and TLR-9 bind single-strand RNA and hypomethylated CpG-rich DNA, respectively (146). Signaling through these two receptors on B cells results in autoantibody production and lupus nephritis (186). It has been demonstrated that type I IFN signaling is essential for the process of up-regulation TLRs and activation through TLR-7 and -9 on B cells resulting in autoantibody production (187). Moreover, dual inhibition of TLR-7 and TLR-9 results in a significant improvement of lupus nephritis and decreased the level of autoantibodies in lupus-prone mice.

Increased levels of type I IFN were observed in patients with SLE (188). The association between the levels of type I IFN and the titers of autoantibodies, and disease activity have also been described. An accumulation of plasmacytoid dendritic cells, which are major sources of type I IFN production, has been reported in the epidermal layer of cutaneous lupus lesions and the glomeruli of lupus nephritis (189). In addition, the expression of IFN-inducible genes in PBMC isolated from

patients with SLE correlates with disease activity (190). There are several identified IFN-inducible genes associated with increased risk of SLE. For example, many functional variants of Interferon regulatory factor 5 (IRF5), together with a number of SNPs have been identified (191). The resulting haplotypes derived from these variants correlate with high serum levels of IFN-α and autoantibodies, which are associated with heightened risk of SLE. One SNP in the third intron of signal transducer and activator of transcription 4 (STAT4), associates with a severe SLE phenotype, that is characterized by young age of onset with severe clinical manifestations and the presence of typical autoantibodies (192). This SNP correlates with IFN-α hypersensitivity and anti-dsDNA autoantibody production in SLE patients. Moreover, a combination of these variants from different genes (e.g. IRF5 and STAT4) results in an additive risk factor of the disease (193).

An anti-IFN-α monoclonal antibody, Sifalimumab, has been studied in a phase I clinical trail (194) for the treatment of active SLE. The results showed an improvement of the disease, in terms of disease activity, disease flare and immunosuppressive drug usage.

1.4.4 Tumour necrosis factor-α (TNF-α)

TNF-α is another important cytokine involved in the pathogenesis of SLE, but its actual role is still unclear (195). Immune-regulatory and pro-inflammatory functions of TNF-α have been reported, including

different effects on a range of cells (e.g. B cells, T cells and dendritic cells) (196).

It has been demonstrated that immune complexes can activate lupus macrophages to produce high levels of TNF-α (197). There are two forms of TNF-α; a membrane-bound form expressed as a trimer on the cell membrane and a soluble form which is secreted to the extracellular compartment (83). Both forms are involved in the generation of B cell follicles of the secondary lymphoid organ structures, such as lymph nodes and Peyer's patches and they are growth factors for B cells, inducing IL-1 and IL-6 production (198–200). TNF-α is also involved in the proliferation of T cells responding to certain stimuli (e.g. IL-2) (201). However, the duration of T cells exposure to TNF-α is crucial: short-term stimulation leads to proliferation, further activation of T cells and induction of IFN-γ production, whilst long-term stimulation promotes a loss of T cell receptors (TCRs) on the cell membrane and results in a decreased response of T cells (195, 201, 202). In addition, TNF-α is directly involved in both apoptotic and anti-apoptotic pathways depending on the TNF-α receptors engaged (TNFR) (34). TNFR1 mediates both apoptotic and anti-apoptotic, and inflammatory responses while TNFR2 is involved only in the apoptotic pathway. Therefore, TNF-α and their receptors determine may regulate cell survival, differentiation, inflammatory responses or cell apoptosis.

Decreased production of TNF-α has been reported to correlate with severe disease in lupus mice (NZB/W) (203). The mice with intact TNF-α had only mild disease activity, and these findings suggest that TNF-α deficiency may be a trigger factor in the development of SLE. In contrast, another study described increased serum levels of TNF-α in lupus mice (MRL/lpr) and the levels correlated with the severity of renal disease (204). High concentrations of TNF-α were also observed in renal tissue. Furthermore, treatment of lupus mice with anti-TNF-α resulted in an improvement of the disease, including decreased arthritis and pulmonary disease (205, 206). As a result, the role of TNF-α in the pathogenesis of murine SLE is still questionable in murine lupus mice.

Many studies have demonstrated that serum levels of TNF-α are markedly high in patients with SLE, compared to healthy controls and higher in patients with active disease (207–210). Increased levels of TNF-α in renal tissue from patients with lupus nephritis, particularly in glomeruli, have also been observed (209), with levels correlated with urine TNF-α levels and renal disease activity. These findings suggest that TNF-α plays a pro-inflammatory role in the development of lupus nephritis.

In addition, many studies have described TNF-α gene polymorphisms associated with susceptibility to SLE (195). Most of these studies have demonstrated microsatellites (single sequence repeat; SSRs)

and SNPs in the promoter regions of TNF-α gene, and showed a correlation with specific clinical features. For example, the microsatellites of TNF-α2, TNF-β3 and TNF-δ2 alleles were associated with mucocutaneous manifestations (e.g. photosensitivity and Raynaud's phenomenon) (211). The -308A/G polymorphism correlated with mucocutaneous manifestations (e.g. malar rash, oral ulcers and classic discoid LE) and hematological involvement (212). Furthermore, an abnormal TNF-α expression correlating with disease activity has been reported in PBMC and bone marrow cells from patients with SLE (213, 214).

Anti-TNF-α therapy has been used in clinical studies in patients with SLE, but shows contradicting results. Several lines of evidence have demonstrated that patients with other rheumatic diseases who received TNF-α blockers, have developed lupus-like symptoms, such as malar rash, oral ulcers and discoid rash, and a presence of autoantibodies (e.g. ANA and anti-dsDNA antibodies) (215). Therefore, the role of TNF-α and the application of TNF-α antagonists for patients with SLE are still unclear.

1.5 Summary

Neutrophils have a pathological role in the progression of autoimmune diseases (11). It has been reported that neutrophils are an important contributing factor in disease pathology of SLE, including JSLE. JSLE is a subgroup of SLE, and patients usually have more severe clinical features and major organ involvements (e.g. kidneys and CNS) (55, 60, 61). Increased numbers of apoptotic neutrophils in patients with JSLE have been reported and this factor could can be a major cause of exposure of autoantigens leading to autoantibody production and subsequently induction of tissue and organ damage (49, 99, 100, 103). Moreover, an imbalance in the production of cytokines may be involved in the disease pathology, particularly TNF-α, type I IFNs, IL-6 and IL-17 (83, 146, 216–219) these factors could also be responsible for increased neutrophil apoptosis in JSLE. In addition, neutrophil functional defects have been reported in patients with SLE, leading to susceptibility to infections. However, there are only a few reports describing defective neutrophil functions in JSLE patients (50–52, 95, 112). As the pathogenesis of the disease is still unclear, supportive treatments are only the option that prevent organ or system damage. Current treatments include corticosteroids, anti-malarial drugs (e.g. hydroxychloroquine), immunosuppressive drugs and new biologics. All medications have side effects, and so their clinical applications, particularly in juvenile patients, need to be carefully considered.

1.6 Research Aims

The main objectives of my study were to:

1. Determine if sera from patients with JSLE was defective in regulating neutrophil functions, such as cell survival, and opsonisation of bacteria for effective phagocytosis and activation of the respiratory burst.

2. Investigate the effects of recombinant-cytokines, which have been reported as being imbalanced in patient serum, on neutrophil apoptosis and to measure cytokines in the sera of JSLE patients.

3. Determine whether adding GM-CSF (a potent cytokine that delays neutrophil apoptosis) is capable of protecting neutrophils against apoptosis in JSLE patients.

4. Determine the molecular events that regulate neutrophil responses to TNF-α signaling, as the role of TNF-α in disease pathology remains unclear.

5. Investigate JSLE neutrophil functions in terms of cell survival, phagocytosis, ROS production and chemotaxis.

6. Evaluate whether hydroxychloroquine, a commonly-used anti-malarial agent for patients with JSLE, has any effects on neutrophil functions.

Chapter 2: Materials and Methods

2.1 Materials

Table 2.1 Materials for neutrophil isolation and culture

Materials	Supplier
Lithium heparin vacutainers	Grenier Bio-one (Gloucestershire, UK)
Polymorphprep^{TM}	AxisShield (Cambs, UK)
RPMI 1640 (+25mM Hepes with L-glutamine)	Gibco (Paisley, UK)
Ammonium chloride lysis buffer - Ammonium chloride (NH_4Cl) - Potassium hydrogen carbonate ($KHCO_3$) - Ethylenediaminetetracetic acid (EDTA)	Sigma (Poole, UK)
Isoton	Beckman Coulter Inc. (Florida, USA)
Rapid Romanowsky stain	HD Supplies (Aylesbury, UK)
Human pooled AB serum	Sigma (Poole, UK)
Fetal bovine serum	Invitrogen (Paisley, UK)
Phosphate buffered saline (PBS) tablets	Oxid Ltd (Basingstoke, UK)

Table 2.2 Materials for preparation of protein lysates and Western blotting

Materials	Supplier
Laemelli lysis buffer - Glycerol - Sodium dodecyl sulphate - Tris - Bromophenol blue	Fisher Scientific (Loughborough, UK) Sigma (Poole, UK)
Dithiothreitol (DTT) Glycine Sodium chloride (NaCl)	Sigma (Poole, UK)
Hydrochloric acid (HCl)	VWR International (Leicestershire, UK)
Ammonium persulphate (APS) Isopropanol Methanol Tetramethylethylenediamine (TEMED) Tween-20	Sigma (Poole, UK)
Polyacrylamide	Severn Biotech (Kidderminster, UK)
Biotinylated protein ladder detection pack	Cell-Signalling Technology (USA)
Polyvinylidene fluoride (PVDF) membrane	Millipore (Hertfordshire, UK)

Whatman filter paper	Sigma (Poole, UK)
Marvel non-fat powered milk	Home Bargain (Liverpool, UK)
Bovine serum albumin (BSA) Ponceau S	Sigma (Poole, UK)
Immobilon Western Chemiluminescent HRP substrate	Millipore (Billerica, UK)
Enhanced chemiluminescence hyperfilm	Amersham Life Science (Bucks, UK)
Film developing (developer and fixer)	Sigma (Poole, UK)

Table 2.3 Cytokines and stimulants

Materials	Supplier
Recombinant human GM-CSF	Roche Diagnostics Ltd. (East Sussex, UK)
Recombinant human TNF-α Recombinant human IFN-γ	Calbiochem (Nottingham, UK)
Recombinant human IL-1β Recombinant human IL-8 Recombinant human IL-17	Invitrogen (Paisley, UK)
Recombinant human IL-6 N-formylmethionyl-leucyl-phenylalanine (fMLP) Phorbol 12 myristate 13-acetate (PMA)	Sigma (Poole, UK)

Bacterial lipopolysaccharide (LPS) Cycloheximide	Sigma (Poole, UK)

Table 2.4 Dyes and antibodies

Materials: dyes and antibodies	Supplier
Hanks balanced salt solution (HBSS)	Gibco (Paisley, UK)
FITC-conjugated Annexin V	Invitrogen (Paisley, UK)
Propidium iodide (PI)	Sigma (Poole, UK)
Western blotting antibodies - anti-human Mcl-1 - anti-human caspase 3 - anti-human caspase 7 - anti-human caspase 8 - anti-human caspase 9 - anti-human caspase 10 - anti-human FLIP - anti-human TNFR1 - anti-human TNFR2 - anti-human FADD - anti-human TRADD - anti-human β-actin	Cell-Signalling Technology (USA)

- anti-human GAPDH	Cell-Signalling Technology (USA)

Table 2.5 Materials for isolation neutrophil mRNA, cDNA synthesis and Quantitative PCR

Materials	Supplier
Trizol® reagent	Gibco (Paisley, UK)
Chloroform Isopropanol Ethanol	Sigma (Poole, UK)
RNeasy kit DNA digestion solution	Qiagen (Crawley, UK)
Superscript III first strand cDNA synthesis kit RNase OUT RNase inhibitors	Invitrogen (Paisley, UK)
Random primers	Promega (Southampton, UK)
Quantitect SYBR green PCR kit	Qiagen (Crawley, UK)
Primers	Eurofins (UK)

Table 2.6 Materials for measurement of neutrophil phagocytic assay and respiratory burst

Materials	Supplier
Staphylococcus aureus (Oxford strain)	Department of Medical Microbiology, University of Liverpool, UK
LB agar and LB broth	Merck (Feltham, UK)
Propidium iodide, Gelatin	Sigma (Poole,UK)
Hanks balanced salt solution (HBSS)	Gibco (Paisley, UK)
Dihydrorhodamine 123 (DHR123)	Invitrogen (Paisley, UK)

Table 2.7 Materials for neutrophil chemotaxis assay and measurement of neutrophil ROS production

Materials	Supplier
Hanging cell culture inserts	Millipore (Hertfordshire, UK)
Luminol	Sigma (Poole, UK)

Table 2.8 Other materials

Other materials	**Supplier**
Serum vacutainers	Grenier Bio-one (Gloucestershire, UK)
Reacti-bind™ Protein L coated, clear 96-well plate (Blocking buffer)	Thermo scientific (Illinois, USA)
Hydroxychloroquine (HCQ)	Sigma (Poole, UK)

2.2 Methods

2.2.1 Isolation of human neutrophils from whole blood

For the UK cohort, the study of adult healthy controls was approved by the University of Liverpool Committee for Research Ethics (CORE), while the juvenile healthy controls and JSLE patient study was approved by the Liverpool Paediatric Research Ethic Committee. For the Thai cohort, the study of juvenile healthy controls and JSLE patients was approved by Chulalongkorn University Human Research Ethic Committee. The diagnosis of JSLE for both population is based on at least 4 out of the 11 criteria from the American College of Rheumatology (1997 revision) guidelines before the age of 17 years (59). The disease activity for the Thai cohort was classified into disease active and inactive

using clinical assessment and/or Systemic Lupus Eryhtematosus Disease Aactivity Index (SLEDAI) score (220). For example, JSLE patients with disease active usually present with skin manifestations (e.g. malar rash, oral ulcers, cutaneous vasculitis and diffuse hair loss) and renal involvement (e.g. lupus nephritis, proteinuria and hypertension), and increase in SLEDAI score of more than 3. Written informed consent and/or assent form were obtained from all participating subjects and/or their parents. Whole blood was collected into heparinized vacuette containers by venipuncture and was processed within 2 h of collection. Neutrophils were isolated by one-step centrifugation using Polymorphprep, according to the manufacturer's instructions (221). The principle of neutrophil isolation by Polymorphprep is based on an isosmotic density barrier that separates the peripheral blood mononuclear cells and granulocytes (including neutrophils) into two layers after centrifugation, whereas erythrocytes sediment to the bottom of the solution (221). In brief, whole blood was layered in a 1:1 to 1.5:1 ratio onto Polymorphprep and centrifuged at 500 g for 30-35 min in room temperature. Plasma and peripheral blood mononuclear cell layers were discarded. The granulocyte later containing including neutrophils was harvested by a Pasteur pipette and resuspended in RPMI 1640 media plus 25 mM Hepes with L-glutamine (2mM) then centrifuged at 500 g for 5 min. The cells were resuspended in media and contaminating erythrocytes were removed by hypotonic lysis (ammonium chloride lysis

buffer: 13.4 mM $KHCO_3$, 155 mM NH_4Cl and 96.7 mM EDTA in 500 mL distilled water) used in a ratio of 1:9 media:lysis buffer for 3 min before centrifugation at 500 g for 3 min. Ammonium chloride lysis buffer has little effect on neutrophil function (222). Neutrophils were resuspended in media and counted using a Multisizer 3 cell counter (Beckman Coulter), and the suspension volume was adjusted to give a final concentration of 5 x 10^6 cells/mL. The purity of samples (> 95% neutrophils) was assessed by examination of cytospins prepared using a Shandon 3 cytospin (20 µL cells in 180 µL PBS+EDTA). After centrifugation at 30 g for 5 min, neutrophils were stained with Rapid Romanowsky staining. Neutrophils were further resuspended in RPMI 1640 media plus 25 mM Hepes supplemented with 10% (v/v) human AB serum or 10% (v/v) child healthy control serum or JSLE serum for specific experiments, as indicated in the text. Neutrophils were incubated in a 5% CO_2 incubator at 37°C with gentle agitation.

2.2.2 Serum collection

Sera were collected from the peripheral blood samples of JSLE patients and healthy juvenile controls by centrifugation of clotted blood at 500 g for 5 min. The sera were frozen in -80°C freezer for later use.

2.2.3 Flow cytometry for neutrophil apoptosis

Neutrophil apoptosis and necrosis was measured using flow cytometry. Phosphatidylserine expressed on the membrane of apoptotic neutrophils was stained with annexin V-FITC and DNA of necrotic neutrophils in late apoptosis (with leaky plasma membrane) was stained with propidium iodide (PI). 1 x 10^5 cells of neutrophils in 100 μL HBSS were incubated with annexin V-FITC (10 μL/mL) for 15 min in the dark, at room temperature and PI (1 μg/mL) was added just prior to the flow analysis. 5,000 gated cells were analyzed using the Guava Easycyte flow cytometer.

In all experiments measuring apoptosis by annexin V/PI staining and flow cytometry, absolute numbers of neutrophils were routinely counted after each experiment. Simply measuring the % of neutrophils that expressed markers of apoptosis (e.g. annexin V binding) could underestimate the level of cell death if some neutrophils had become necrotic and lysed. Therefore, in all experiments measuring neutrophil apoptosis, both the % number of apoptotic cells and the actual number of cells in the incubation were routinely measured. It was found that in all experiments, the numbers of neutrophils recovered after incubation was always > 95% of the initial incubation (1x10^6 cells/mL). Adjustment of this small loss of cells did not affect the values for % apoptosis and so in all experiments in this thesis, "% Apoptosis" values are presented.

2.2.4 Preparation of protein lysates

Isolated neutrophils were centrifuged at 1,000 g for 3 min after incubation and/or treatment (as described in the text), the supernatant was discarded and the pellet was washed with PBS. The neutrophils were re-centrifuged at 1,000 g for another 3 min. The supernatant was again discarded and the pellet was rapidly lysed in boiling Laemelli lysis buffer, consisting of 10% (v/v) glycerol, 1 M Tris-HCl (pH 6.8), 100 mM DTT, 3% (w/v) SDS and 0.001% (w/v) bromophenol blue, to a final concentration of $1x10^4$ cells/µL. All samples were immediately boiled for 5 min with occasional vortexing. Protein lysates were frozen at -20°C for later use.

2.2.5 Western blotting

Protein lysate samples were boiled, briefly centrifuged in a microfuge and 10-15 µL loaded onto 8-12% resolving polyacrylamide gels (dependent on the size of the protein of interest) with a 4.5% polyacrylamide stacking gel. The resolving gels were composed of 8-12% (v/v) acrylamide, 1% (w/v) SDS, 370 mM Tris-HCl pH 8.8, 1% (w/v) APS and 0.1% (v/v) TEMED. The APS and TEMED were added last to aid polymerization. The stacking gels comprised 4.5% (v/v) acrylamide, 1% (w/v) SDS, 122 mM Tris-HCl pH 6.7, 1% (v/v) APS and 0.1% (v/v) TEMED. A biotinylated protein molecular weight marker mixture was loaded alongside the samples. Gels were electrophoresed for

45-50 min at 200V in SDS running buffer (25 mM Tris, 0.1% (w/v) SDS and 192 mM glycine) using a Bio-rad Mini Protean II Electrophoresis kit. Proteins were transferred to polyvinylidenedifluoride membranes in transfer buffer (20% (v/v) methanol, 12.5 mM Tris and 95 mM glycine) by electrophoresis at 100V for 80 min using the same apparatus. Transferred proteins on the membranes were checked with Ponceau S staining (0.1% (w/v) Ponceau S and 5% (v/v) acetic acid). The stain was removed from the membranes by washing with wash buffer (150 mM NaCl, 0.1% (v/v) Tween-20 and 10 mM Tris-HCl pH 8) and the membranes were incubated with blocking buffer (5% (w/v) Marvel or non-fat skimmed milk in wash buffer) for at least 1 h on an orbital shaker at room temperature in order to prevent non-specific antibody binding. Membranes were then rinsed with wash buffer for 30 sec and incubated with the appropriate primary antibody in either blocking buffer or 5% (w/v) BSA in wash buffer at the appropriate concentration overnight at 4°C on an orbital shaker. Following washing with wash buffer for 10 min three times, the membranes were incubated with the appropriate HRP-conjugated secondary antibody in blocking buffer for at least 1 h at room temperature on an orbital shaker. Secondary antibody was removed by washing with wash buffer for 10 min (three times) and the membranes were stained with Enhanced Chemiluminescence (ECL) reagents (Millipore) and then were carefully exposed to Hyperfilm in the dark to detect bound antibodies, avoiding signal saturation. Densitometry

analysis of Western blots was performed using the AQM Advanced 6 Kinetic Imaging System. Re-probing of membranes with other antibodies, such as β-actin or GAPDH, was carried out following stripping the membranes with stripping buffer (50 mM glycine, 0.1% (v/v) Tween-20, 150 mM NaCl and HCl to pH 2.5), followed by blocking with blocking buffer for 1 h. The band density of β-actin or GAPDH was measured and analyzed alongside with the band density of the protein of interest in order to confirm equivalent loading of proteins in each sample. Table 2.9 summarises antibodies used for Western blotting.

Table 2.9 Western blot antibodies

Protein of interest	**Primary antibody**	**Secondary antibody**
Mcl-1	Rabbit anti-human Mcl-1 (1:1,000)	HRP-conjugated donkey anti-rabbit IgG (1:10,000)
Caspase 3	Rabbit anti-human Caspase 3 (1:1,000)	HRP-conjugated donkey anti-rabbit IgG (1:10,000)
Caspase 7	Rabbit anti-human Caspase 7 (1:1,000)	HRP-conjugated donkey anti-rabbit IgG (1:10,000)
Caspase 8	Mouse anti-human Caspase 8 (1:1,000)	HRP-conjugated sheep anti-mouse IgG (1:10,000)
Caspase 9	Rabbit anti-human Caspase 9 (1:1,000)	HRP-conjugated donkey anti-rabbit IgG (1:10,000)

Caspase 10	Rabbit anti-human Caspase 10 (1:1,000)	HRP-conjugated donkey anti-rabbit IgG (1:10,000)
FLIP	Rabbit anti-human FLIP (1:1,000)	HRP-conjugated donkey anti-rabbit IgG (1:10,000)
TNFR1	Rabbit anti-human TNFR1 (1:1,000)	HRP-conjugated donkey anti-rabbit IgG (1:10,000)
TNFR2	Rabbit anti-human TNFR2 (1:1,000)	HRP-conjugated donkey anti-rabbit IgG (1:10,000)
FADD	Rabbit anti-human FADD (1:1,000)	HRP-conjugated donkey anti-rabbit IgG (1:10,000)
TRADD	Rabbit anti-human TRADD (1:1,000)	HRP-conjugated donkey anti-rabbit IgG (1:10,000)
β-actin	Mouse anti-human actin (1:10,000)	HRP-conjugated sheep anti-mouse IgG (1:10,000)
GAPDH	Mouse anti-human GAPDH (1:10,000)	HRP-conjugated sheep anti-mouse IgG (1:10,000)

2.2.6 Isolation of neutrophil mRNA

1-2 x 10^7 isolated neutrophils were centrifuged at 1,000 g for 3 min and the supernatant was discarded. The cell pellet was lysed using 1 mL Trizol Reagent and the pellet was disrupted by repeatedly pipetting to obtain a homogeneous solution, which was then allowed to stand for at least 5 min at room temperature. 200 μL of chloroform was added and

mixed by vortexing the samples vigorously for 15 sec. The solution was allowed to stand for another 2-3 min at room temperature before centrifugation at 10,000 g for 15 min at 4°C. The aqueous layer containing RNA was removed and an approximately equal amount of isopropanol was added to precipitate the RNA at -20°C overnight. The RNA precipitate was centrifuged at 10,000 g for 30 min at 4°C to form a pellet and the supernatant was discarded. The pellet was washed using 70% (v/v) ethanol/DEPC treated water and allowed to dry before resuspension in 100 µL RNase-free water. RNA was further cleaned to remove remaining contaminants using the Qiagen RNeasy kit, according to the manufacturers instructions. Genomic DNA contamination was eliminated by adding Qiagen DNase digestion solution in the column kit for 15 min at room temperature. RNA was finally eluted in 30 µL RNase-free water. The quality and quantity of RNA was measured using a Nanodrop ND-100 spectrophotometer. The RNA samples were stored at -80°C for later use.

2.2.7 cDNA synthesis

cDNA was synthesized from total RNA using the Superscript III First Strand cDNA synthesis kit (Qiagen) and RNase OUT (Qiagen), according to the manufacturer's instructions. The total amount of each template RNA used in this study was usually 200-250 ng, and all RNA samples were adjusted to an equal amount within each experiment before

cDNA synthesis. Each RNA sample was added to 1 μL (250 ng or 20 μM) random primer, 1 μL (10 mM) dNTPs and RNase free-water to a total volume of 13 μL before heating at 65°C for 5 min on heat block and then cooling on ice for at least 1 min. A mixture of 4 μL First-Strand buffer, 1 μL RNase OUT RNase inhibitor, 1 μL (0.1 M) DTT and 1 μL (200 units/μL) Superscript III reverse transcriptase was added into each sample on ice before incubation in a Thermo PX2 thermal cycler. cDNA synthesis was initiated at 25°C for 5 min, 50°C for 60 min and then heating to 70°C for 15 min. cDNA samples were stored at -20°C for further use. Table 2.10 summarises the cDNA synthesis procedure.

2.2.8 Quantitative PCR (real-time PCR or qPCR)

Quantitative PCR was carried out using the QuantiTect SYBR Green detection kit (Qiagen), according to the manufacturers instructions. 1 μL cDNA was added to 10 μL QuantiTect, 0.8 μL (10 pM) of each forward and reverse primer and RNase-free water to a total reaction volume of 20 μL. Each sample was prepared in triplicate and Quantitative PCR was performed using the Roche LightCycler 480 qPCR machine. Levels of gene expression in samples were normalized to β-actin by comparing the Ct values, according to the Pfaffl method (223). Table 2.11 and 2.12 summarise the Quantitative PCR cycle settings protocol and primer sequences.

Table 2.10 cDNA synthesis procedure

Reagents	Volume (µL)	Concentration	Temp (°C)	Time (min)
RNA+RNase free-water	11	<10 ng/µL	60	5
Random primers	1	20 µM		
dNTPs	1	10 mM	On ice	>1
First-Strand buffer	4	5X	25	5
RNase OUT	1	40 units/µL	50	60
DTT	1	0.1 M		
Superscript III RT	1	200 units/µL	70	15
Total volume	20			

Table 2.11 Quantitative PCR cycle settings

Step	Procedure	Temp (°C)	Time	Cycles
1.	Taq activation	95	15 min	1
2.	Denaturation	95	1 min	45

	Annealing	55	30 sec	
	Elongation	72	30 sec	
3.	Generating melt curves and checking product specificity	60	30 sec	1

Table 2.12 Primer sequences

Gene symbol	Product length (bp)	Forward Sequence (5'-3') Reverse Sequence (5'-3')
Mcl-1_L	170	TTATCTCTCGGTACCTTCGG TGCTTCGGAAACTGGACATC
Mcl-1_S	164	CCTTCCAAGGATGGGTTTG GGAGCTGGTTTGGCATATC
TNF	219	CAGAGGGCCTGTACCTCATC GGAAGACCCCTCCCAGATAG
BCL2A1	82	GAATAACACAGGAGAATGGATAAGG CCAGCCAGATTTAGGTTCAAAC
CFLAR	93	ATAGATGTGGTTCCACCTAATGTC GTAGAGCAGTTCAGCCAAGTC
TNFAIP3	113	AACATTTTGCTGCTGCCTCA TCCTTCAAACATGGTGCTTCC
APAF1	94	CTTCCAGCCAACCTATTTTCCT CCTGATTAACCTTGGAGATAAAAGAA

BAX	101	ATGGAGCTGCAGAGGATGAT CAGTTGAAGTTGCCGTCAGA
CASP10	124	GGAACGGACACACAACTCTC AGCCCACTCACTTACAGACTAA
CASP8	95	AGTAAGCAACAAGGATGACAAGA ATCAATCAGAAGGGAAGACAAGTT
FADD	176	CACAGACCACCTGCTTCTGA CTGGACACGGTTCCAACTTT
FAS	104	TGTAGTATGAATGTAATCAGTGTATGT GATATTTCAGCAAAAGGTCATAGC
TNFRSF1A	88	TGTGTCTCCTGTAGTAACTGTAAG AGTCCTCAGTGCCCTTAACA
TNFRSF1B	145	GTCCACACGATCCCAACAC CACACCCACAATCAGTCCAA
TRADD	88	GGTGCATCATTGGGGATTCT GGGAGAAGGTGAGGCTGAT
GAPDH	106	CTCAACGACCACTTTGTCAAGCTCA GGTCTTACTCCTTGGAGGCCATGTG
ACTB	211	CATCGAGCACGGCATCGTCA TAGCACAGCCTGGACAGCAAC
B2M	114	ACTGAATTCACCCCCACTGA CCTCCATGATGCTGCTTACA
PPIA	60	GCTTTGGGTCCAGGAATGG GTTGTCCACAGTCAGCCATGGT

2.2.9 RNA-sequencing and Bioinformatics

RNA-sequencing and Bioinformatic analysis were performed as previously described by H. Wright and H. Thomas (224). In brief, 3 x 10^7 isolated neutrophils were pelleted, and RNA from the pellet was extracted with Trizol reagent using an RNAeasy kit, which included a DNA digestion step (as described in section *2.2.6*). Total RNA concentration and integrity were assessed using the Agilent 2100 Bioanalyser RNA Nano chip. The total amount of RNA was >1 μg with an RNA integrity number (RIN) of 8 or more. Total RNA samples were enriched for mRNA using either poly-A selection or ribosomal depletion. Standard Illumina protocols were used to generate 50 bp single-end read libraries, which were then sequenced by the Illumina HiSeq 2000 Analyser sequencing platform at the Beijing Genomics Institute (BGI). The human genome (hg19) was applied as a standard genome for read mapping using TopHat (225, 226) and Bowtie (227). Relative expression values (RPKM) were calculated using Cufflinks (228). Statistical analysis was performed using Cuffdiff to determine differential gene expression (228). Bioinformatics analysis was performed using Ingenuity Pathway Analysis (IPA) to identify how differentially expressed genes are mapped onto specific biochemical pathways.

2.2.10 Staphylococcus aureus (S. aureus) culture

A glycerol stock of *S. aureus* (Oxford strain) in a cryovial tube stored at -80°C was streaked onto a sterile LB agar plate using aseptic technique. The plate was incubated at 37°C overnight to obtain single bacterial colonies and was then stored at 4°C for later use. A streak plate from the glycerol stock was discarded every 4 weeks, and a new fresh streak plate was prepared to ensure optimal bacterial growth.

A single colony of *S. aureus* from the streak plate was inoculated into 10 mL of sterile LB broth and incubated at 37°C overnight on a gentle orbital shaker to mid-exponential growth phase of growth. An aliquot of culture was serially diluted with sterile LB broth and the absorbance of each dilution was recorded at 540 nm using a spectrophotometer. Known aliquots of each dilution were then plated and grown overnight. The number of colonies was counted and calibration curve of numbers of viable bacteria versus A_{540nm} was prepared. The A540 of overnight cultures was then measured the concentration of viable bacteria determined (from the calibration curve) and the culture was centrifuged at 2,000 g for 10 min at room temperature. The supernatant was discarded and the bacterial cells were washed with PBS three times. The bacterial cells were then adjusted to a final concentration of 1 x 10^{10} cells /mL and resuspended in PBS for immediate use.

2.2.11 Opsonisation of Staphylococcus aureus and PI labelling

1 x 10^{10} bacterial cells/mL in PBS were heated-killed by incubation at 60°C for 30 min in a water bath. The bacteria were centrifuged at 2,000 g for 10 min and the supernatant was discarded. The bacteria were then washed with PBS three times and stained with 120 μM propidium iodide (PI) in PBS at 4°C for 2 h on an orbital shaker in the dark. *S. aureus* stained with PI were centrifuged at 2,000 g for 10 min and the supernatant was removed. The PI-stained bacteria were washed 3 times with 0.1% gelatin in HBSS and then opsonised with 30% human AB serum or JSLE serum in PBS at 37°C for 1 h in a shaking incubator. The opsonised, PI-stained bacteria were washed with PBS three times and resuspended in PBS at a final concentration of 1 x 10^{10} bacteria/mL and were stored at 4°C.

2.2.12 Measurement of neutrophil phagocytosis and respiratory burst

1 x 10^6 neutrophils/mL were resuspended in RPMI 1640 media plus 25 mM Hepes (without human serum), and incubated with opsonised PI-stained *S. aureus* stained with PI at a 1:20 neutrophil:bacteria ratio, at 37°C for 30 min with gentle agitation in a 5% CO_2 incubator. Neutrophils were then washed with HBSS and centrifuged at 1,000 g for 3 min. The supernatant was discarded and the cells were resuspended in 2 mL HBSS. 200 μL cells were added to well of a 96-well microtitre plate. For respiratory burst measurement, 1 x 10^6 neutrophils/mL were incubated

with opsonised PI-stained *S. aureus* for 15 min before 25 nM of Dihydrorhodamine 123 (DHR123) was added for a further 15 min. The percentage of neutrophils phagocytosing bacteria and showing respiratory burst activity was measured by flow cytometry.

2.2.13 Neutrophil chemotaxis (transmigration assay)

The chemotaxis assay was performed using Millipore Hanging Cell Culture plate inserts. 24-well tissue culture well plates were used and coated with poly (2-hydroxyethyl methacrylate) to prevent cell adhesion following transmigration, at a concentration of 12 mg/mL in ethanol. The plates were kept in a 37°C incubator overnight or until the ethanol evaporated. 800 μL RPMI 1640 plus 25 mM Hepes with or without chemoattractanct (IL-8) was added into each well. The hanging inserts with a 3 μm pore-size filter at their base, were suspended in the culture media and left to equilibrate for at least 10 min at room temperature. 1 x 10^6 neutrophils in 200 μL culture media were added into the hanging inserts and incubated at 37°C in a 5% CO_2 incubator for 90 min. The hanging inserts were then removed and migrated neutrophils in each well were counted using the Multisizer 3 cell counter (Beckman Coulter) after a dilution of 1:1,000 in Isoton II. The count number of migrated neutrophils was calculated as a percentage of the total number of cells originally added.

2.2.14 Measurement of neutrophil ROS production

Luminol-dependent chemiluminescence was performed to measure total ROS production from neutrophils. The luminol can pass through cell membranes and so can be used to detect both intra- and extracellular oxidant production. The molecule is oxidized by free radicals and then releases energy in the form of light (229). 5 x 10^6 cells/mL in RPMI 1640 plus 25 mM Hepes were incubated with or without different concentrations of hydroxychloroquine (2, 10, 20 and 40 μM) for 1 h. The cells were washed and then primed with TNF-α (10 ng/mL) for 30 min. The respiratory burst was induced in either a receptor-dependent manner by addition of fMLP, or a receptor-independent manner using phorbol-ester PMA. 2 x 10^5 cells in 40 μL culture media were added to 50 μM luminol and stimulated the respiratory burst was stimulated by either 1 μM fMLP or 0.1 μg/mL phorbol-ester PMA, with HBSS in a total volume of 200 μL in a 96-well white microtitre plate. The produced oxidants were measured using the FLUOstar Omega plate reader from BMG Labtech.

2.2.15 Cytokine assays

Human Cytokine 10-Plex Panel from Invitrogen was used to measure the cytokine levels in healthy juvenile control serum and JSLE serum. The assay measured the levels of GM-CSF, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IFN-γ and TNF-α plus IL-17 and IFN-α. The

technology uses polystyrene beads stained with fluorophores of different intensities, each of which has a unique spectral property. Each bead is conjugated to a protein-specific capture antibody and added along with control or patient samples into the 96-well filter microplate where cytokines in each sample bind to the protein-specific capture antibody. Following this, protein-specific biotinylated detector antibodies are added and allowed to bind to the specific cytokines attached to the beads. Excess biotinylated detector antibodies are then removed, and streptavidin conjugated to the fluorescent protein (Streptavidin-R-Phycoerythrin) is added and allowed to bind to biotinylated detector antibody-cytokine-bead complexes establishing a four-member solid phase sandwich. Each complex is quantified by a Bio-Plex Suspension Array System and analyzed using a flow cytometry, model Luminex (230) (Bio-Rad, Hemel Hempstead, UK).

Each 50 μL from healthy juvenile control serum and JSLE serum was pre-incubated with 50 μL blocking buffer for 30 min at room temperature to prevent non-specific antibody binding to the beads using Reacti-bind™ Protein L coated into clear 96-well plate. All samples were transferred to a 96-well filter microplate and the Luminex assay was conducted using duplicate assays of protein standard. 50 μL blocked serum was incubated with 25 μL of the beads conjugated to a protein-specific capture protein for 2 h on an orbital shaker. The samples were

washed with wash buffer following incubation with 100 μL protein-specific biotinylated detector antibodies for 1 h at room temperature. The samples were washed with wash buffer and 100 μL Streptavidin-R-Phycoerythrin was added and incubated for further 30 min. The wells were washed with wash buffer and four-member solid phase sandwiches in each sample were resuspended in 125 μL wash buffer. The plate was analyzed using a Bio-Plex Suspension Array System and the levels of cytokine in each sample were measured and analyzed using reference solutions to generate a standard curve for each cytokine.

2.2.16 Statistical analysis

Statistical analysis was performed by using a combination of parametric and non-parametric statistical analyses. Normal distributed data were analyzed using the parametric Student's t-test or One-way ANOVA followed by Bonferroni's post hoc comparisons tests whilst non-normally distributed data were analyzed using the Mann-Whitney U test and Kruskal-Wallis test and post-hoc testing using Dunn's Multiple Comparison test. Data were expressed as mean ± SEM or median with interquartile range, and differences were considered significant for values of $p \leq 0.05$.

Chapter 3: Effects of JSLE Serum on Neutrophil Function

3.1 Introduction

Increased neutrophil apoptosis plays an important role in the pathogenesis of SLE, particularly JSLE patients (104). The expression of antigens on the surface of apoptotic cells has been suggested as a major source of autoantigens (50, 72, 103). These expressed autoantigens may then induce autoantibody production, leading to the abnormal immune responses that underpin the disease in these patients. However, the actual causes of increased neutrophil apoptosis in lupus patients are still unclear, but there are several possibilities: for example, an imbalance of cytokines in patient serum that normally regulate neutrophil apoptosis (e.g. GM-CSF and TNF-α); an increased expression of death receptors and death receptor associated proteins on the cell membrane (e.g. Fas and FADD); the production of autoantibodies against neutrophil nuclear components (e.g. anti-dsDNA and anti-Ro antibodies) (100–102). These factors may trigger either the *extrinsic* and/or *intrinsic* apoptotic pathways, and subsequently induce neutrophil apoptosis.

The *extrinsic pathway* is activated by factor(s) (e.g. FasL, TRAIL and high dose TNF-α) in serum, which bind to death receptors on the neutrophil cell surface, thereby regulating apoptotic proteins and the caspase cascade inside the cells (42). The caspase cascade is initiated by the activation of initiator caspase 8 into an active (cleaved) form. The

active form of caspase 8 then directly activates executioner caspases (e.g. caspase 3 and 7) into active forms, leading to neutrophil apoptosis, and/or indirectly activates the *intrinsic pathway* via Bid (Figure 1.4). Bcl-2 family member proteins and the mitochondria mainly control the *intrinsic pathway,* which may involve activated Bid (truncated Bid; tBid) which induces the release of cytochrome c from mitochondria into the cytoplasm causing cell death via apoptosome formation (39, 42, 43). Cytochrome c induces Apaf-1 protein to recruit and cleave caspase 9 into an active form. The recruitment of Apaf-1 and the active form of caspase 9 generates the formation of apoptosome which subsequently activates executioner caspases (3 and 7) (42). In human neutrophils, the release of cytochrome c from mitochondria via the intrinsic pathway can be prevented by an important anti-apoptotic protein, Mcl-1 (39).

There are only a few studies that have investigated serum factors in JSLE that may regulate neutrophil apoptosis. Recently, it was demonstrated that addition of JSLE serum to healthy control neutrophils significantly increased neutrophil apoptosis (103, 105). The mRNA levels of caspases 7, 8 and 9 were significantly increased in JSLE neutrophils compared to healthy children controls, and the expression of cleaved caspase 3 was increased in JSLE neutrophils (103). Furthermore, caspase 3 activation and Bid protein expression were further increased upon addition of JSLE sera. These findings suggest that extrinsic and/or

intrinsic apoptotic pathways are activated in neutrophils from patients with JSLE.

Other impairments of neutrophil function have been reported in lupus patients, particularly in adult-onset SLE, leading to susceptibility to infections by different types of microbes, especially bacteria (e.g. *S. aureus* and *E. coli*) (95, 112). Neutrophil phagocytosis is an important process to eliminate bacteria, which requires effective opsonisation of bacteria (by serum proteins), phagocytosis (via binding to neutrophil opsonic receptors) and activation of killing mechanisms. Opsonin receptors on neutrophils include Fcγ receptors and complement receptors (11) which bind to the opsonised pathogens and facilitate that process of phagocytosis. Previous studies have demonstrated decreased neutrophil phagocytosis in SLE patients, possibly because serum autoantibodies directed against CD11b/CD18 (Mac-1) interfered with opsonin receptors on the neutrophil cell surface, or decreased the expression of complement receptor (CR1) on neutrophils, leading to impaired bacterial recognition (113). Other reports have claimed that factor(s) in patient serum could promote neutrophil aggregation by activating neutrophils to overexpress adhesion molecules and subsequently hamper phagocytosis (114, 115).

Bacterial opsonisation by serum is an essential step for efficient killing by neutrophils prior to phagocytosis (231). Pathogenic bacteria, particularly Gram-positive bacteria (e.g. *S. aureus*) are generally

opsonised by factor(s) in serum, such as complement fragments, immunoglobulins or acute-phase reactant proteins. Opsonised bacteria are eventually targeted and eliminated by neutrophils via the process opsono-phagocytosis (4, 232). In lupus patients, dysregulation of serum factor(s) has been described (112). For example, levels of complement components are decreased in lupus patients because the circulating autoantigen-autoantibody complexes consume complement proteins and then activate an abnormal immune response. This condition generally occurs in patients when their disease is active (233). Transiently or permanently decreased levels of immunoglobulin have also been reported, although lupus patients usually present with hyperglobulinemia and increased levels of antibody due to autoantibody production (234). As a result, deficiencies in essential factor(s) in patient serum may contribute to decreased neutrophil phagocytosis. In addition, previous studies showed that JSLE serum significantly induced neutrophil apoptosis compared to healthy juvenile control serum (103), suggesting that enhanced neutrophil apoptosis could be another contributing factor involved in defective neutrophil phagocytosis.

Apoptotic neutrophils have altered cell surface receptors and decreased functions compared to non-apoptotic neutrophils (26). For example, apoptotic neutrophils express lower levels of FcγRIII compared to normal neutrophils (235). Moreover, disruption of cytoskeletal

integrity in apoptotic neutrophils can negatively affect functions such as cell adhesion, migration, and degranulation, as well as phagocytic ability (235). These findings suggest that serum defects and enhanced numbers of apoptotic neutrophils in SLE patients impair their functions and can result in enhanced susceptibility to infections.

Dysregulation of ROS production in SLE has been described in several reports, but the results are still controversial and somewhat contradictory (130, 131). Some reports showed decreased superoxide production in patients compared to healthy controls, whilst other reports demonstrated increased levels of ROS production (124, 131). Therefore, ROS production in JSLE patients needs to be further investigated. In this Chapter, the effects of JSLE serum on neutrophil apoptosis, phagocytosis and ROS production were studied.

3.2 Aims

The aims of this Chapter were to determine if sera from a cohort of Thai patients with JSLE was defective, compared to healthy control sera, in regulating neutrophil functions, such as cell survival and opsonisation of bacteria for effective phagocytosis and activation of the respiratory burst.

Objectives

- To compare rates of apoptosis of healthy control neutrophils after incubation with healthy juvenile control serum, or serum from Thai patients with active or inactive JSLE, as defined by clinical assessment (as described in Materials and Methods).

- To measure the expression of activated caspases (initiator and executioner) and the anti-apoptotic protein, Mcl-1, after incubation of neutrophils with control or patient serum.

- To determine the ability of JSLE serum to opsonise bacteria for subsequent phagocytosis and activation of the respiratory burst.

3.3 Results

3.3.1 JSLE serum induced neutrophil apoptosis

Sera from inactive JSLE (iJSLE) patients (n=12), active JSLE (aJSLE) patients (n=7) and healthy juvenile controls (HC) (n=9) were collected from patients attending clinics at Chulalongkorn University, Thailand. Isolated neutrophils from healthy adult controls were incubated with 10% iJSLE serum, 10% aJSLE serum or 10% HC serum for up to 6h and the percentage of neutrophil apoptosis (and the absolute numbers of apoptotic neutrophils) was measured by flow cytometry. Neutrophils incubated with iJSLE sera or aJSLE sera showed increased apoptosis at 2h, but this did not reach statistical significance (iJSLE sera: median

8.7%, IQ range 6.3-10.1%; aJSLE sera: Median 10.0%, IQ range 5.7-17.1%) compared to HC sera (Median 5.5%, IQ range 4.9-6.4%; $p>0.05$). After 6 h incubation, the levels of apoptosis from neutrophils incubated with iJSLE sera (21.6%, IQ range 19.2-24.7%) and aJSLE sera (22.3%, IQ range 18.6-28.6%) were significantly higher compared to HC sera (15.6%, IQ range 12.2%-18.9%, $p<0.05$). However, there was no significant difference in the mean values and the median levels of apoptosis between neutrophils incubated with iJSLE and aJSLE sera at 2 h and 6 h ($p>0.05$) (Figure 3.1).

3.3.2 Caspase activation in JSLE serum-induced neutrophil apoptosis

To identify whether the intrinsic or extrinsic (or both) apoptotic pathways were activated by JSLE sera, healthy control neutrophils were incubated with inactive iJSLE, aJSLE and HC sera for 6 h, and the levels of expression of activated caspases were measured. Sera from iJSLE, aJSLE and HC that induced levels of apoptosis of 20.5%, 35.5% and 7.1% apoptosis, respectively, were chosen for this study. Levels of activation of caspases -3, -7, -8 and -9 were measured by Western blotting. After incubation with the aJSLE serum, high levels of activated caspases -3 and -7 were detected, compared to levels after incubation with iJSLE serum or HC serum. Both iJSLE and aJSLE sera resulted in activation of caspase -8 compared to HC, but

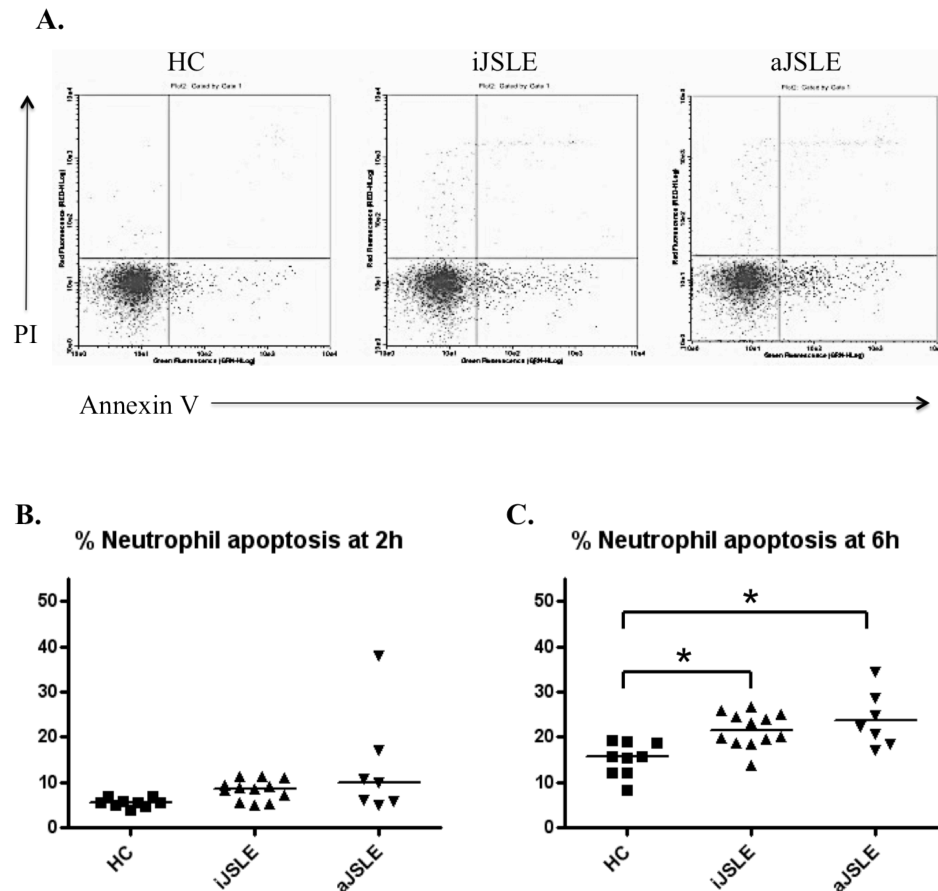


Figure 3.1 JSLE serum-induced neutrophil apoptosis. (**A**) Representative flow cytometry results showing increased apoptosis (upper and lower right quadrant) after incubation of healthy control neutrophils with inactive JSLE sera (iJSLE), active JSLE sera (aJSLE) and healthy juvenile control sera (HC) for 6 h. **(B-C)** Show the percent of apoptosis from neutrophils incubated with inactive JSLE sera (n=12), active JSLE sera (n=7) and healthy juvenile control sera (n=9) at 2 h (**B**) and 6 h incubation (**C**). Data represent median values of three independent experiments using the same sera (* $p<0.05$, Non-parametric One-way ANOVA using Kruskal-Wallis test with Post-hoc using Dunn's Multiple Comparison test).

smaller differences were detected in levels of activation of caspase -9 (Figure 3.2).

The effects of these three sera samples were repeated in three independent experiments on neutrophil preparations from different donors and the band densities of the western blots of each these activated caspases were measured using densitometry (Figure 3.3). The results showed that expression of the active form of caspase -3 in neutrophils incubated with aJSLE serum (0.7 ± 0.05) was significantly increased, compared to iJSLE serum (0.4 ± 0.03, $p<0.005$) and control serum (0.1 ± 0.01, $p<0.005$), whilst there was no significant difference between iJSLE serum and HC serum ($p>0.05$). A similar result was obtained for caspase -7 expression, in that neutrophils incubated with aJSLE serum (0.02 ± 0.005) had significantly increased levels of activated enzyme, compared to those after incubation with iJSLE serum (0.007 ± 0.005, $p<0.05$) or HC serum (0.004 ± 0.0006, $p<0.01$). There was no significant difference in activation levels following incubation with iJSLE serum or HC serum ($p>0.05$). Neutrophils incubated with active aJSLE serum had significantly elevated levels of caspase -8 (0.7 ± 0.05) compared to incubation with iJSLE serum (0.4 ± 0.03, $p<0.005$) or HC serum (0.12 ± 0.01, $p<0.005$). The expression of the active form of caspase -9 expression was elevated after incubation with aJSLE serum (1.45 ± 0.08) compared to iJSLE serum (1.1 ± 0.08, $p<0.005$), whilst there was no

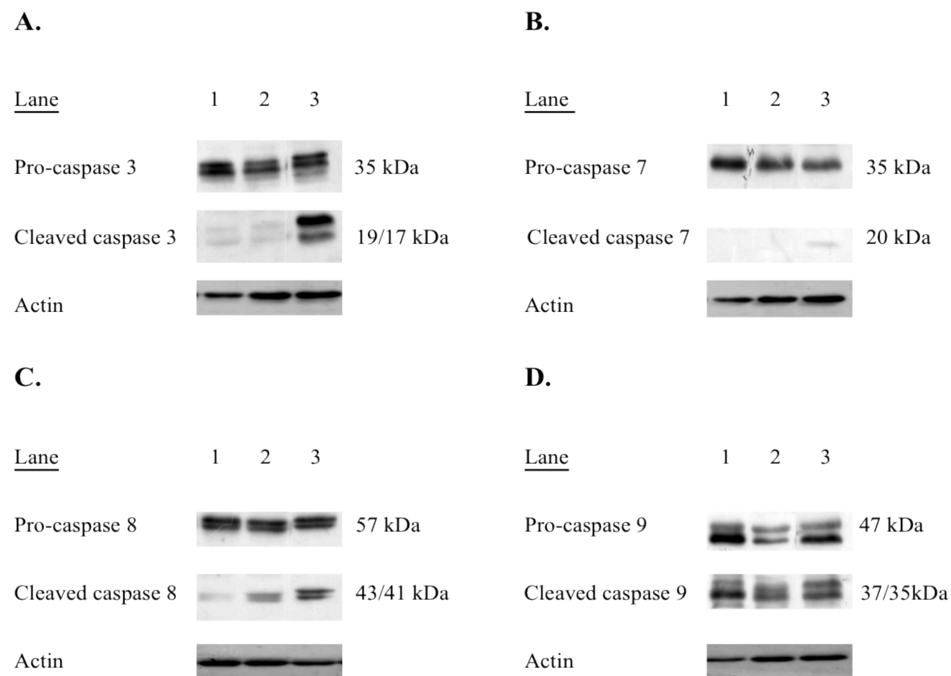


Figure 3.2 Caspase activation in JSLE serum-induced neutrophil apoptosis at 6 h. Representative western blots of protein lysates from neutrophils probed for: **A,** pro-form of caspase -3 (35 kDa) and cleaved form (19/17 kDa); **B,** pro-form of caspase -7 (35 kDa) and cleaved form (20 kDa); **C**, pro-form of caspase -8 (57 kDa) and cleaved form (43/41 kDa) and **D**, pro-form of caspase -9 (47 kDa) and cleaved form (37/35 kDa). Lane 1, neutrophils were incubated with a HC serum (7.1% of neutrophil apoptosis); lane 2, neutrophils were incubated with iJSLE serum (20.5% of neutrophil apoptosis); lane 3, neutrophils were incubated with aJSLE serum (35.5% of neutrophil apoptosis). Also shown is the actin blot to confirm equal protein loading in each lane.

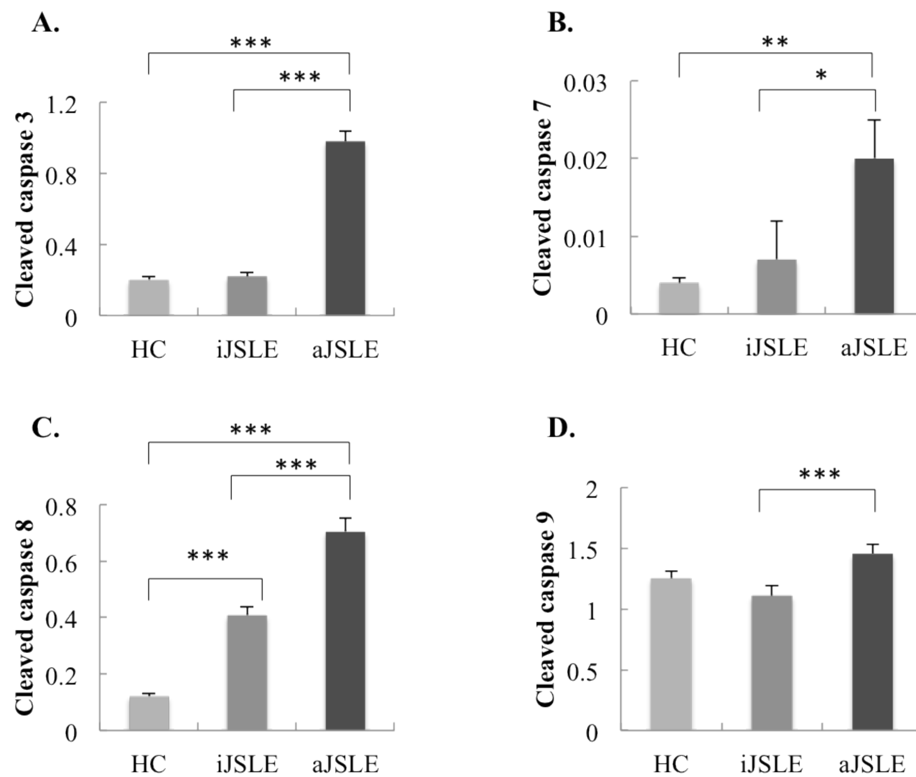


Figure 3.3 Relative expression of activated caspases (normalised to actin) from neutrophils incubated with HC serum, iJSLE serum and aJSLE serum. **A**, cleaved form of caspase -3; **B**, cleaved form of caspase -7; **C**, cleaved form of caspase -8 expression and **D**, cleaved form of caspase -9. Data represent mean ± SEM of three independent experiments (* $p<0.05$, ** $p<0.01$ and *** $p<0.005$, One-way ANOVA followed by Bonferroni's post-hoc comparisons tests).

significant difference between HC serum (1.25 ± 0.06) and inactive or active JSLE serum ($p>0.05$) (Figure 3.3).

3.3.3 Mcl-1 protein expression in JSLE serum-induced neutrophil apoptosis

The anti-apoptotic protein Mcl-1 plays a key role in neutrophil survival, and its levels of expression regulate whether the neutrophil survives or undergoes apoptosis. Neutrophils were incubated with iJSLE and HC sera for up to 6 h and protein lysates were collected (insufficient aJSLE sera were available for these experiments). The expression levels of Mcl-1 were determined by western blotting (normalised to actin). Mcl-1 expression in neutrophils incubated with iJSLE sera (n=8) was lower (at 0.43 ± 0.18) compared to the HC sera, but this was not statistically-significant (n=4, 0.47 ± 0.13, $p>0.05$) (Figure 3.4).

3.3.4 Effect of JSLE serum on bacterial opsonisation and phagocytic ability

Isolated neutrophils from healthy donors, were incubated with or without opsonised PI-labelled *S. aureus* (SAPI), and were stained by Rapid Romanowsky staining (following cytospins) to visualise numbers of engulfed *S. aureus* (Figure 3.5). Quantification of phagocytosed SAPI was achieved using flow cytometry and showed that phagocytosis of SAPI opsonised with iJSLE (28.6 ± 8.3%) and aJSLE (21 ± 8.7%) was

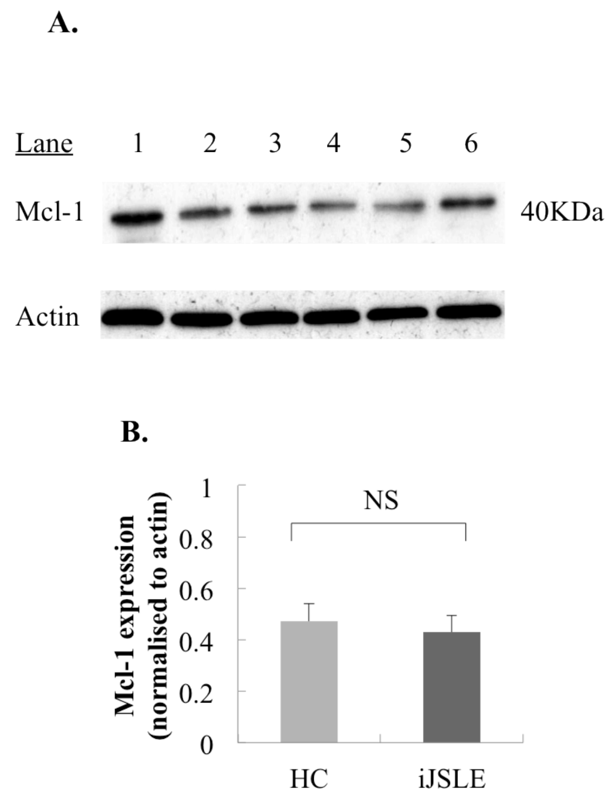
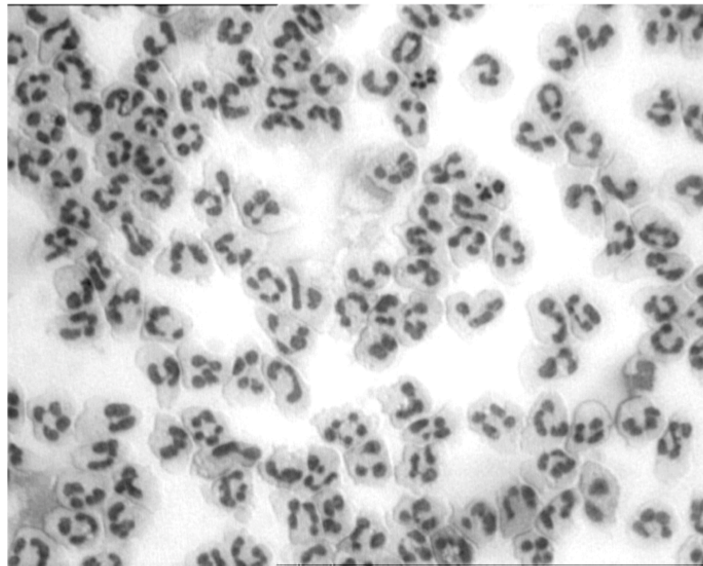


Figure 3.4 Mcl-1 protein expression. **(A)** Representative western blot of neutrophil protein lysates after incubation of control neutrophils with HC sera (lane 1) and iJSLE sera (lanes 2-6) and probed for Mcl-1 (40 kDa). **(B)** Relative expression of Mcl-1 (normalised to actin) from neutrophils incubated with iJSLE sera (n=8) and HC sera (n=4) at 6h. (NS $p>0.05$, Mann-Whitney U test).

A.



B.

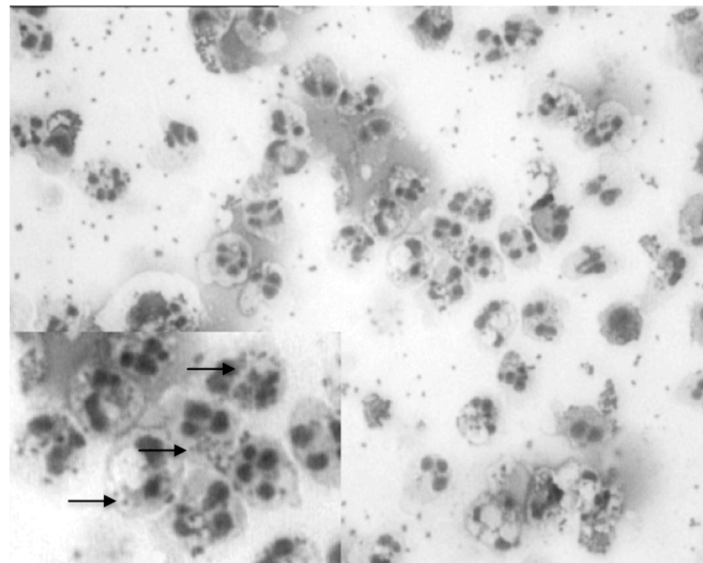


Figure 3.5 Cytospins of isolated neutrophils from healthy donors incubated without (**A**) or with (**B**) opsonised *S. aureus*-PI (SAPI). B shows *S. aureus* were engulfed by neutrophils (small black dots inside neutrophils).

decreased compared to HC serum (37.6 ± 18.6%), but these values did not reach statistical significance ($p>0.05$). Neutrophils only phagocytosed low levels on non-opsonised SAPI (Figure 3.6A).

Figure 3.6B compares phagocytosis by freshly-isolated neutrophils and apoptotic (aged) neutrophils incubated with opsonised SAPI. Neutrophils incubated with 10% human AB serum overnight became apoptotic (70.1 ± 8.5%), and showed lower rates of phagocytosis of SAPI compared to freshly-isolated neutrophils, but these values did not reach statistical significance ($p>0.05$). However, apoptotic neutrophils showed decreased phagocytic ability when incubated with SAPI opsonised with aJSLE (5.0 ± 2.1%) and iJSLE (13.3 ± 1.3%) serum compared to HC serum (21.5 ± 3.3%, $p<0.005$ and $p<0.05$, respectively). Moreover, the phagocytic ability was significantly decreased when apoptotic neutrophils were incubated with SAPI opsonised with aJSLE serum compared with iJSLE serum ($p<0.05$).

3.3.5 Effect of JSLE serum on ROS production

Levels of ROS production by freshly-isolated neutrophils incubated with SAPI opsonised with iJSLE (37.1 ± 4.7%) and aJSLE (33.5 ± 1.4%) were decreased compared to HC serum (51.2 ± 8.9%), but this difference did not reach statistical significance ($p>0.05$) (Figure 3.7). The level of ROS production by freshly-isolated neutrophils incubated with SAPI without opsonisation was lower than for opsonised SAPI, but

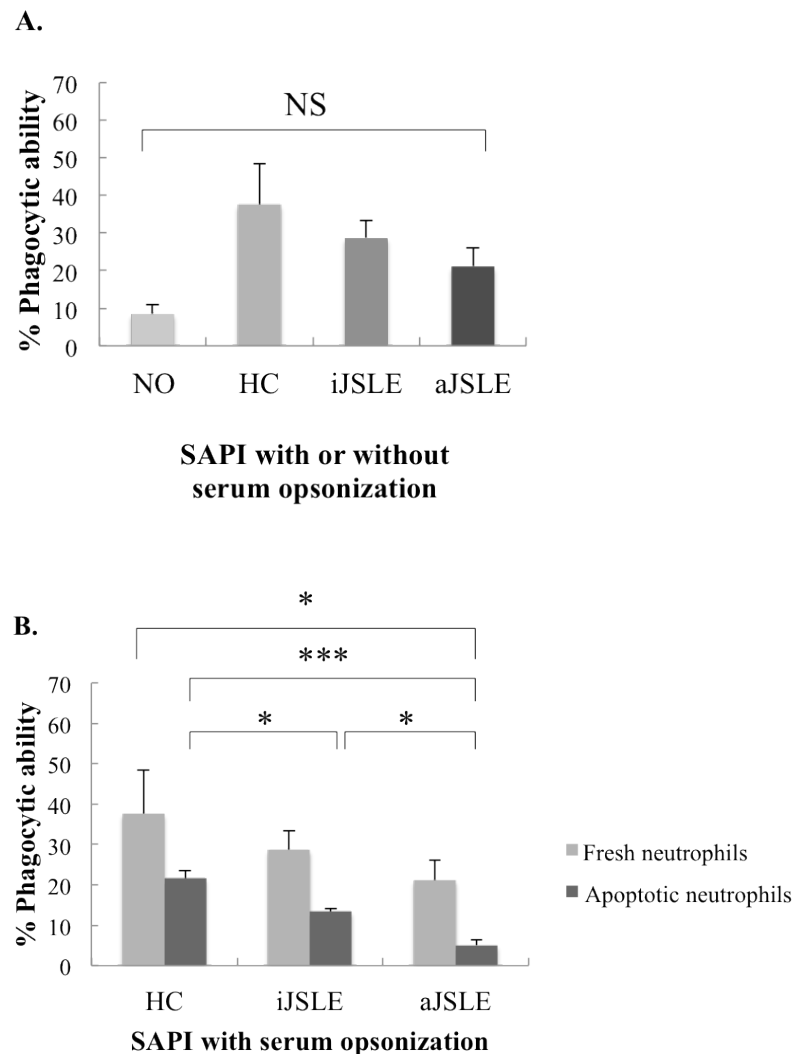


Figure 3.6 (A) Phagocytic ability of freshly-isolated neutrophils incubated with *S. aureus*-PI (SAPI) with or without serum opsonisation. There was a decrease in the level of phagocytosis of SAPI opsonised with iJSLE or aJSLE serum compared to HC serum (HC). Phagocytosis, in the absence of serum opsonisation (NO) was further decreased compared to all other opsonisation conditions. (**B**) Phagocytic ability of freshly-isolated neutrophils or apoptotic (aged) neutrophils incubated with SAPI opsonised with different sera. The level of SAPI phagocytosis was decreased in apoptotic neutrophils compared to freshly-isolated cells. Opsonisation of SAPI with iJSLE serum resulted in significantly lower numbers of phagocytosed SAPI, compared to levels obtained with HC serum, and was further decreased in aJSLE serum (* $p<0.05$, *** $p<0.005$, One-way ANOVA followed by Bonferroni's post-hoc comparisons tests).

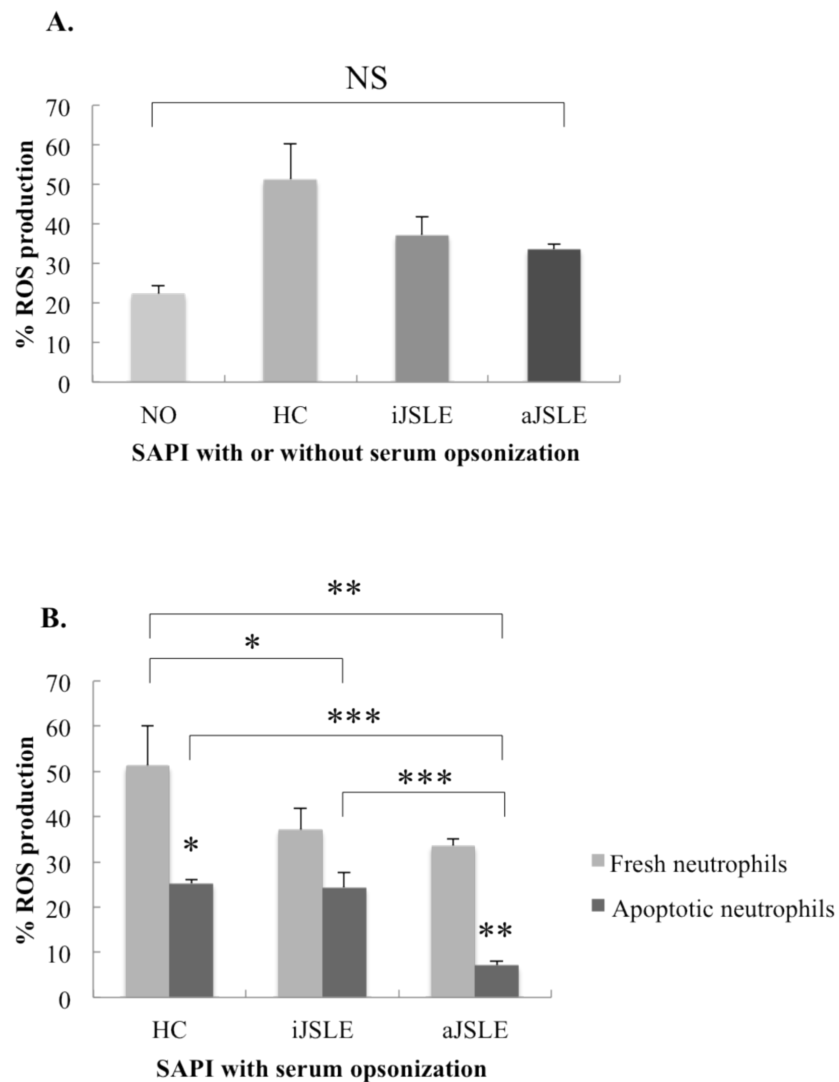


Figure 3.7 (A) ROS production by freshly-isolated neutrophils incubated with *S. aureus*-PI (SAPI) with or without serum opsonisation. There was decreased ROS production with SAPI opsonised with iJSLE or aJSLE sera compared to HC serum. ROS production in the absence of serum opsonisation was further decreased. **(B)** ROS production of freshly-isolated neutrophils or apoptotic (aged) neutrophils incubated with SAPI opsonised with different sera. ROS production was significantly decreased in apoptotic neutrophils incubated with opsonised SAPI particularly with aJSLE and HC. Opsonisation of SAPI with aJSLE serum resulted in significantly decreased ROS production by apoptotic neutrophils, compared to iJSLE or HC serum opsonised SAPI. (* $p<0.05$, ** $p<0.01$, *** $p<0.005$, One-way ANOVA followed by Bonferroni's post-hoc comparisons tests).

no statistical significance was demonstrated ($p>0.05$) (Figure 3.7A).

Apoptotic (aged) neutrophils showed lower levels of ROS production compared to freshly-isolated neutrophils, particularly neutrophils incubated with opsonised SAPI with aJSLE and HC serum ($p<0.05$) (Figure 3.7B). Apoptotic neutrophils incubated with SAPI opsonised with aJSLE serum had significantly decreased ROS production ($7.0 \pm 0.9\%$) compared to iJSLE ($24.3 \pm 3.3\%$) or HC sera ($25.2 \pm 0.8\%$, $p<0.005$). However, there were no statistically-significant differences in ROS production between apoptotic neutrophils incubated with SAPI opsonised in iJSLE serum and HC serum ($p>0.05$).

3.4 Discussion

In a recent study, it was shown that JSLE serum induced increased in apoptosis of healthy control neutrophils, suggesting that factor(s) in patient serum could induce neutrophil cell death (103, 105). Initial experiments in this Chapter confirmed and considerably extended these findings using serum from JSLE patient with a different genetic background i.e. a JSLE population from Thailand. Initial results were consistent with the previous study and showed that JSLE serum from a Thai population increased the level of apoptosis in healthy control neutrophils compared to healthy juvenile control serum. Results in this Chapter show that serum from patients with active JSLE induce

significantly greater levels of apoptosis, as assessed by caspase activation than serum from patients with inactive disease.

A previous study in adult-onset SLE showed that increased levels of neutrophil apoptosis were correlated with disease activity (101). However, the correlation between the level of neutrophil apoptosis and disease activity in juvenile patients has not until now been investigated. In my study, sera from patients with active disease induced higher levels of neutrophil apoptosis compared with sera from inactive JSLE patients. Although the results of annexin V binding assays did not reach statistical significance, this probably reflects a relatively low sample size, and the difficulty in assessing JSLE disease as “active” or “inactive”. Ongoing studies in Thailand are recording SLEDAI scores of patients in preparation for an extension of this study.

JSLE serum has been previously shown to activate both the *extrinsic* and *intrinsic* apoptotic pathways in neutrophils (103, 105) based on the types of caspases that were activated. These previous studies demonstrated that caspase activity was increased after adding JSLE serum, in particular caspases -8 and -9 and the levels of activation of these two caspases by JSLE serum closely correlated with the level of apoptosis (105). Furthermore, the induction of apoptosis observed following addition of JSLE serum, was significantly decreased by inhibitors of caspase -8 and -9 (105). These observations were matched

by the finding that levels of mRNA for caspase -7 and -9 (but not caspase -3) were elevated in JSLE neutrophils, compared to healthy controls. In contrast, mRNA levels for the inhibitors of apoptosis, IAP1, IAP2 and XIAP were all decreased in JSLE neutrophils (105).

The results in this Chapter confirm that JSLE serum induces neutrophil apoptosis and activates caspases, but additionally that serum from patients with active disease induce higher levels of apoptosis and caspase activation, compared to healthy control and serum from JSLE patients with inactive disease. Caspases -3, -7 and -8 (but not -9) were activated by this serum. These findings suggest that JSLE serum induces neutrophil apoptosis largely via the extrinsic pathway (regulated by caspase -8) rather than the intrinsic pathway (largely regulated by caspase -9). To determine whether the intrinsic pathway was activated by JSLE serum, expression of the major anti-apoptotic protein, Mcl-1, was investigated. Unfortunately, serum samples from active JSLE patients were very limited. Only sera from inactive JSLE patients and healthy controls were compared and the results showed the protein expression of Mcl-1 in neutrophils incubated with inactive JSLE serum was lower, but the decrease in expression did not reach statistical significance, compared to expression induced by incubation with healthy juvenile control serum. This suggests that JSLE serum had a tendency to activate via intrinsic

pathway but the testing of serum samples from active JSLE patients would be required to confirm this finding.

Apart from JSLE serum-induced neutrophil apoptosis, another function of neutrophils affected by JSLE serum, is their phagocytic ability. Previous studies have shown that factor(s) in serum from patients with lupus induced neutrophil aggregation leading to decreased neutrophil phagocytosis (114, 115). The reported deficiencies in complement components and immunoglobulins in patient serum, particularly during disease active (233, 234), may contribute to decreased efficiency of bacterial opsonisation. In my study, bacterial opsonisation with JSLE serum and subsequent phagocytic ability by neutrophils were measured. Phagocytosis of *S. aureus* opsonised with JSLE serum and healthy juvenile control serum was compared, and the results showed that the level of phagocytosis of *S. aureus* opsonised with JSLE serum was decreased, and activity was further decreased when the bacteria were opsonised with active JSLE serum. Although the levels did not reach statistical significance, this suggests that JSLE serum (particularly in patients with disease active) is likely to decrease bacterial opsonisation leading to defective neutrophil phagocytosis and hence increased susceptibility to infections.

Defective neutrophil phagocytosis was also observed when freshly-isolated neutrophils were compared with apoptotic (aged)

neutrophils. *S. aureus* opsonised with inactive JSLE serum showed significantly decreased phagocytosis compared to bacteria opsonised with healthy control serum. Moreover, a further decrease of neutrophil phagocytosis was observed in *S. aureus* opsonised with active JSLE serum. Taken together, these findings suggest that defective neutrophil phagocytosis in JSLE patients is probably caused by factors in JSLE serum. These are likely to represent deficiencies in opsonising factors, but alternatively may be an increase in factors that inhibit opsonisation. This observation was supported by results that showed decreased neutrophil phagocytosis by apoptotic neutrophils incubated with bacteria that were opsonised with active JSLE serum.

ROS production was evaluated alongside measurements of phagocytic ability and the results showed similar patterns. For example, the levels of ROS production by freshly-isolated neutrophils incubated with unopsonised SAPI were decreased compared to those observed in response to opsonised bacteria. Apoptotic neutrophils showed lower levels of ROS production compared to freshly-isolated neutrophils. ROS production was further decreased in apoptotic neutrophils incubated with SAPI opsonised with JSLE serum, particularly active JSLE serum. Significantly higher levels of ROS production by freshly-isolated neutrophils incubated with SAPI opsonised by healthy control serum were observed, compared to levels measured in apoptotic neutrophils

incubated with SAPI opsonised by inactive JSLE serum. This finding suggests that ROS production in JSLE patients is decreased probably because of JSLE serum induces neutrophil apoptosis and is less effective in bacterial opsonisation.

Notably, JSLE patients with active disease usually present with decreased level of complement factors in serum (233). Thus, further studies are required to correlate levels of serum complement factors and the efficiency of serum to opsonise bacteria for subsequent neutrophil phagocytosis and ROS production.

In conclusion, the composition and function of JSLE serum is likely to be a major factor involved in the pathogenesis of the disease. It has several effects on neutrophil function. Neutrophil apoptosis induced by JSLE serum, particularly from patients with active disease, is one important pathogenic feature. JSLE serum also a negative effect on neutrophil phagocytosis and ROS production. Therefore, it is important to further investigate the factor(s) in JSLE serum that induce neutrophil apoptosis. In the following Chapter, cytokines that are capable of regulating of neutrophil apoptosis will be studied in the serum of JSLE patients.

Chapter 4: JSLE serum analysis of cytokines and effect of recombinant-cytokines on neutrophils

4.1 Introduction

4.1.1 Role of cytokines in JSLE and effects on neutrophils

Cytokines are major regulators of the immune system which activate cell proliferation, differentiation and maturation (83). An imbalance in the production of cytokines is associated with the immune-pathogenesis of JSLE and adult-onset SLE (216–218). Cytokines are involved in almost every step of normal and abnormal immune responses. They act as mediators connecting one cell to other cells, and can ultimately lead to the tissue and organ damage that is observed in lupus. Increased production of cytokines, such as IL-1, IL-6, IL-17, type I IFNs, TNF-α and B lymphocyte stimulator (BLys) has been described in lupus and their levels usually correlate with the degree of disease activity (146, 219), particularly IL-6, IL-17, type I IFNs and TNF-α (83).

The precise role of cytokines in the pathogenesis of SLE is not fully defined, despite the fact that these molecules have been studied in this disease for many decades (146). IL-6 is one of the first cytokines that was described in SLE, as it is involved in the process of B cell maturation. An important role of IL-6 is to activate the differentiation of B cells into plasma cells producing autoantibodies. IL-6 also down-

regulates levels of membrane CD5 expression in B cells, which allows for the expansion of autoreactive B cells (236). IL-17 is another cytokine that has been recently described in SLE pathogenesis and the role of IL-17 is believed to be similar to IL-6, as it can also activate B cell proliferation and autoantibody production (76). Levels of Type I IFNs have been reported as abnormal in SLE, and are produced by plasmacytoid dendritic cells. The effects of type I IFNs (particularly IFN-α) are to promote dendritic cell maturation and aid the process of antigen presentation by dendritic cells to T-cells. Moreover, they can activate B cell class-switching, B cell maturation and autoantibody production by plasma cells (237). While the role of IL-6, IL-17 and type I IFNs in the pathogenesis of SLE is fairly well defined, the role of TNF-α in the immune-pathogenesis of SLE is still controversial. It has been reported that the production of this cytokine is increased and that levels correlate with disease activity. In contrast, administration of anti-TNF-α therapy in some autoimmune patients, elevates the levels of autoantibody causing lupus-like symptoms (195).

A recent study of JSLE patients has shown a contrasting finding to adult-onset of SLE, in terms of serum cytokine levels. It was reported that certain cytokines in serum, such as IL-6, TNF-α and GM-CSF, are significantly decreased compared to healthy juvenile controls (103). This study suggested that decreased levels of GM-CSF probably have a direct

effect on increased neutrophil apoptosis, leading to autoantigen production and an abnormal immune response in JSLE patients. However, the effects of decreased levels of IL-6 and TNF-α on delayed neutrophil apoptosis are still questionable.

The effects of cytokines on neutrophil apoptosis have been widely studied (238). Evidence shows that IL-1β, IL-2, IL-15, IL-8, IL-18, type II IFNs, G-CSF and GM-CSF can delay neutrophil apoptosis (239, 240). It has been reported that increased levels of these cytokines correlate with delayed neutrophil apoptosis in several diseases, such as rheumatoid arthritis, acute respiratory distress syndrome (ARDS), cystic fibrosis, septicemia and acute pneumonia (241–244). In contrast, the effects of IL-6 on neutrophil apoptosis are more controversial. An *in vitro* study showed that increased rates of apoptosis were observed when neutrophils were incubated with IL-6, but another study showed a contrasting result as neutrophils treated with IL-6 had a lower rate of apoptosis (245, 246). However, it has been reported that IL-6 can prolong neutrophil survival after coronary bypass in patients with unstable angina and acute myocardial infarction (247). Similarly, the effects of TNF-α on neutrophil apoptosis is biphasic (248); high concentrations (>30 ng/mL) promote apoptosis whereas lower concentrations (~10 ng/mL) delay this phenomenon. Therefore, the effect of these cytokines on neutrophil

apoptosis in the pathogenesis of adult-onset SLE and JSLE needs further investigation.

4.1.2 Effect of GM-CSF on JSLE serum-induced neutrophil apoptosis

Granulocyte-macrophage colony-stimulating factor (GM-CSF) is a hematopoietic cytokine produced by activated T cells, macrophages, endothelium, fibroblasts and bone marrow stromal cells (249). It normally functions as an essential growth factor for leukocytes, particularly myeloid lineages. Hematopoietic myeloid progenitors (stem cells), stimulated by GM-CSF together with other cytokines, such as IL-3, IL-4 and IL-5, develop into granulocytes (including neutrophils), monocytes/macrophages and dendritic cells (249).

GM-CSF is a member of the short-chain 4-α-helical bundle family of cytokines that binds to a specific cytokine receptor on the cell membrane (250). The receptors are heterodimeric and composed of two subunits: a common β receptor (βc) subunit and a unique α receptor (GM-CSFRα). GM-CSF firstly binds to the GM-CSFRα on the cell surface and then recruits the βc subunit to initiate intracellular signaling pathways and physiological responses (249). There are at least three major intracellular pathways that have been described following GM-CSF activation: (1) the Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway; (2) the mitogen-activated protein kinase (MAPK) pathway and (3) the phosphatidylinositol 3-kinase (PI3-K) pathway (251). The three

pathways are mainly involved in the processes of cell proliferation/differentiation.

Apart from GM-CSF activation of cell proliferation/differentiation, another of its important roles is to regulate the survival of immune cells, particularly mature neutrophils. It has been demonstrated that GM-CSF significantly delays neutrophil apoptosis by enhancing expression of the anti-apoptotic protein, Mcl-1 (41, 238, 252). The levels of Mcl-1 are increased because GM-CSF stabilizes the protein and also decreases its turnover rate via Erk/Mek and PI3-K/Akt pathways. However, the activation of Erk and Akt is only transient and other kinases (e.g. p38MAPK) may be responsible for regulation of Mcl-1 turnover rate. Conversely, a decrease in expression and increased phosphorylation of the pro-apoptotic protein, Bax via PI3K/Act activation, has also been described in neutrophils treated with GM-CSF, which may also contribute to maintaining neutrophil survival (241). Furthermore, there are many other proteins involved in the control of apoptosis that are regulated by GM-CSF treatment (238). For example, increased phosphorylation of the pro-apoptotic protein, Bad which disrupts its binding to the anti-apoptotic protein Bcl-X_L which then allows Bcl-X_L to protect against the release of cytochrome c from mitochondria: this prevents caspase activation, and prevents neutrophil apoptosis (253). Taken together, these studies clearly show that GM-CSF

can regulate both anti- and pro- apoptotic proteins, including the anti-apoptotic protein Mcl-1 of the intrinsic apoptotic pathway, and prevent neutrophil apoptosis.

4.1.3 Effect of TNF-α on neutrophil apoptosis and JSLE serum-induced apoptosis

It has been reported that serum levels of TNF-α are significantly increased in patients with SLE, particularly those with active disease (207–210). Increased levels of TNF-α have also been observed in glomerular tissue from lupus nephritic patients (209), suggesting that TNF-α plays an inflammatory role in patients with SLE. Moreover, levels of TNF-α are correlated with disease activity. However, one study has shown that SLE patients with inactive disease had higher serum levels of TNF-α compared to healthy controls and patients with active disease, suggesting a protective effect of TNF-α in these patients (207). Therefore, the precise role of TNF-α in the pathogenesis of SLE, is still unclear (195). In addition, anti-TNF-α therapy is largely ineffective in patients with SLE. Furthermore, several studies have shown that patients with other autoimmune diseases who received TNF-α blockers, have developed lupus-like symptoms and increase serum autoantibodies (215). Therefore, anti-TNF-α therapy for patients with SLE, particularly juvenile patients, needs to be carefully considered.

A previous study in JSLE patients showed that serum levels of TNF-α were lower compared to juvenile healthy control sera and low concentrations of TNF-α can delay neutrophil apoptosis (29). Therefore, TNF-α was added to JSLE serum to determine whether TNF-α could protect neutrophils from apoptosis, but no significant difference was observed (103).

TNF-α has dual effects on neutrophil apoptosis: low concentrations (~10 ng/mL) protect against neutrophil apoptosis whilst higher concentrations (>30 ng/mL) promote apoptosis (248). Furthermore, the effects of TNF-α on target cells are varied, and regulated by the different types of surface receptors, TNFR1 (p55/p60) and TNFR2 (p75/p80). Ligation of these receptors can lead to activation of a range of cellular responses, including cell survival, apoptosis, differentiation and proliferation (254, 255). For example, TNF-α signaling can lead to the generation of an apoptotic signal via FADD-mediated activation of caspase 8, whereas TRADD/TRAF-2 (TNF receptor associated factor-2) activation can lead to activation of NF-κB and triggering of downstream events that result in activated gene expression leading to an anti-apoptotic or a proliferative response (256–260). In view of these varied neutrophil activities triggered by TNF-α, and its uncertain role in the pathology of SLE, it is important to determine its role on neutrophil function under conditions that mimic

pathological conditions. It is important to understand their responses to this molecule under conditions that mimic acute and chronic inflammation (e.g. in systemic lupus erythematosus). On the one hand, neutrophils can express TNF-α on their cell surface and release this molecule into the extra cellular environment, thereby triggering events in adjacent cells. On the other hand, they can themselves be stimulated by TNF-α, into either anti- or pro-apoptotic pathways, depending on the local concentration of this cytokine.

4.2 Aims

In the previous Chapter, it was found that JSLE serum significantly induced neutrophil apoptosis compared to healthy control serum. Imbalance in the levels of cytokines in serum from SLE patients, including JSLE, has been reported. It is important to determine the effects of these cytokines on neutrophil apoptosis and measure the levels of these cytokines in the serum. A previous study demonstrated decreased serum levels of GM-CSF in JSLE patients compared to healthy juvenile controls (103). Furthermore, JSLE serum decreased the levels of Mcl-1 expression (as shown in previous chapter). As GM-CSF stabilizes the anti-apoptotic protein, Mcl-1 leading to delayed neutrophil apoptosis (41, 238, 261), it is important to determine whether adding GM-CSF to neutrophils incubated with JSLE serum can increase the levels of Mcl-1 in neutrophils and protect them against apoptosis. In addition, the role of

TNF-α in the pathogenesis of SLE is still unclear (195). Therefore, it is necessary to more clearly understand the role of TNF-α in the pathogenesis of JSLE and other conditions in which this cytokine may play a role in pathology.

Objectives

- To determine the potential of recombinant-cytokines to affect neutrophil apoptosis and to measure the levels of cytokines in the serum of patients with active JSLE, inactive JSLE and healthy controls.
- To establish whether adding GM-CSF to neutrophils incubated with JSLE serum can protect against apoptosis and "reverse" the pro-apoptotic effect of the serum.
- To determine whether GM-CSF can reverse the decrease in Mcl-1 in neutrophils incubated with JSLE serum.
- To determine if GM-CSF could reverse the activation of caspases that is induced by JSLE serum.
- To determine the molecular events that regulate neutrophil responses to TNF-α signaling.

4.3 Results

4.3.1 Effects of recombinant-cytokines on neutrophil apoptosis

Neutrophils isolated from healthy adult controls were treated with the following cytokines; GM-CSF, IL-1β, IL-6, IL-17, IFNα and TNF-α (low and high concentrations) for up to 22 h incubation. The absolute numbers of neutrophils and the percentage of neutrophil apoptosis were compared between untreated and treated cells using flow cytometry (as described in Materials and Methods section). Neutrophils were isolated from healthy adult controls (n=5) and incubated with 10% human AB serum with or without GM-CSF (5 ng/mL), IL-1β (10 ng/mL), IL-6 (10 ng/mL), IL-17 (100 ng/mL), IFN-α (10 ng/mL) and TNF-α (10 or 100 ng/mL) to determine whether any of these cytokines could either save or induce apoptosis. It was found that neutrophils were significantly protected against apoptosis after 22 h incubation with GM-CSF (31.5 ± 4.4%), IL-1β (49.3 ± 3.1%) or low concentrations of TNF-α (55.4 ± 2.5%), compared to untreated controls (70.1 ± 3.7%, $p<0.05$). Neutrophils incubated with IL-6 (69.4 ± 3.9%), IL-17 (72.1 ± 3.6%), IFN-α (75.4 ± 2.1%) and high concentrations of TNF-α (76.2 ± 0.8%) had no effect on apoptosis compared to the control ($p>0.05$) (Figure 4.1).

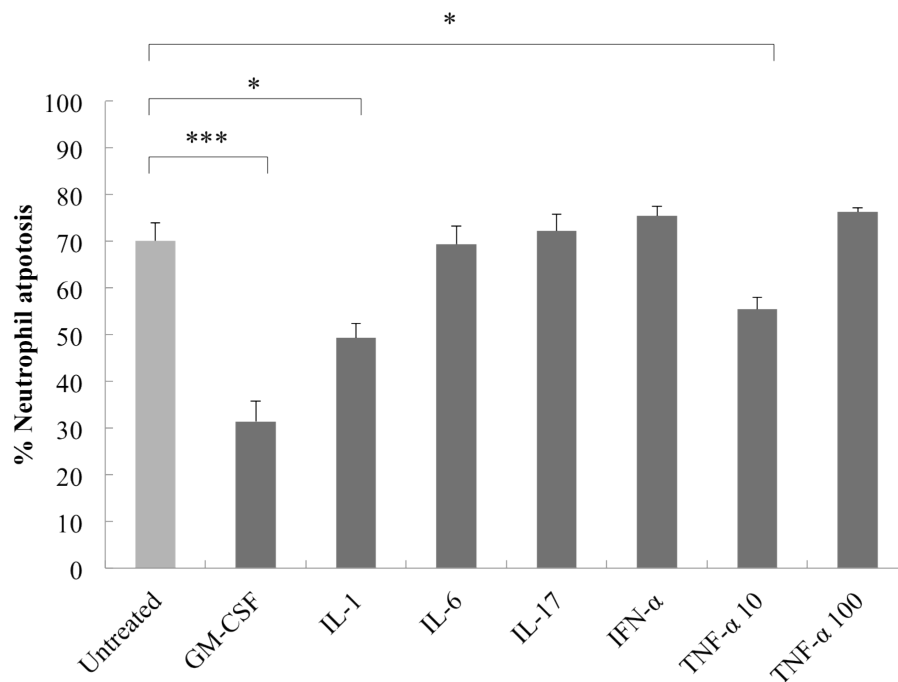


Figure 4.1 Effects of cytokines on neutrophil apoptosis. The graph shows the percent of neutrophil apoptosis after incubation with 10% human AB serum with or without cytokines at 22 h (5 replicates). TNF-α 10 = low concentrations of TNF-α (10 ng/mL) and TNF-α 100 = high concentrations of TNF-α (100 ng/mL) (* $p<0.05$, *** $p<0.005$, Student's t-test).

4.3.2 Analysis of cytokines in JSLE serum

The levels of cytokines in JSLE serum were measured using a Human Cytokine 10-Plex panel and compared between inactive JSLE, active JSLE and healthy juvenile control sera. Table 4.1 summarises the levels of measurable cytokines in JSLE and control sera. The results show that the levels of IL-2, IL-4, IL-8 and IL-10 were detectable in both JSLE and control sera, but only IL-8 levels were significantly higher in active JSLE sera (Median 21.34, IQ range 11.30-58.35) compared to inactive JSLE sera (Median 0, IQ range 0-1.11, $p<0.05$) (Figure 4.2). The levels of GM-CSF, IL-1β, IL-5, IL-6, IL-17, IFN-α, IFN-γ and TNF-α were undetectable in all groups (i.e. GM-CSF < 26.34 pg/mL, IL-1β < 11.25 pg/mL, IL-5 < 7.2 pg/mL, IL-6 < 7.06 pg/mL, IL-17 < 34.48 pg/mL, IFN-α < 19.07 pg/mL, IFN-γ < 21.96 pg/mL and TNF-α < 14.98 pg/mL).

4.3.3 Protective effect of GM-CSF on JSLE serum-induced neutrophil apoptosis

Previous results in this thesis showed that inactive and active JSLE sera significantly induced apoptosis in healthy adult neutrophils at 6h, and Figure 4.1 showed that, of the cytokines studied, GM-CSF was the most potent in delaying neutrophil apoptosis ($p<0.005$). As amounts of active JSLE sera were very limited, sera from inactive JSLE patients (n=3) were used for this study. Isolated neutrophils from a healthy adult

Table 4.1 Concentrations of measurable cytokines in serum (pg/mL) from healthy juvenile controls (n=5), inactive JSLE (n=5) and active JSLE (n=5), as measured by a Luminex assay. Data shown are median values plus interquartile range (IQ range). Statistical analysis carried out using a non-parametric one-way ANOVA, using Kruskal-Wallis with Post-hoc test using Dunn's multiple comparison test (* $p<0.05$).

Cytokines	**Control**	**iJSLE**	**aJSLE**	**Kruskal-Wallis**	**Post-hoc**
IL-2	0.71 (0.68-1.73)	0.66 (0.58-0.71)	0.71 (0.68-0.73)	NS	-
IL-4	4.68 (4.34-5.02)	4.01 (4.01-4.34)	4.01 (3.84-4.34)	NS	-
IL-8	0 (0-1.10)	0 (0-1.11)	21.34 (11.30-58.35)	*	iJSLE VS aJSLE
IL-10	0 (0-2.22)	0.13 (0.05-0.79)	0.07 (0-1.69)	NS	-

Note: The levels of GM-CSF, IL-1β, IL-5, IL-6, IL-17, IFN-α, IFN-γ and TNF-α were below the lower limit of detection amongst all groups (data not shown).

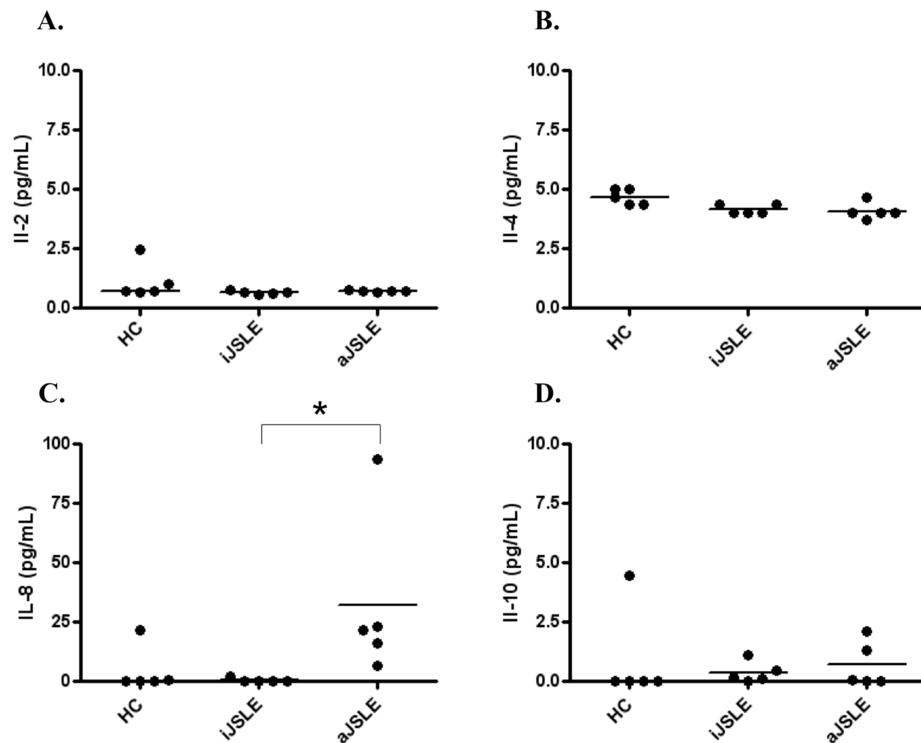


Figure 4.2 Cytokine concentrations in sera from healthy juvenile controls (HC, n=5), inactive JSLE (iJSLE, n=5) and active JSLE (aJSLE, n=5) measured by the Luminex assay; **(A)** IL-2, **(B)** IL-4, **(C)** IL-8 and **(D)** IL-10. Data shown are the median concentrations of each cytokine in serum (represented by horizontal bar), (* $p<0.05$, Non-parametric one-way ANOVA carried out using Kruskal-Wallis with Post-hoc test using Dunn's multiple comparison test).

control were incubated with 10% inactive JSLE serum with or without GM-CSF treatment (5 ng/mL) for up to 6 h. The result showed that adding GM-CSF to neutrophils incubated with inactive JSLE sera resulted in significantly decreased apoptosis at 6 h incubation (6.92 ± 0.92%) compared to the neutrophils incubated in inactive JSLE sera without GM-CSF supplementation (21.79 ± 3.7%, $p<0.05$) (Figure 4.3).

4.3.4 Effect of GM-CSF on JSLE serum-induced loss of Mcl-1 expression

Neutrophils incubated with inactive JSLE serum exhibited significantly increased neutrophil apoptosis and decreased levels of Mcl-1 protein expression (Figure 3.1 and Figure 3.4). GM-CSF stabilizes the levels of Mcl-1 protein expression in parallel with delaying neutrophil apoptosis (41, 238). Therefore, the effects of GM-CSF on Mcl-1 protein expression were determined in neutrophils that were incubated with JSLE serum. Isolated neutrophils from healthy adult controls were incubated with inactive JSLE serum (n=3) with or without GM-CSF treatment for up to 6 h. Protein lysates were prepared and the expression of Mcl-1 was quantified using Western blotting (normalised to actin) and densitometry. The results showed that the relative expression of Mcl-1 in neutrophils incubated with inactive JSLE sera and GM-CSF treatment were significantly higher (relative expression of 0.37± 0.05) compared to neutrophils incubated with inactive JSLE sera without GM-CSF (relative expression of 0.2 ± 0.01, $p <0.05$) (Figure 4.4).

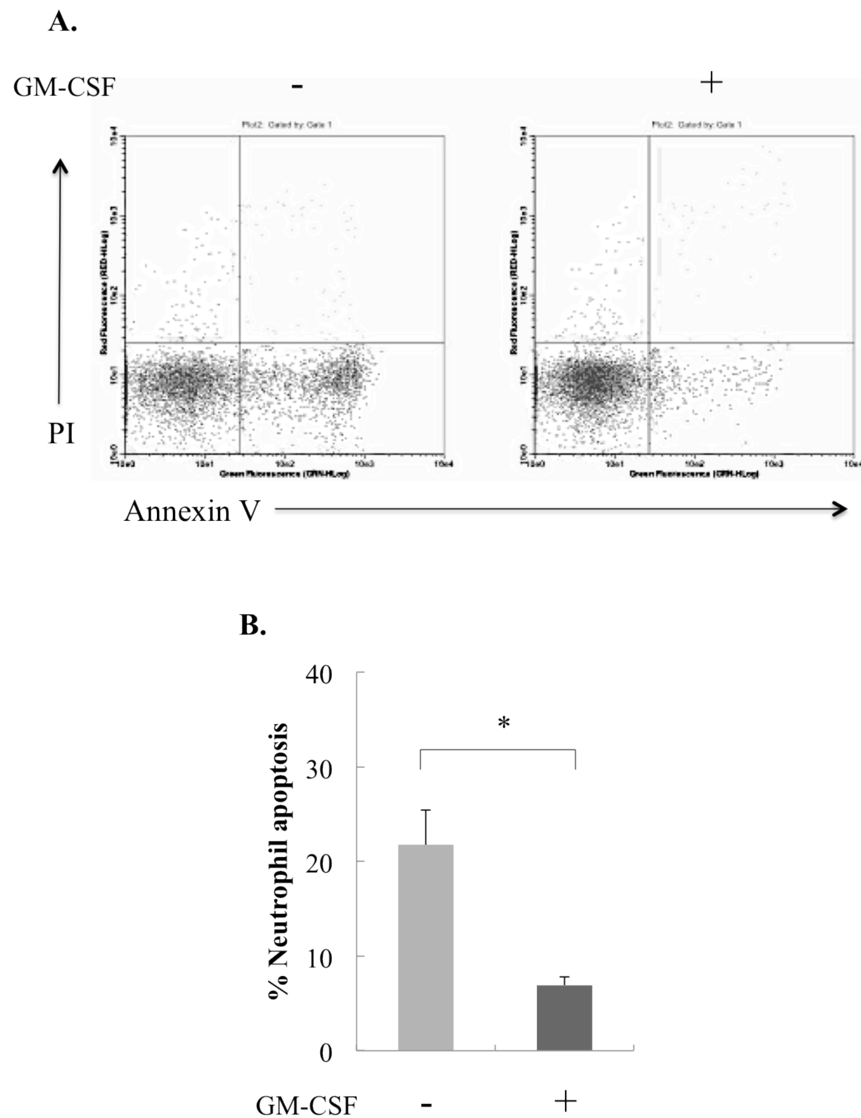


Figure 4.3 Protective effect of GM-CSF on JSLE serum-induced neutrophil apoptosis. (**A**) Representative flow cytometry result showing decreased apoptosis (upper and lower right quadrant) after addition of inactive JSLE serum with GM-CSF to neutrophils at 6 h. **(B)** The graphs show the percent of apoptosis from neutrophils incubated with inactive JSLE sera (n=3) with or without GM-CSF treatment at 6 h incubation, data represent mean ± SEM of three independent experiments (* $p<0.05$, Student's t-test).

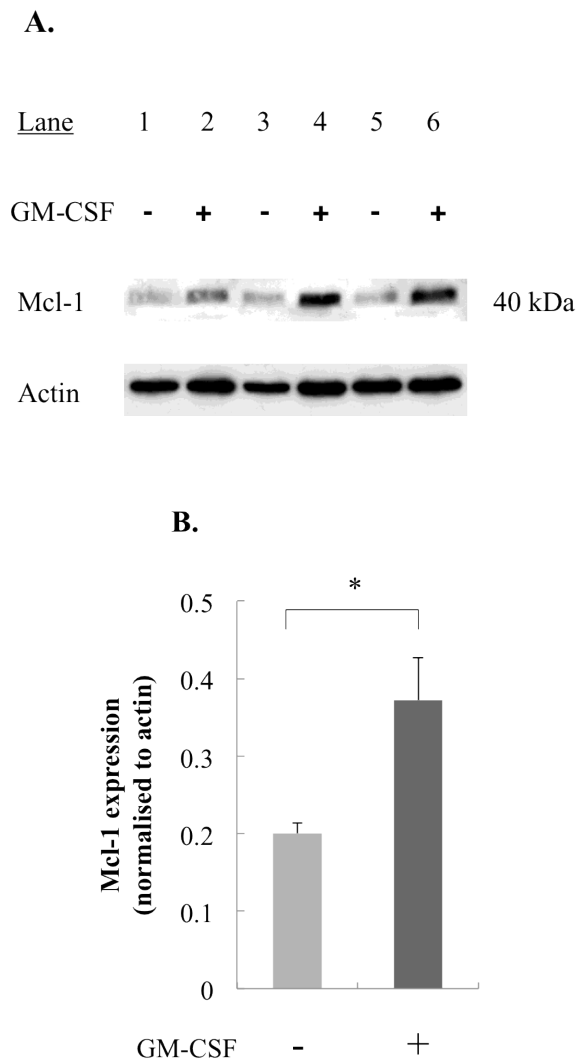


Figure 4.4 Mcl-1 protein expression in neutrophils incubated with JSLE serum and GM-CSF. **(A)** Representative Western Blot of Mcl-1 levels after incubation of neutrophils with inactive JSLE sera, either with (lane 2, 4 and 6) or without (lane 1, 3 and 5) GM-CSF. **(B)** Relative expression of Mcl-1 (normalised to actin) from neutrophils incubated with inactive JSLE sera (n=3) with or without GM-CSF treatment at 6 h. (* $p<0.05$, Student's t-test)

4.3.5 Effect of GM-CSF on JSLE serum-induced caspase activation

Previous work in this thesis has shown that JSLE serum induced apoptosis via the extrinsic and/or intrinsic apoptotic pathways by activating the caspase cascade, via activation of caspases -3, -7, -8 and -9. Therefore, it was important to determine whether GM-CSF could overcome the effect of JSLE serum-induced apoptosis and prevent caspase activation. Neutrophils were incubated with inactive JSLE serum in the presence or absence of GM-CSF for up to 6 h, and protein lysates were prepared. The expression of activated caspases -3, -7, -8 and -9 was measured using Western blotting (normalised to actin) and densitometry. The results showed that neutrophils incubated with inactive JSLE serum and GM-CSF treatment resulted in lower levels of activation of caspases -3, -7, -8 and -9, compared to neutrophils incubated without GM-CSF treatment (Figure 4.5A). The relative expression of the active form of caspase -3 in neutrophils incubated with inactive JSLE serum with GM-CSF treatment was lower than that observed with inactive JSLE serum alone (relative expression 0.08 vs 0.23). This protection against activation was also observed for other caspases; caspase -7 (0.0007 vs 0.08), caspase -8 (0.42 vs 0.52) and caspase -9 (0.04 vs 0.17). Because the low availability of JSLE serum, only single experiments were performed. (Figure 4.5 B, C and D).

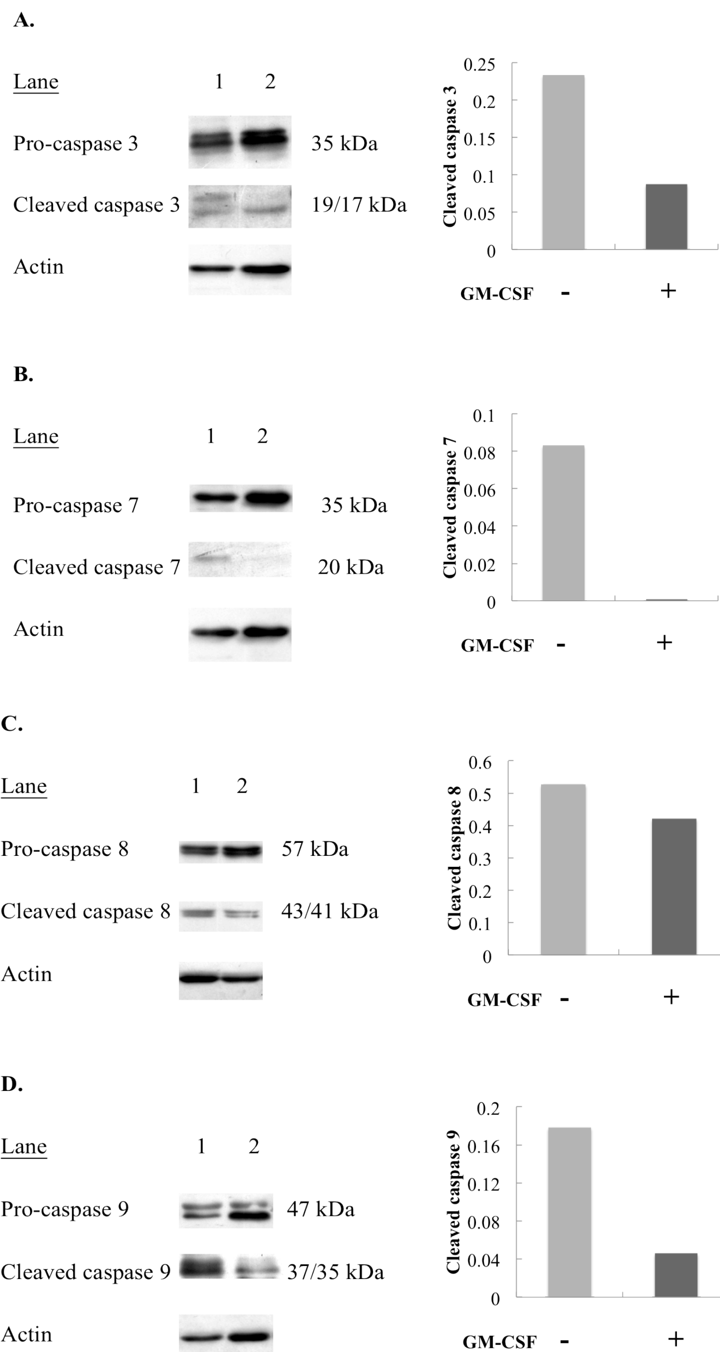


Figure 4.5 Caspase activation in neutrophils incubated with JSLE serum with or without GM-CSF treatment at 6 h. Representative western blots of protein lysates from neutrophils probed for: **A,** Caspase -3; **B,** Caspase -7; **C**, Caspase -8 and **D**, Caspase -9. Lane 1 is neutrophils incubated with inactive serum without GM-CSF, and lane 2 is neutrophils incubated with inactive JSLE serum with GM-CSF. Relative expression of caspases (normalised to actin) is represented in bar graphs.

4.3.6 Changes in expression of genes involved in death receptor signaling

The effects of TNF-α on neutrophil apoptosis have been demonstrated previously (e.g. Figure 4.1), showing that after 22 h incubation of untreated cells, levels of apoptosis were approximately 70.1% (± 3.7 %) whereas in TNF-α-treated cells, the level of apoptosis was significantly lower (55.4 ± 2.5 %, n = 5, $p < 0.05$). It has been previously reported that this concentration of TNF-α also induces gene expression (248, 262) and so a PCR array was initially performed using a Human Apoptosis RT^2 Profiler PCR Array (SA Biosciences) to detect neutrophil transcripts whose levels were regulated by TNF-α treatment (this assay was performed by Dr. Connie Lam and Dr. Kate Roberts). The relative expression of 84 key genes involved the regulation of apoptosis was measured, and 23 were selected for further analysis, based either on their relative transcript abundance in neutrophils (Ct values of 20 or less), or on the basis that their relative levels were affected, positively or negatively, by TNF-α treatment. Repeat experiments on neutrophils isolated from different donors were then performed for individual genes by quantitative PCR experiments (Figure 4.6).

Following the addition of TNF-α (10 ng/mL) to neutrophils for 1 h, one of the key genes whose expression was up-regulated was TNF-α itself, expression of which increased by over 20-fold (Figure 4.6). In addition, mRNA levels for TNFAIP3 (A20) increased by similar amounts

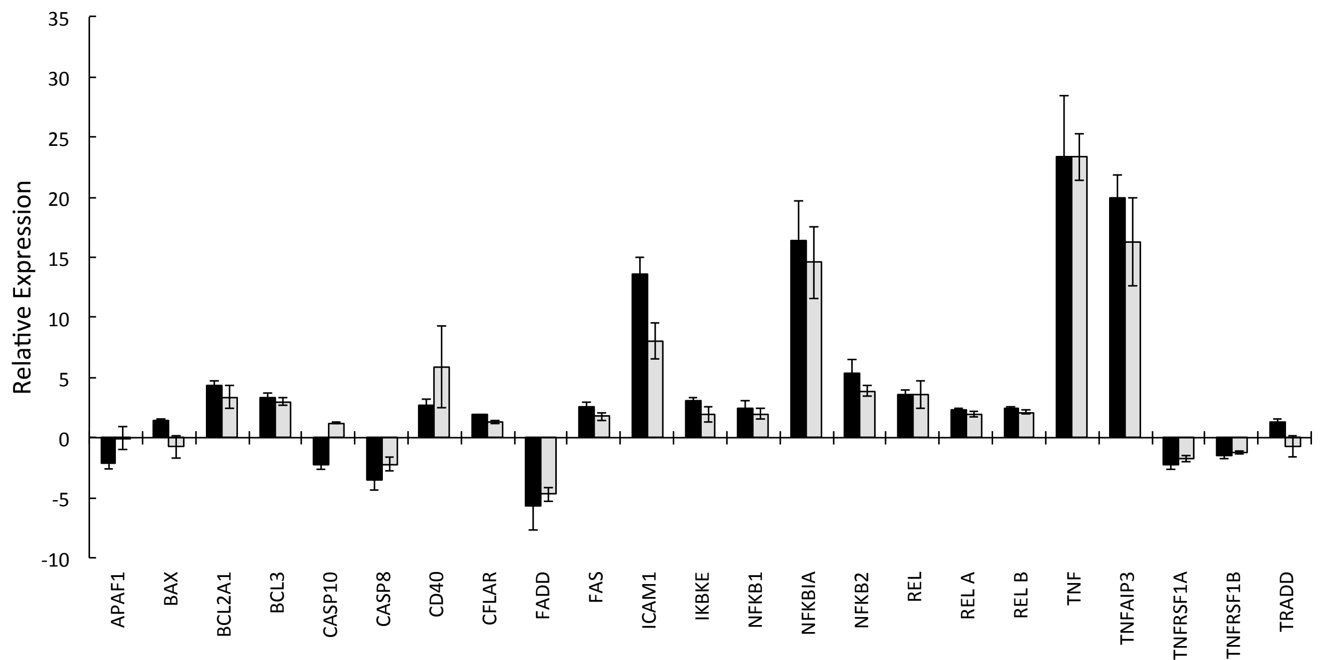


Figure 4.6 Low concentrations of TNF-α stimulate neutrophil gene expression. Neutrophils were incubated for 1 h in the absence (control) or presence of TNF-α (10 ng/mL). Fold changes in mRNA levels were quantified by real-time PCR (normalized to GAPDH) (black bar) and RNA-sequencing (grey bar) (mean fold change ± SEM, n = 3 for each dataset). Abbreviations: BCL2A1 (BFL-1)= B-cell lymphoma 2-related protein A1 or; CFLAR (FLIP)=CASP8 and FAS-like apoptosis regulator of FLICE-like inhibitory protein; TNFAIP3=Tumor necrosis factor α-induced protein 3; Apaf-1=Apoptotic protease activating factor 1; BAX= Bcl-2-associated X protein; CASP10= Caspase 10; CASP8= Caspase 8; FADD= Fas-Associated protein with Death Domain; TNFRSF1A&1B (TNFR1&2)=Tumor necrosis factor receptor superfamily member 1A&1B or Tumor necrosis factor receptor 1&2; TRADD=Tumor necrosis factor receptor type 1-associated DEATH domain protein.

following TNF-α treatment. Transcripts for BCL2A1, CFLAR (FLIP), and FAS were also up-regulated. However, mRNA for some genes involved in apoptosis control was significantly down-regulated following TNF-α treatment. These down-regulated genes included APAF1, CASP8, CASP10, FADD, TNFRSF1A and TNFRSF1B.

In a parallel study in the laboratory, RNA-sequencing was performed to measure the transcriptome of human neutrophils and identify changes induced by similar TNF-α treatment (conducted by Dr. Helen Wright). The real-time PCR results were therefore compared with results of these RNA-sequencing experiments, and both the qualitative and quantitative profiles of altered gene expression observed following TNF-α treatment using these two independent methods, were very similar (Figure 4.6).

Bioinformatics analyses of the changes in gene expression following stimulation with TNF-α for 1 h are shown in Figure 4.7. This scheme was generated by Ingenuity Pathway Analysis software (Ingenuity® Systems, www.ingenuity.com) in which the fold change in gene expression values between the control samples and the TNF-α treated samples are projected onto the Death Receptor Signaling pathway. In this scheme, mRNA levels that decreased following TNF-α treatment are shown in green, while those that increased are shown in red. This scheme predicts that signaling of apoptosis via a range of death receptors

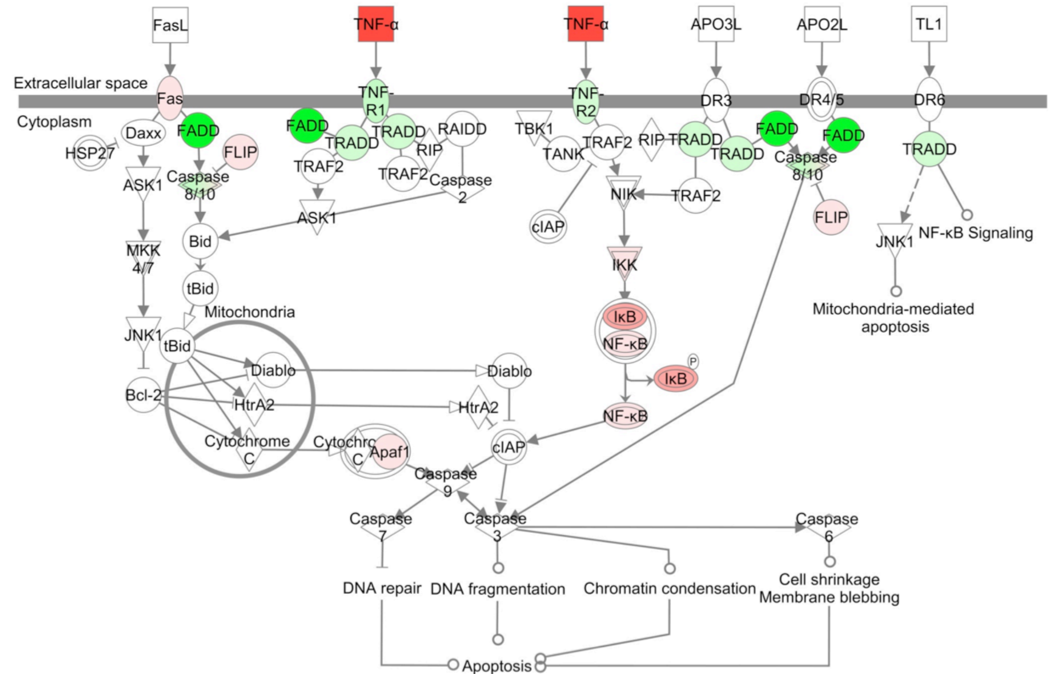


Figure 4.7 Bioinformatics analyses reveal changes in expression of genes associated with the Death Receptor Pathway. Data represent the mean fold change (n=3) in expression following stimulation with TNF-α for 1 h compared to untreated control measured using real-time PCR. Up-regulated genes are shown in red and down-regulated genes are shown in green, the analysis was generated using IPA (Ingenuity). Similar results were obtained when the RNA-sequencing data were analyzed in this way. This analysis was performed by Dr. Helen Wright and Huw Thomas.

would be down-regulated as a result of the re-programming of gene expression following TNF-α treatment of neutrophils.

4.3.7 Time course and concentration dependence of TNF-α induced mRNA changes

The above transcriptome analyses were performed after 1 h incubation in the presence and absence of TNF-α used at 10 ng/mL. It was therefore necessary to determine (a) whether similar changes in gene expression were obtained at higher concentrations of TNF-α and (b) if 1 h was the optimal incubation time to observe these changes in neutrophil mRNA levels following stimulation. To address the first question, neutrophils were incubated for 1 h with 40 ng/mL of TNF-α and changes in gene expression were determined by real-time PCR and compared to the data observed following addition of this cytokine at 10 ng/mL. Figure 4.8 shows no significant differences in mRNA levels after incubation at either concentration of TNF-α. Next, the relative expression of a number of genes that were up- or down-regulated over a period of 6 h following TNF-α exposure was measured. Maximal changes in gene expression were detected by 1 h incubation with 10 ng/mL TNF-α, and extension of the incubation time to either 3 h or 6 h did not result in any changes in expression of those genes of interest, above those observed after 1 h incubation (Figure 4.9).

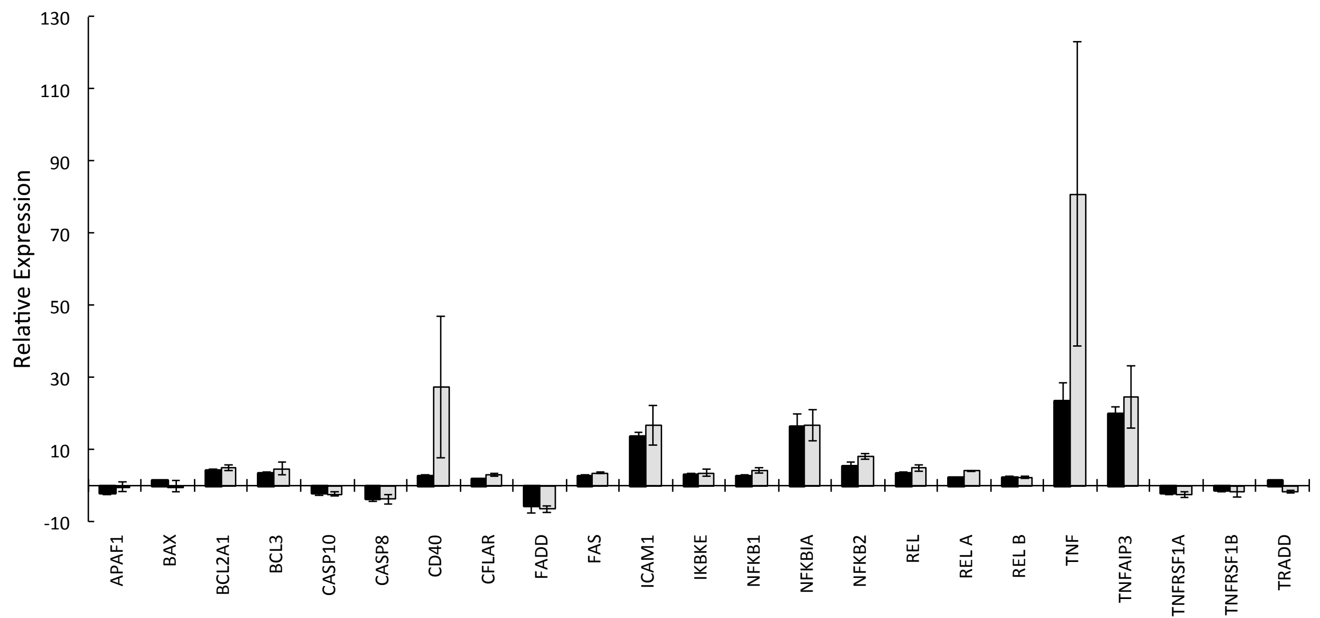


Figure 4.8 Neutrophil gene expression is independent of the concentration of TNF-α. Neutrophils were incubated exactly as described in the legend to Figure 4.6, except that the concentration of TNF-α was 40 ng/mL. After quantitation of transcript levels by real-time PCR, expression levels after incubation for 1 h with 40 ng/mL TNF-α (grey bars) were compared with those measured after incubation for 1 h with 10 ng/mL (black bars) (mean fold change ± SEM, n=3 of each dataset).

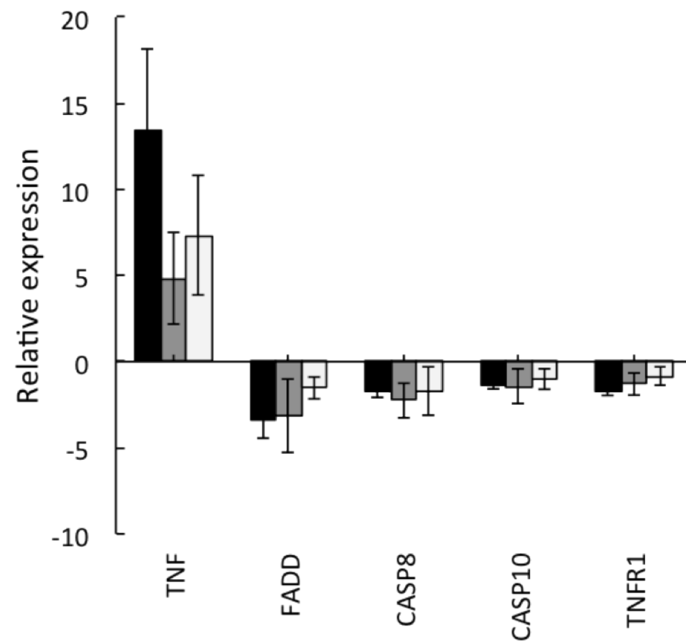


Figure 4.9 Neutrophils were incubated for up to 6 h in the absence or presence of TNF-α (10 ng/mL). Gene expression was analyzed by real-time PCR (normalized to GAPDH). Samples were collected 1 h (black bar), 3 h (grey bar) and 6 h (open bar) after addition of TNF-α. Values shown are mean fold changes (± SEM) of three separate experiments.

4.3.8 Changes in protein levels following TNF-α treatment

To determine if these TNF-α-induced changes in mRNA levels were accompanied by corresponding changes in protein levels, neutrophils were incubated for up to 6 h in the absence and presence of TNF-α (10 ng/mL) and protein lysates were prepared for measurement of protein levels by Western blotting. Very few changes in protein levels were detected following 2 h incubation, but for most proteins under investigation maximal changes in expression were detected by 4-6 h incubation. Figure 4.10 shows representative Western blot data and a summary of quantified data for FLIP, FADD and caspase 10 measured 4 h after TNF-α treatment, while that for TNFR1, TNFR2, TRADD and caspase 8 are shown 6 h after TNF-α treatment. In line with the RNA-sequencing data, expression of FLIP was significantly enhanced following TNF-α treatment ($p<0.05$), while the expression of all other proteins investigated significantly decreased following TNF-α treatment ($p<0.05$). Thus, levels of expression of TNFR1, TNFR2, TRADD and FADD were all significantly decreased after TNF-α treatment.

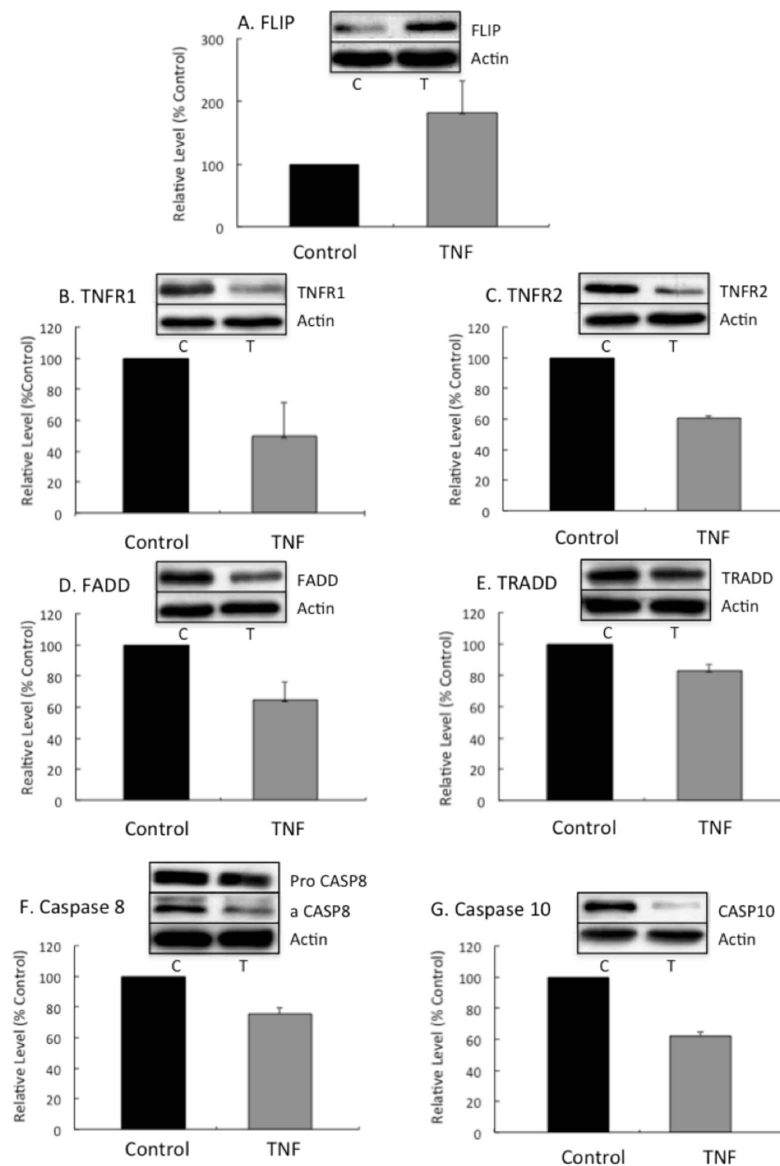


Figure 4.10 Changes in mRNA levels after TNF-α treatment result in parallel changes in expression of pro- and anti-apoptotic proteins. Neutrophils were incubated with TNF-α (10 ng/mL) and at 2 h, 4 h and 6 h incubation, protein extracts were made and subject to Western blotting. In each panel, representative Western blots of the protein of interest are shown, together with the corresponding actin control blot to ensure equivalence of protein load. Underneath each blot in each panel is summary data of three separate experiments showing mean values (± SEM). For all data shown, there was a statistically significant difference between control (untreated) and TNF-α-treated neutrophils ($p<0.05$). Panels A, D and G are analyses after 4 h incubation with TNF-α, while for the remaining panels, samples were analyzed after 6 h incubation.

4.4 Discussion

An imbalance in the production of cytokines in the immune-pathogenesis of JSLE and adult-onset SLE has been previously described (216–218) and such cytokines are major factors involved in the regulation of autoantibody production. The serum levels of cytokines such as IL-1, IL-6, IL-17, type I IFNs, TNF-α and B lymphocytes stimulators (BLys) have been reported to be increased and often show a strong correlation with disease activity (146, 219). For example, IL-6 and IL-17 play a role in B cell proliferation and autoantibody production, whilst type I IFNs support dendritic cell maturation and autoantigen presentation (76, 83, 236, 263). However, the role of TNF-α is still questionable and one study on JSLE patients demonstrated that levels of IL-6, TNF-α were significantly decreased in patient sera (103). This study also showed decreased levels of GM-CSF in JSLE patient sera.

This Chapter firstly set out to determine whether the reported imbalance in production of cytokines also had an effect on neutrophil apoptosis, as it has been proposed that neutrophil apoptosis is one of a major sources of autoantigen in JSLE patients (104). The effect of each of the dysregulated cytokines reported in adult-onset SLE and JSLE were studied. The results showed that GM-CSF, IL-1 and low concentrations of TNF-α significantly delayed neutrophil apoptosis, particularly GM-CSF which showed the strongest protective effect on neutrophil apoptosis

(Figure 4.1). Next, levels of cytokines in JSLE serum were measured to determine whether the serum levels of these cytokine are dysregulated in JSLE patients, in line with some other previous studies. In my study, the levels of GM-CSF, IL-1β, IL-6, IL-17, IFN-α, and TNF-α were below the lower limit of detection of the assay used (i.e. GM-CSF < 26.34 pg/mL). However, the levels of IL-2, IL-4, IL-8 and IL-10 were detectable, but only IL-8 was significantly increased in active JSLE serum, compared to inactive JSLE serum. It has been reported that increased serum IL-8 levels in adult-onset SLE significantly correlated with disease activity, and the levels of IL-8 may serve as an indicator of disease activity in adult-onset SLE (264). Therefore, this finding suggests that the increased levels of IL-8 may be useful to predict disease flare in inactive JSLE patients. However, one previous study could not detect increased serum levels of IL-8 in patients with adult-onset SLE (265); thus, the correlation of serum IL-8 and SLE disease activity needs further studies, and analysis of more serum samples, particularly from patients with active disease is required.

As GM-CSF had the strongest protective effect on neutrophil apoptosis (showed in Figure 4.1), it was important to determine whether GM-CSF could protect neutrophils from the pro-apoptotic effects of JSLE serum. Unfortunately, serum samples from JSLE patients with active disease were very limited; therefore, sera from inactive JSLE

patients (which also induced neutrophil apoptosis), and from healthy juvenile controls were compared. The results showed that GM-CSF was able to prevent the effects of JSLE serum-induced neutrophil apoptosis as it significantly decreased the rate of apoptosis from neutrophils incubated with inactive JSLE serum. In addition, it significantly increased Mcl-1 protein expression and decreased caspase activation; both initiator caspases (-8 and -9) and executioner caspases (-3 and -7). GM-CSF can delay neutrophil apoptosis by stabilizing Mcl-1 protein levels, and this finding showed that adding GM-CSF to neutrophils incubated with JSLE serum overcomes the pro-apoptotic effect of JSLE serum-induced neutrophil apoptosis that may be triggered via the extrinsic and/or intrinsic apoptotic pathways. This may be important for future studies that could consider the use of GM-CSF as an alternative treatment in JSLE patients.

In spite of the fact that IL-1 and low concentrations of TNF-α also significantly delayed neutrophil apoptosis (as shown in Figure 4.1), the possibility of using IL-1 and TNF-α for the treatment in SLE patients is highly unlikely, as both cytokines seem to play an important role in the pathogenesis of inflammation in kidneys (202, 266). Therefore, therapeutic IL-1 and TNF-α for JSLE patients is inappropriate, particularly in view of their underlying kidney problems. As mentioned previously, the actual role of TNF-α in the pathogenesis of SLE is still

controversial. Many studies have shown that serum levels of TNF-α are significantly increased in patients with SLE and are correlated with disease activity (207–210). Nevertheless, another study has shown a contrasting finding of increased serum levels of TNF-α in SLE patients with inactive disease, suggesting a protective effect of TNF-α in disease pathology (207). Therefore, the role of TNF-α in the pathogenesis of SLE, including neutrophil apoptosis, needs to be further studied.

Because of the dual effects of TNF-α on neutrophil apoptosis (248), previous reports have, until now, only provided part of the explanation as to how neutrophils respond to TNF-α. In the experiments described in this Chapter, the molecular events that regulate neutrophil responses to TNF-α signaling were investigated. Understanding the TNF-α signaling pathway could lead to the development of new target therapies, in view of the success of TNF-α blockers in the pathology of many inflammatory conditions. The results showed a novel mechanism whereby human neutrophils undergo a re-programming of gene expression to protect themselves from TNF-α-induced cell death. They develop this protection from cell death by down-regulating the expression of key proteins involved in death receptor signaling (FADD, TRADD, TNFR1, TNFR2, caspase 8 and caspase 10) and in parallel, up-regulate expression of a number of anti-apoptotic genes (BCL2A1, CFLAR and TNFAIP3). These mechanisms would allow neutrophils to survive and

function in TNF-α-rich environments, and enable them to perform their role in inflammation. As TNF-α itself is one of the genes that is markedly up-regulated when neutrophils are exposed to TNF-α, neutrophils can actively contribute to the TNF-α signaling network in inflammation (e.g. in renal tissue of lupus nephritis) without the possibility of autocrine-induced cell death. Taken together, these findings suggest that the role of TNF-α in JSLE may be highly tissue-specific. High concentrations of TNF-α (e.g. in serum) may induce neutrophil apoptosis whilst low concentrations (e.g. in renal tissue) may increase neutrophil survival leading to tissue inflammation and organ damage.

Another important finding of my study was that TNF-α itself was one of the most abundant transcripts expressed by neutrophils exposed to this cytokine. This autocrine production of TNF-α would provide a positive feedback loop for neutrophils to actively contribute to TNF-α signaling in inflammatory conditions, such as SLE and more importantly provide an explanation for their resistance to TNF-α-induced death. Significant down-regulation of a number of genes that control responsiveness to death signals was shown in Figure 4.6. While changes in mRNA levels were maximal 1 h after stimulation with TNF-α, incubation periods in excess of 4 h were required before corresponding changes in protein levels were detected (Figure 4.10). This time course may help explain observations of TNF-α-mediated neutrophil death,

followed by survival. At early time points following TNF-α addition, neutrophils will not have had sufficient time to down-regulate their death-receptor signaling pathways and so will be susceptible to apoptosis.

Quantitative PCR and RNA-sequencing to measure transcript levels in neutrophils following TNF-α treatment were performed and showed a very close correlation between the sets of data generated by these two independent methods. The former method is commonly-used to measure expression levels of specific transcripts in human neutrophils whilst the latter has not been used extensively to quantify the transcriptome of these cells. The benefits of RNA-sequencing over other transcriptome methods (qPCR arrays) are many-fold (226, 228), and together with the ever-decreasing costs of this technology, make this approach an effective and cost-effective way to study neutrophil function, both *in vitro* and *ex vivo*.

In conclusion, the results presented in this Chapter have demonstrated the importance of an imbalance of cytokine production in JSLE serum and have determined the effects of each cytokine on neutrophil apoptosis. The results suggest that serum IL-8 could probably be useful as an indicator of disease activity in JSLE patients, while GM-CSF could potentially be used as an alternative treatment in patients with JSLE. TNF-α has dual effects on neutrophil apoptosis and its effects are probably tissue-specific. The clinical application of TNF-α/ant-TNF-α

treatment in JSLE patients needs to be carefully considered. Understanding the molecular basis of TNF-α signaling will shed new light on the pathogenesis of SLE.

Chapter 5: JSLE neutrophil apoptosis and function

5.1 Introduction

As autoantigens are a major cause of abnormal immune responses leading to tissue and organ damage in adult-onset SLE and JSLE, the source of autoantigens has been widely studied for many years. It has been found that autoantigens are produced and modified inside the apoptotic cells from lupus patients, following by the release of these modified autoantigens into the extracellular compartment (72). Autoantigens are subsequently processed by antigen presenting cells (e.g. dendritic cells) and then presented to CD4+ T cells and B cells resulting in autoantibody production.

Apoptosis or programmed cell death is a fundamental process as cells complete their lifespan, or when they are exposed/attacked by various types of pathogens and mechanical stresses from the environment. This controlled form of cell death allows apoptotic cells to limit the release of their intracellular components into surrounding tissues (25). The apoptotic cells are usually recognized and cleared by phagocytic cells (e.g. macrophages), otherwise the cells will undergo secondary necrosis and their intracellular components will be released to activate macrophages to produce pro-inflammatory cytokines (e.g. TNF-α and IL-8) leading to local tissue inflammation (267).

Abnormal apoptotic cells and their defective clearance by phagocytic cells have been described in SLE (72, 268). These abnormal apoptotic cells can escape from phagocytic clearance and undergo secondary necrosis (267), in which their intracellular components including autoantigens (e.g. DNA, chromosome and histone) are released into extracellular compartment. The autoantigens are subsequently captured by immunoglobulins and complement factors forming immune complexes. These immune complexes activate antigen-presenting cells and B cells to produce further autoantibodies (72). On the other hand, the abnormal apoptotic cells may be engulfed by phagocytic cells (i.e. macrophages) and transferred to lysosomes for elimination. However, if there are defects in the degradation process or if it is overloaded, the degraded products may be able to activate innate immune cells, particularly plasmacytoid dendritic cells, leading to pro-inflammatory cytokine production (e.g. type I IFN and TNF-α) and abnormal immune responses (267).

There are only a few reports describing the role of apoptotic cells particularly neutrophils (the most abundant white blood cells) in the pathogenesis of JSLE. However, one study has demonstrated that JSLE neutrophils are altered and have increased rates of constitutive apoptosis (103). Moreover, an imbalance of pro- and anti- apoptotic protein expression inside JSLE neutrophils has been described. The altered

apoptotic neutrophils from JSLE patients activate peripheral blood mononuclear cells to produce type I IFN, a key mediator in the pathogenesis of SLE, leading to abnormal inflammatory responses (50).

One of the most important intracellular molecules regulating neutrophil apoptosis is the anti-apoptotic protein, Mcl-1. This anti-apoptotic protein inhibits neutrophil apoptosis by blocking the release of cytochrome c from the mitochondria. The expression of this protein is controlled by numbers of cytokines and growth factors (39). In response to cell activation by these factors, the Mcl-1 gene will be transcribed into mRNA, and subsequently translated into anti-apoptotic protein Mcl-1. However, mRNA Mcl-1 can exist in two isoforms, which are Mcl-1 long form (Mcl-1_L) and Mcl-1 short form (Mcl-1_S). Mcl-1_L is the product of normal transcription of the Mcl-1 gene and translation of the full length mRNA. It prevents apoptosis by binding to intracellular pro-apoptotic proteins (such as Bak and Bax). On the other hand, Mcl-1_S is formed by alternative splicing and loss of exon 2 from the full length mRNA of the Mcl-1 gene (which contains 3 exons) (39). The loss of exon 2 generates a short protein Mcl-1_S that can bind to and inhibit full length Mcl-1 and induce apoptosis (269). Mcl-1_S has the properties of a BH3-only protein. Overexpression of Mcl-1_L has been described in hematological malignancies (such as chronic myeloid leukemia), whilst up-regulation of

$Mcl\text{-}1_S$ has been reported during infections (270, 271). The expression of these two forms in JSLE neutrophils has never been reported.

Apart from abnormal neutrophil apoptosis, impaired neutrophil functions have also been described in SLE patients. These defects include: decreased phagocytic ability (112); overexpression of inflammatory receptors on the cell surface, such as FcγRI (CD64) and complement receptor CR3 (CD11b/ITGAM), leading to increased downstream inflammatory responses (115, 120, 121); abnormal neutrophil chemotaxis (97, 123, 124).

In my previous Chapter, I showed that factor(s) in serum from patients with JSLE demonstrated several effects on healthy control neutrophils, such as cell survival, phagocytosis and ROS production. In this Chapter, the functions of JSLE neutrophils were characterized.

5.2 Aims

The aims of this Chapter were to determine if neutrophil functions from JSLE patients were defective, compared to healthy juvenile controls.

Objectives

- To compare rates of neutrophil apoptosis

- To measure the mRNA expression level of the anti-apoptotic gene, Mcl-1 long and short forms.
- To quantify JSLE neutrophil phagocytosis and chemotaxis.

5.3 Results

5.3.1 JSLE neutrophil apoptosis

Neutrophils isolated from JSLE patients with inactive disease (iJSLE, n=5) and healthy juvenile controls (HC, n=5) were cultured with human AB serum for up to 2 h. The rate of neutrophil apoptosis was measured at time 0 and 2 h incubation using flow cytometry. The results showed increased rate of neutrophil apoptosis in iJSLE patients (3.4 ± 0.7%) compared to HC (5.4 ± 0.7%) at time 0; however, this did not reach statistical significance ($p>0.05$). Following 2 h incubation, there was no significant difference in the rate of neutrophil apoptosis between iJSLE patients (8.1 ± 1.8%) and HC (9 ± 2.4%, $p>0.05$) (Figure 5.1).

5.3.2 Mcl-1 mRNA expression in JSLE neutrophils

To determine if increased constitutive apoptosis if JSLE neutrophils was associated with increased expression of the pro-apoptotic Mcl-1_S, mRNA was extracted from JSLE neutrophils (n=4) and healthy control neutrophils (n=4). Mcl-1_L and Mcl-1_S expression were determined by real-time PCR and normalised to actin mRNA. There was no difference in the relative expression levels of Mcl-1_L between JSLE

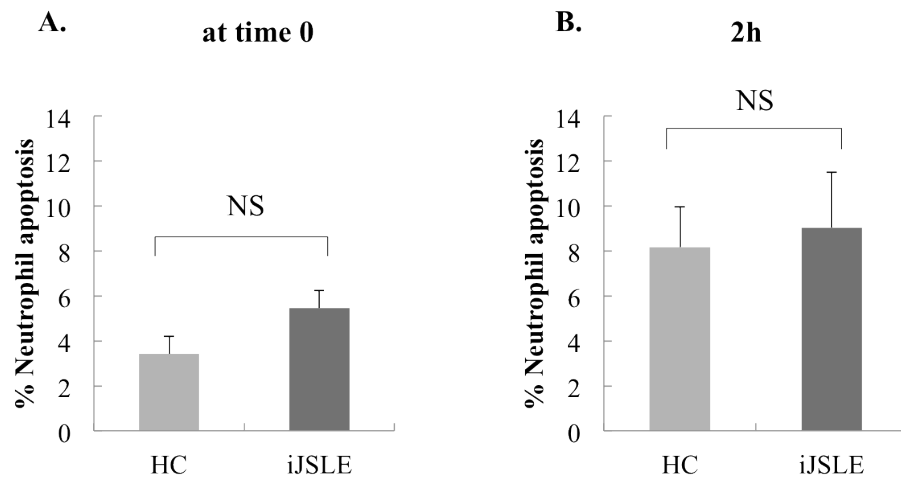


Figure 5.1 JSLE neutrophil apoptosis. The graphs show the percentages of neutrophil apoptosis from inactive JSLE patients (iJSLE, n=5) and healthy juvenile controls (HC, n=5) at time 0 (**A**) and 2 h incubation (**B**). Data represent mean ± SEM (NS $p>0.05$, Student's t-test).

neutrophils (1.33 ± 0.51) and healthy control neutrophils (1.31 ± 0.54, p>0.05). The relative expression of Mcl-1_S in JSLE neutrophils was 0.99 ± 0.40, compared to the healthy control neutrophils (1.14 ± 0.67, p>0.05) (Figure 5.2).

5.3.3 JSLE neutrophil phagocytosis

Figure 5.3 compares phagocytosis by neutrophils from inactive JSLE patients (iJSLE) and healthy juvenile control neutrophils (HC) incubated with *S. aureus* that had been stained with PI and opsonised by human AB serum (opsonised SAPI) for 30 min. The percentage of neutrophils showing phagocytic activity was measured using flow cytometry. The results showed that iJSLE neutrophils incubated with opsonised SAPI had lower rates of phagocytosis of SAPI (69.3 ± 5%) compared to HC neutrophils (74.9 ± 1.5%), but these values did not reach statistical significance (p>0.05).

5.3.4 JSLE neutrophil chemotaxis

To determine whether JSLE neutrophils decrease chemotactic activity, chemotaxis assay was performed using Millipore Hanging Cell Culture plate inserts in 24-well tissue culture well plates pre-coated with poly (2-hydroxyethyl methacrylate). IL-8 (100 ng/mL) was added into each bottom well as a chemoattractant. Freshly-isolated neutrophils from inactive JSLE patients (iJSLE, n=5) and healthy juvenile controls (HC, n=5) were added into hanging inserts and they were incubated in an

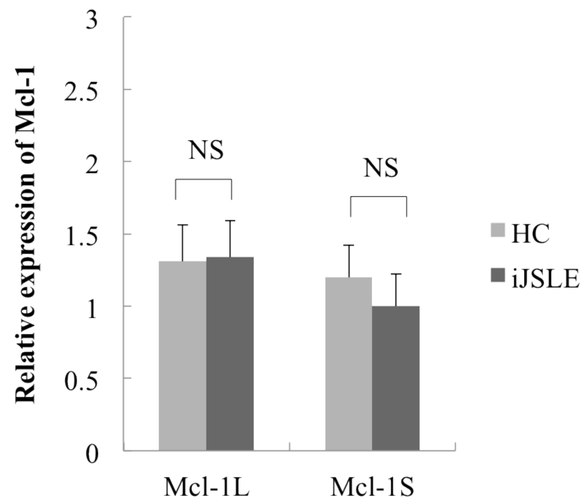


Figure 5.2 Relative expression of Mcl-1_L and Mcl-1_S (normalised to actin). There was no significant difference in relative expression of Mcl-1_L and Mcl-1_S between JSLE neutrophils (n=4) and healthy control neutrophils (n=4), Data represent mean ± SEM (NS p>0.05, Student's t-test).

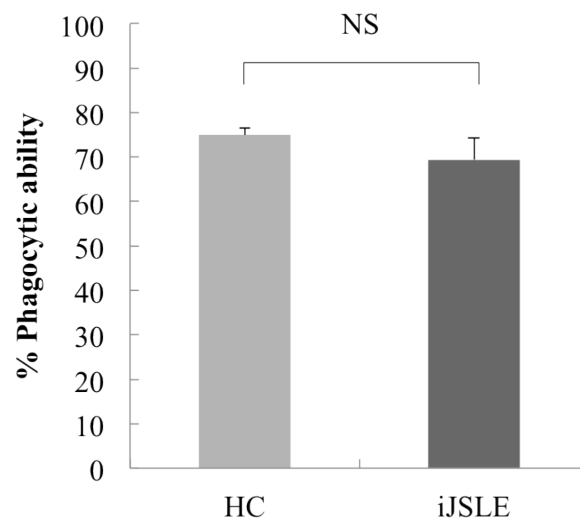


Figure 5.3 JSLE neutrophil phagocytosis. Phagocytic ability of inactive JSLE neutrophils (iJSLE) incubated with opsonised *S. aureus*-PI (SAPI). There was no significant difference in the level of phagocytosis of opsonised SAPI by iJSLE neutrophils (n=3) compared to healthy juvenile control neutrophils (HC, n=3). Data represent mean ± SEM (NS $p>0.05$, Student's t-test).

incubator at 37°C for 90 min. Migrated neutrophils in each well were counted and calculated as a percentage of the total number of cells originally added. Figure 5.4A demonstrates the chemotaxis assay used in this study.

The result showed that neutrophils isolated from both iJSLE patients (25 ± 2.3%) and HC (21 ± 2.8%) showed chemotactic responses to IL-8 and the cells moved through the hanging inserts into the bottom wells, compared with cells without IL-8 treatment ($p<0.05$). However, the percentages of migrated neutrophils responding to IL-8 from iJSLE patients and healthy juvenile controls were not statistically-significant ($p>0.05$) (Figure 5.4B).

5.4 Discussion

Dysregulated neutrophil apoptosis has been reported as a key event in the pathogenesis of JSLE (50, 103, 104). It has been proposed that increased neutrophil apoptosis is a major source of autoantigen exposure, inducing T and B cell activation, leading to abnormal immune responses in adult-onset SLE and JSLE patients (72, 268). In my previous Chapter, it was demonstrated that JSLE serum significantly induced neutrophil apoptosis, particularly JSLE serum from patients with active disease. However, the results in this Chapter showed that freshly-isolated JSLE neutrophils had slightly higher rates of apoptosis immediately after isolation compared to healthy juvenile control neutrophils, but this did

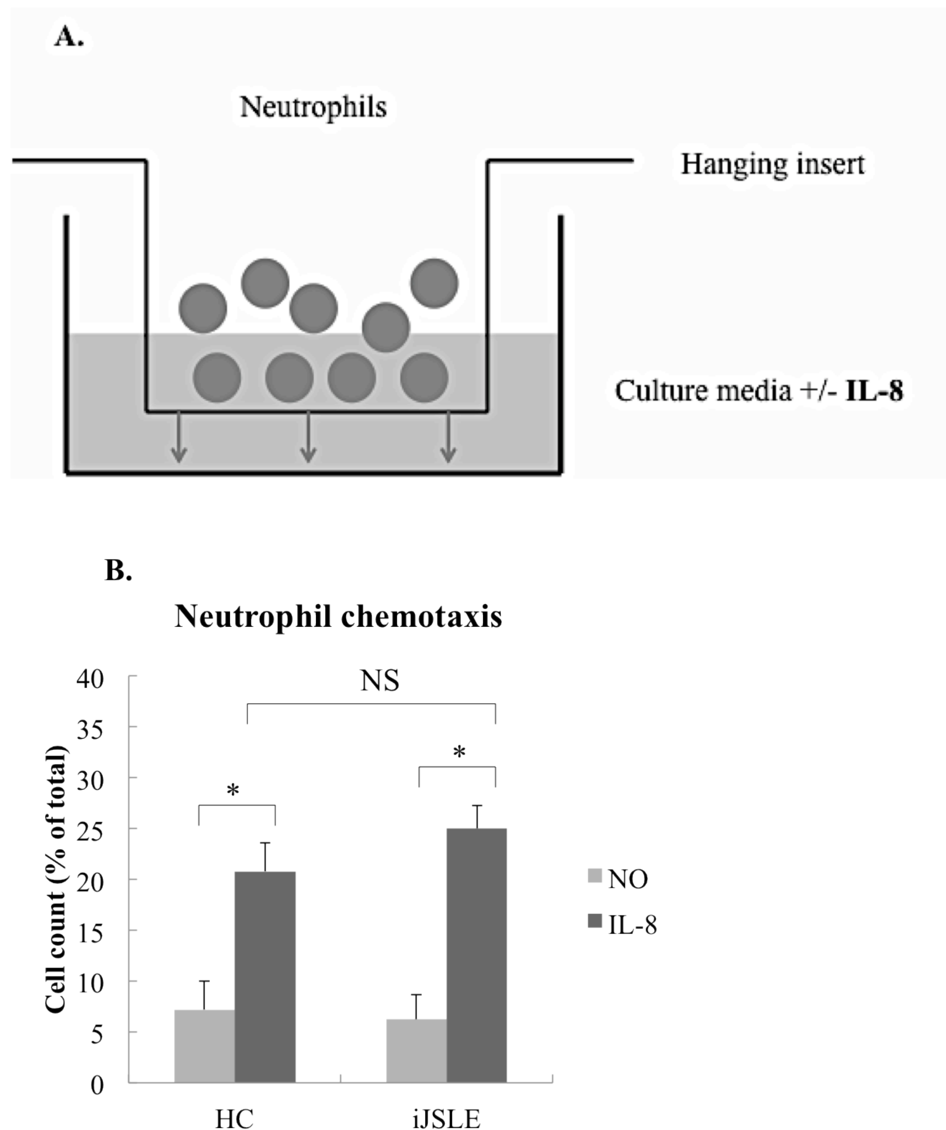


Figure 5.4 JSLE neutrophil chemotaxis. **(A)** Neutrophil chemotaxis assay was performed using Millipore Hanging Cell Culture plate inserts. IL-8 (10 ng/mL) was added as a chemoattractant. Neutrophils were added into hanging insert and incubated for 90 min. **(B)** Migrated neutrophils from inactive JSLE patients (iJSLE, n=5) and healthy juvenile controls (HC, n=5) in the bottom wells with chemoattractant (IL-8) or without treatment (NO) were counted and calculated as a percentage of the total number of cell originally added. Data represent mean ± SEM (* $p<0.05$, Student's t-test).

not reach statistical significance. This finding suggests that JSLE serum is a major actor that induces neutrophil apoptosis in JSLE patients rather than JSLE neutrophils themselves. Nevertheless, Figure 5.1 shows higher rate of apoptosis in JSLE neutrophils at time 0, compared to healthy juvenile control neutrophils while the rate of apoptosis at 2 h incubation was similar between both groups. Although, this higher rate of JSLE neutrophil apoptosis at time 0 did not reach statistical significance, this may represent higher rates of apoptosis *in vivo*. After 2h incubation, JSLE neutrophils were incubated with human AB serum, that probably reversed the effect of JSLE serum *in vivo,* and subsequently protected JSLE neutrophils from apoptosis at 2 h. However, longer time points and experiments using healthy juvenile control serum are required to support the assumption that JSLE neutrophils could be saved by human AB serum and/or healthy juvenile control serum. It would also be interesting to have performed similar experiments with neutrophils isolated from JSLE with active disease, and this should be investigated in future studies.

Mcl-1 is an important intrinsic anti-apoptotic protein regulated by expression of the *Mcl-1* gene. Processing of the mRNA can generate 2 isoforms via alternative splicing, Mcl-1_L and Mcl-1_S. Interestingly, both isoforms have different functions. Mcl-1_L is derived from full length mRNA and the protein delays apoptosis, whilst Mcl-1_S is a splicing

variant and it induces apoptosis (39, 269). Abnormal expression of Mcl-1_L and Mcl-1_S has been reported in several conditions, but it has never been reported in JSLE patients (270, 271). Therefore, it was interesting to determine whether the expression of these two isoforms in neutrophils isolated from JSLE patients is dysregulated. However, the results showed that there was no significant difference in the expression of both isoforms in JSLE neutrophils compared to healthy juvenile control neutrophils.

Decreased phagocytic ability by neutrophils has been reported in SLE patients (112). In the previous Chapter, it was demonstrated that defective neutrophil phagocytosis in JSLE patients is probably caused by factor(s) in JSLE serum, particularly when apoptotic neutrophils were incubated with opsonised bacteria with active JSLE serum. The aim of this Chapter focused on JSLE neutrophil function; however, the results showed that there were no significant differences in the percentages of phagocytic neutrophils between the two groups.

Another crucial function of neutrophil is chemotaxis and it has been reported that neutrophil chemotaxis in patients with SLE is impaired (97, 123). Nevertheless, the results are still controversial and not confirmed in other reports. Neutrophil chemotactic activity has been reported as significantly decreased and correlated with a high incidence of infection in SLE patients, while hyperactivity of neutrophil chemotaxis was described in another study (124). In my results, the chemotactic

activity of JSLE neutrophils was not significantly different to healthy controls, suggesting that the susceptibility to infection in JSLE patients is not caused by defective JSLE neutrophil chemotaxis, but probably results from JSLE serum-induced neutrophil apoptosis and ineffective bacterial opsonisation by JSLE serum.

However, the JSLE neutrophils in this Chapter were only isolated from patients with inactive disease, and further studies using neutrophils from patients with active disease should be performed.

In conclusion, the results in this Chapter have demonstrated that JSLE neutrophils themselves have normal functions as the rates of neutrophil apoptosis, phagocytosis and chemotaxis were not significantly changed from healthy juvenile control neutrophils. These findings suggest that dysregulated neutrophil functions are mainly caused by the factor(s) in JSLE serum.

Chapter 6: Effects of anti-malarial agents on neutrophil function

6.1 Introduction

Anti-malarial agents, alongside corticosteroids, are amongst the oldest drugs used for the initial treatment of JSLE and adult-onset SLE. They used to treat patients with malarial infection and for anti-malarial prophylaxis (272) but it was observed that soldiers in the Second World War who received anti-malarial prophylaxis, showed as improvement in their underlying rheumatic conditions, such as inflammatory arthritis and cutaneous lupus lesions. Consequently, these incidental findings leads to the beginning of systematic studies to investigate the beneficial effects of these agents for the treatment of rheumatic diseases, including SLE.

The role and efficacy of anti-malarial agents for the treatment of SLE has been evaluated over many decades of their use (273–276). It has been shown that lupus patients treated with anti-malarial agents (particularly chloroquine and hydroxychloroquine) experienced decreased disease progression, improved clinical outcome and increased survival rate (90). However, the actual mechanisms responsible for the beneficial effects anti-malarial agents on this disease remain unclear. Anti-malarial agents also have an important role in the treatment of JSLE patients, and are very effective, particularly for patients presenting with

cutaneous lupus lesions and inflammatory joints (55, 60, 61). The majority of JSLE patients who receive hydroxychloroquine show decreased disease flares and slower progression of major organ damage (64). Although anti-malarial agents are commonly and successfully used in combination with corticosteroids for the initial treatment for JSLE patients, the most serious and irreversible side effect is ocular toxicity (i.e. retinopathy) (91). JSLE patients who receive anti-malarial agents require routine eye examination (every 6-12 months), and ocular toxicity needs to be closely monitored.

The major anti-malarial agents currently used in JSLE and adult-onset SLE patients are chloroquine and hydroxychloroquine (55, 60, 61). Because of the higher (x3) toxicity of chloroquine, hydroxychloroquine is more commonly prescribed in JSLE patients (90). Both chloroquine and hydroxychloroquine are 4-amino-quinolines with lipophilic properties, and they can easily pass through cell membranes into intracellular compartments, including cell organelles (i.e. vesicles and granules) (272). Although the mechanisms of action of these agents are still unclear, both drugs show clearly-demonstrable anti-inflammatory effects, immunosuppression and photo-protection in patients with SLE (90). It has been reported that chloroquine and hydroxychloroquine suppress autoantigen presentation, and inhibit Toll-like receptor (TLR) signaling and leukocyte activation (277). The alteration of pH and stability of

lysosomes also seems to be an important mechanism of action of these agents in causing decreased binding of autoantigen to MHC class II molecules on the cell membranes, and interference of the TLR signaling pathway in antigen-presenting cells (278). Moreover, decreased production of pro-inflammatory cytokines, such as IL-1β, IL-6, IL-18, IFN-γ and TNF-α has been reported in patients who received anti-malarial agents (279, 280).

One interesting observation is that hydroxychloroquine induces apoptosis of peripheral blood lymphocytes of lupus patients (281). The increased apoptosis of lymphocytes, including T and B cells, can lead to decreased production of autoantibodies in SLE. Furthermore, the effect of hydroxychloroquine on increased synoviocyte apoptosis has been described in patients with rheumatoid arthritis (282). These pro-apoptotic effects on synoviocytes may decrease the production of pro-inflammatory cytokines and pannus formation in the joints of rheumatoid arthritis patients, may explain the anti-inflammatory effects of these agents. However, the effects of these drugs on neutrophil apoptosis have never been reported.

There are only a few reported studies demonstrating the effects of anti-malarial agents on neutrophil function (283–285). It has been shown that neutrophil superoxide production is inhibited by chloroquine and hydroxychloroquine *in vivo* (284). Nevertheless, increased incidence of

bacterial infection has never been reported in patients treated with these agents.

6.2 Aims

The aims of this Chapter were to determine whether hydroxychloroquine, a commonly-used anti-malarial agent for patients with JSLE, has any effects on neutrophil functions, in terms of cell survival, phagocytic ability, ROS production and chemotaxis.

Objectives

- To compare the rate of apoptosis of neutrophils incubated with or without hydroxychloroquine
- To determine whether hydroxychloroquine decreases superoxide production (i.e. ROS production) and phagocytic ability by neutrophils
- To measure chemotactic ability of neutrophils pre-treated with hydroxychloroquine, and to determine the effects of hydroxychloroquine on the efficacy of chemoattractant (IL-8) to attract neutrophils.

6.3 Results

6.3.1 Effects of hydroxychloroquine on apoptosis of neutrophils and neutrophils treated with GM-CSF

Neutrophils were isolated from healthy adult controls (n=5) and incubated with 10% human AB serum with or without hydroxychloroquine (HCQ) to explore whether neutrophil apoptosis was either delayed or enhanced by this drug. The concentrations in serum from patients treated with HCQ are in reported to be within the range of 0-23 µM (286). Therefore, neutrophils were incubated in vitro with concentrations of this drug up to 40 µM. The results showed no significant differences in the percentage apoptosis between neutrophils incubated with 2 µM (69.2 ± 5.4%), 10 µM (74.4 ± 4.4%), 20 µM (76.6 ± 4.3%), 40 µM (77.7 ± 5%) of HCQ and neutrophils without treatment (67.3 ± 6.6%), after 22 h incubation ($p>0.05$) (Figure 6.1A). There was a trend for increased apoptosis as the concentration of HCQ was increased, but this did not reach statistical significance.

HCQ is reported to induce apoptosis of peripheral blood lymphocytes and synoviocytes, but there are no report in its effects on neutrophil apoptosis (281, 282). As GM-CSF can significantly delay neutrophil apoptosis (Figure 4.1), it was therefore important to determine whether HCQ has an effect on apoptosis of neutrophils treated with GM-CSF. Isolated neutrophils (n=5) were incubated with 10% human AB

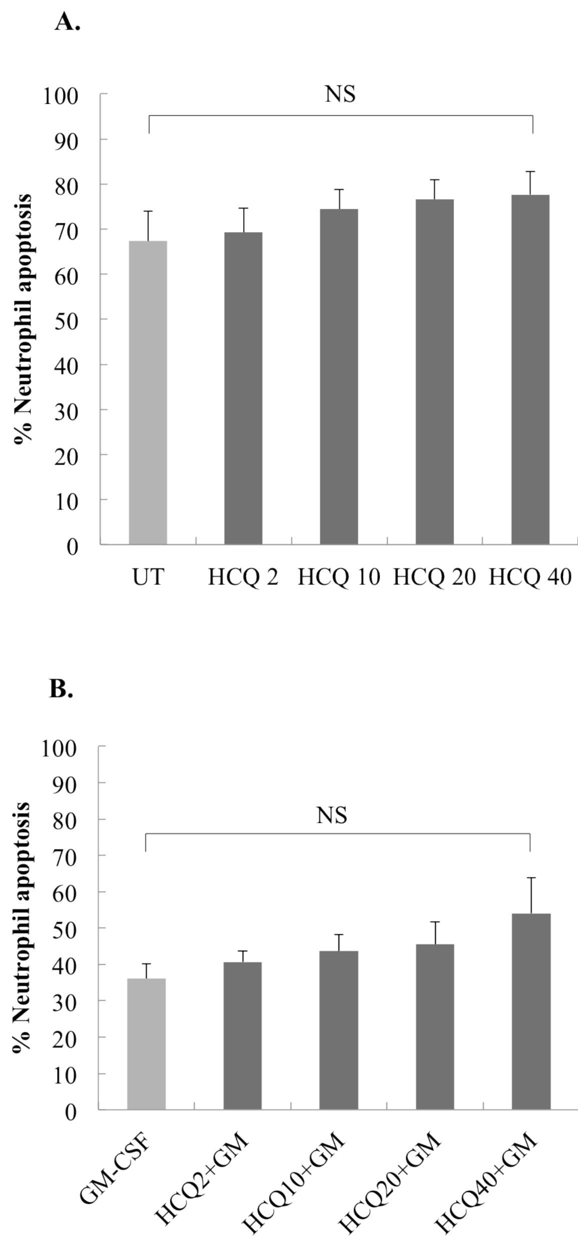


Figure 6.1 Effects of hydroxychloroquine on neutrophil apoptosis. The graph shows the effects of hydroxychloroquine on neutrophil apoptosis after incubation with 10% human AB serum **(A),** or after incubation with 10% human AB serum with GM-CSF (**B)** after 22 h incubation (n=5). HCQ 2, 10, 20 and 40 = hydroxychloroquine at 2 µM, 10 µM, 20 µM and 40 µM, respectively. GM= GM-CSF. Data represent mean ± SEM (NS p>0.05, Student's t-test).

serum plus GM-CSF (5 ng/mL), in the absence or presence of HCQ. The results showed no significant difference in the percent of apoptosis between neutrophils treated with GM-CSF and 2 μM (40 ± 3%), 10 μM (43.6 ± 4.6%), 20 μM (45.4 ± 6.1%), 40 μM (54 ± 9.7%) of HCQ, and neutrophils without treatment (36.1 ± 4%) after 22 h incubation ($p>0.05$) (Figure 6.1B). Again, there was a trend for decreased protection of apoptosis by GM-CSF as the concentration of HCQ was increased, but this did not reach statistical significance.

6.3.2 Effects of hydroxychloroquine on neutrophil phagocytosis and ROS production

Figure 6.2A compares phagocytosis by neutrophils treated with hydroxychloroquine (HCQ) for 1 h with that of untreated neutrophils, following incubation with *S. aureus* that was previously stained with PI and opsonised by human AB serum (SAPI) for 30 min. The percentage neutrophils undergoing phagocytosis was measured using flow cytometry. The results showed that there were no significant differences in the percentage of phagocytosis between neutrophils treated with HCQ at 2 μM (93.4 ± 1.9%), 10 μM (90 ± 3.1%), 20 μM (88.7 ± 2%) and 40 μM (89.6 ± 2.8%), and untreated neutrophils (91.6 ± 2.8%) ($p>0.05$) (Figure 6.2A).

The percentage of neutrophils staining positively for ROS production in the presence and absence of HCQ was analysed using

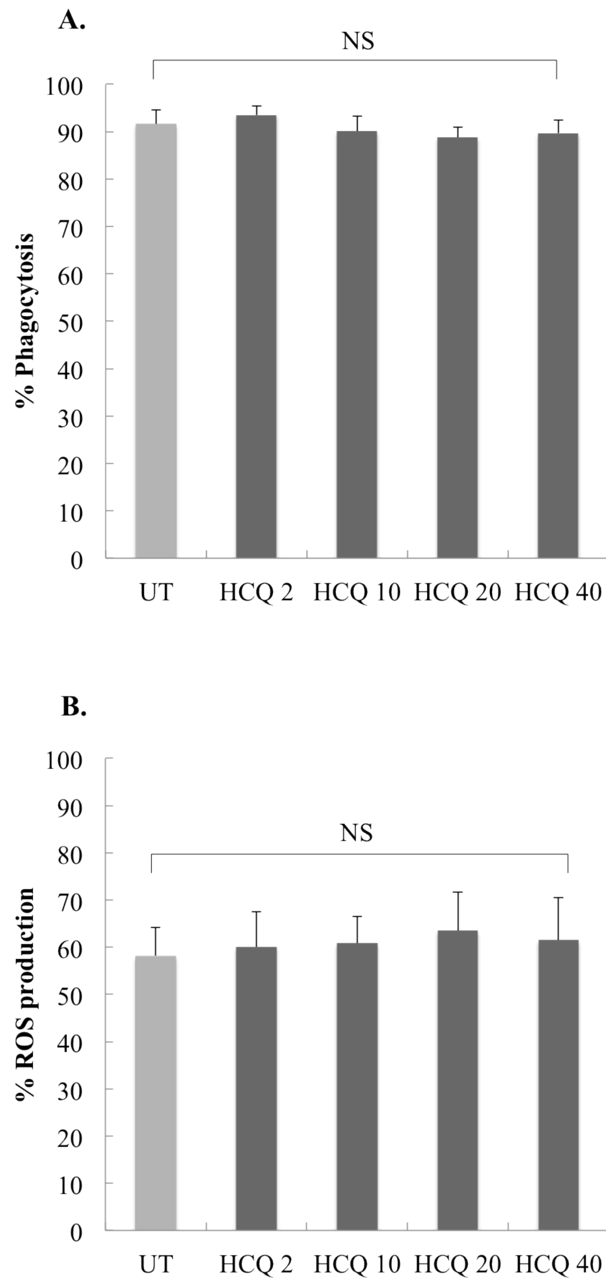


Figure 6.2 (A) Phagocytic ability and **(B)** ROS production of neutrophils treated with increasing concentrations of hydroxychloroquine for 1 h, followed by incubation with PI-stained, opsonised *S. aureus* (n=5). HCQ 2, 10, 20 and 40 = hydroxychloroquine at 2 µM, 10 µM, 20 µM and 40 µM, respectively. Data represent mean ± SEM (NS p>0.05, Student's t-test).

DHR123 (as described in Materials and Methods). Levels of ROS production by neutrophils treated with HCQ, following incubation with SAPI for 30 min were as follows: 2 μM (60 ± 7.4%); 10 μM (60.8 ± 5.6%); 20 μM (63.4 ± 8.2%) and 40 μM (61.4 ± 8.9%). These values were not significantly different from untreated neutrophils (58.1 ± 6.1%), ($p>0.05$) (Figure 6.2B).

6.3.3 Effect of hydroxychloroquine on neutrophil chemotaxis

A chemotaxis assay was performed using Millipore Hanging Cell Culture plate inserts (as previously described in Chapter 5). IL-8 was used as a chemoattractant (100 ng/mL), and hydroxychloroquine was added into each bottom well, together with IL-8 to investigate whether hydroxychloroquine could either increase or decrease the efficacy of IL-8 to attract neutrophils. Freshly-isolated neutrophils (n=5) were added into hanging inserts and incubated in an incubator at 37°C for 90 min. Migrated neutrophils in each well were counted and calculated as a percentage of the total number of cells originally added. The results showed that the percentage of migrated neutrophils in IL-8 with HCQ were as follows: 2 μM (47.2 ± 1.1%); 10 μM (44.5 ± 2%); 20 μM (45.3 ± 1.9%) and 40 μM (46.1 ± 3.2%). These values were not significantly different from migrated neutrophils in IL-8 alone (46.3 ± 3.1%) ($p>0.05$) (Figure 6.3A).

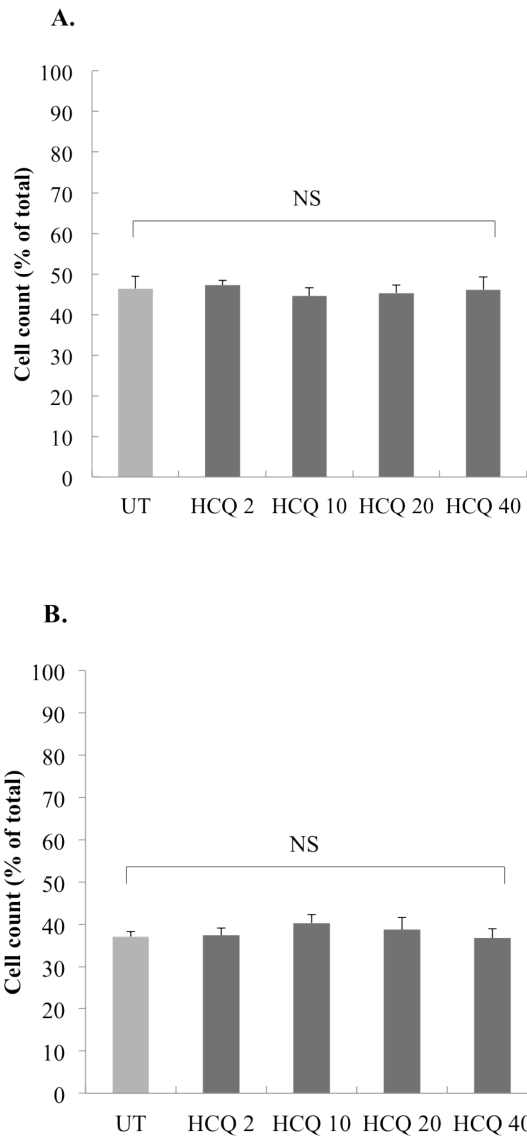


Figure 6.3 Effect of hydroxychloroquine on neutrophil chemotaxis. Neutrophil chemotaxis was performed using Millipore Hanging Cell Culture plate inserts. **(A)** IL-8 (100 ng/mL) and increasing concentrations of hydroxychloroquine (HCQ) were added into the bottom wells. Neutrophils were added into the hanging inserts for 90 min. Migrated neutrophils (n=5) were counted and calculated as a percentage of the total number of cells originally added. **(B)** Isolated neutrophils (n=5) were treated with HCQ for 1 h prior to the chemotaxis assay. IL-8 alone was added into the bottom wells. Migrated neutrophils (n=5) into the bottom well were counted. HCQ 2, 10, 20 and 40 = hydroxychloroquine at 2 μM, 10 μM, 20 μM and 40 μM, respectively. Data represent mean ± SEM (NS p>0.05, Student's t-test).

To determine whether pre-incubation with HCQ had any effect on the chemotactic ability of neutrophils, neutrophils were treated with or without HCQ for 1 h prior to a chemotaxis assay. IL-8 alone was added into each bottom well as a chemoattractant. The results showed that the percentage of migrated neutrophils treated with HCQ at 2 µM (37.4 ± 1.7%), 10 µM (40.3 ± 2%), 20 µM (38.7 ± 2.8%) and 40 µM (36.7 ± 2.2%) were not significantly different from untreated neutrophils (37 ± 1.2%) ($p>0.05$) (Figure 6.3B).

6.4 Discussion

Hydroxychloroquine is an anti-malarial drug that is commonly-used to treat patients with JSLE. The mechanisms of action of this agent is not clearly understood; however, it has been reported that anti-inflammatory effects, immunosuppression and photoprotection are observed in JSLE patients treated with hydroxychloroquine (90). The possible mechanisms of hydroxychloroquine in the treatment of SLE and JSLE are (a) suppression of autoantigen presentation, (b) inhibition of Toll-like receptor signaling pathway and (c) prevention of leukocyte activation (277). However, it has been reported that hydroxychloroquine induces apoptosis of peripheral blood lymphocytes of lupus patients, leading to decreased production of autoantibodies in SLE (281). Furthermore, hydroxychloroquine induces synoviocyte apoptosis in patients with rheumatoid arthritis (282). As neutrophil apoptosis plays a

role in the pathogenesis of JSLE (103), it is important to determine the effects of this drug on neutrophil apoptosis. If hydroxychloroquine exerted a pro-apoptotic effect on neutrophils, this could lead to decreased inflammation, but if these apoptotic neutrophils were not efficiently cleared, then enhanced neutrophil apoptosis may increase autoantigen exposure and hence autoantibody production. This latter phenomenon could explain why some patients respond poorly to these agents and develop side effects (287). The results showed that hydroxychloroquine had no significant pro-apoptotic effects on neutrophils although the concentrations were increased up to 2 times the levels measured in patient serum (0-23 μM) (286). This finding suggests that hydroxychloroquine at normal or double concentrations in patient serum are harmless for neutrophils in terms of cell survival.

One previous study reported that hydroxychloroquine could inhibit superoxide production by neutrophils *in vivo* (284). The study showed that the production of superoxide stimulated by fMLP was decreased in neutrophils incubated with hydroxychloroquine in a dose dependent manner. A 50% decrease in superoxide release was observed in neutrophils treated with hydroxychloroquine at the concentration of 100 μM. In my study, phagocytosis and ROS production by neutrophils treated with hydroxychloroquine were investigated, when intracellular ROS were measured following phagocytosis. The results showed that

hydroxychloroquine at normal (2-20 μM) or double (40 μM) concentrations had no significant effect on phagocytosis and ROS production by neutrophils incubated with SAPI, suggesting that neutrophils exposed to serum concentrations of hydroxychloroquine still function normally. Similarly, the chemotactic ability by neutrophils was also unaffected by this drug. The results showed that hydroxychloroquine did not either interfere the efficacy of the chemoattractive agent (i.e. IL-8) in terms of attracting neutrophils or affect the chemotactic ability of neutrophils pre-treated with hydroxychloroquine. These findings confirm that at the concentrations of hydroxychloroquine attained in patient serum, neutrophils normally respond to IL-8 and their chemotactic ability is still effective.

In conclusion, the results presented in this Chapter have demonstrated that hydroxychloroquine, a commonly-used anti-malarial agent in the treatment of JSLE patients, has no effect on neutrophils in terms of cell survival, phagocytosis, ROS production and chemotaxis at the normal concentrations attained in patient serum. It is therefore very unlikely that the use of hydroxychloroquine in JSLE patients induces any neutrophil dysfunction that can result in a higher incidence of bacterial infections.

Chapter 7: General discussion and conclusions

Neutrophils are the most abundant white blood cells (leukocytes) in the circulation of human blood and play an important role during acute inflammation. Neutrophils are the first leukocytes that rapidly migrate to the inflammatory site, recognize and subsequently remove pathogens (2). There are several steps in the process of pathogen elimination, such as neutrophil priming, neutrophil rolling and adhesion, neutrophil transmigration/chemotaxis, neutrophil opsono-phagocytosis and ROS production, and neutrophil apoptosis and clearance (4). Therefore, effective control of neutrophil function is vital at the site of infection/inflammation.

In contrast, neutrophils also play damaging roles in the pathophysiology of many diseases, including autoimmune diseases (11). Neutrophils have been reported as one of the most important contributing factors in disease pathology of systemic lupus erythematosus (SLE), particularly in terms of neutrophil survival and apoptosis (49). It has been found that there are increased numbers of apoptotic leukocytes (e.g. neutrophils) in patients with SLE (100). It has also been found that autoantigens may be clustered in blebs on the surface of apoptotic cells and trigger abnormal immune responses and autoantibody production which is hypothesised as a major cause of the disease pathology (99). Moreover, neutrophil functional defects have been reported in patients

with SLE. For example, ineffective phagocytosis, decreased cytokine production and dysregulated chemokine response by neutrophils have all been described and can contribute to increased susceptibility of patients to infections, particularly by bacteria and fungi (95, 112).

Juvenile SLE (JSLE) is a subgroup of SLE, and patients usually have more severe clinical features than those with adult-onset SLE (55, 60, 61). JSLE patients usually present with major organ involvement (e.g. lupus nephritis, blood dyscrasia and neuropsychiatric lupus) at the time of diagnosis, which is indicative of a worst prognosis. Although there are only a few reports on disease pathology of JSLE, including a correlation with neutrophil defects, such studies have clearly demonstrated an abnormal neutrophil function that is involved in the immune-pathogenesis of JSLE (50–52). Therefore, the main objective of my study was to characterize abnormal neutrophil function, in terms of cell survival, phagocytosis, ROS production and chemotaxis in JSLE patients. Furthermore, factor(s) that could affect normal neutrophil function (e.g. recombinant human cytokines, commonly-used medications.) were also investigated.

The main findings of my study were as follows:

- Factor(s) in JSLE serum (particularly in active JSLE serum) significantly induced healthy neutrophils become apoptotic via the *extrinsic* and/or *intrinsic* apoptotic pathways

- JSLE serum resulted in decreased bacterial opsonisation, leading to defective neutrophil phagocytosis and ROS production; these defects were, again more marked in serum from patients with active JSLE serum.

- An imbalance of cytokines that are reported in JSLE serum had either protective (e.g. GM-CSF, IL-1 and low concentrations of TNF-α) or neutral effects on neutrophil apoptosis (e.g. IL-6, IL-17 and IFN-α).

- IL-8 levels in JSLE serum could potentially be useful as a biomarker of disease activity in JSLE patients, as the levels of this chemokine were significantly higher in serum from JSLE patients with active disease compared with serum from JSLE patients with inactive disease.

- GM-CSF, a potent cytokine for delaying neutrophil apoptosis, overcame the pro-apoptotic effect of JSLE serum-induced neutrophil apoptosis and it could possibly be used as an alternative treatment in patients with JSLE.

- Although low concentrations of TNF-α significantly saved neutrophil apoptosis, the clinical application of TNF-α/ant-TNF-α treatment in JSLE patients needs to be thoroughly considered

because of the dual effects of this cytokine on neutrophil apoptosis and its tissue-specificity.

- Neutrophils isolated from JSLE patients (with inactive disease) are likely to have normal functions, because the rates of apoptosis, phagocytic ability and chemotaxis were not significantly different from healthy, juvenile control neutrophils.
- Hydroxychloroquine; a commonly-used anti-malarial agent for the treatment of JSLE patients, at concentrations achieved in serum following treatment, had no significant effect on neutrophil functions, in terms of cell survival, phagocytosis, ROS production and chemotaxis.

Results from my study strongly indicate that JSLE serum is possible to be a crucial factor responsible for the dysregulated neutrophil functions. Factor(s) in JSLE serum could activate the caspase cascade (-3, -7 and -8) of either the *extrinsic* and/or *intrinsic* pathways, and decreased the levels of major anti-apoptotic protein; Mcl-1, leading to increased neutrophil apoptosis (as described in Chapter 3). These findings were consistent with previous reports that showed that JSLE serum increased the level of apoptosis in healthy control neutrophils compared to healthy juvenile control serum (103, 105). In addition, my study has

demonstrated that serum from JSLE patients with active disease induce higher levels of apoptosis compared to serum from patients with inactive disease, although the results did not reach statistical significance because of the relatively small sample size. This finding provides new information on the immune-pathogenesis of the disease, suggesting that disease activity in JSLE patient correlates with the degree of neutrophil apoptosis. However, further studies are needed to confirm this finding, and a greater number of serum samples from patients with both active and inactive disease need to be collected to increase the sample size. The difficulty in assessing patients with JSLE disease as "active" or "inactive" is sometime problematic but can be largely overcome by using disease activity scores (i.e SLEDAI scores). Recording SLEDAI scores of JSLE patients in Thailand for an extension of my study is now ongoing and has been initiated by my studies. An extension of my work, therefore, will be to determine if there is a correlation between SLEDAI scores and the rate of neutrophil apoptosis induced by serum from JSLE patients.

Factor(s) in JSLE serum also affected other functions of neutrophils, such as phagocytic ability and ROS production. In my study, both phagocytic ability and ROS production were decreased when neutrophils were incubated with *S. aureus* opsonised with JSLE serum, and further decreased when the bacteria were opsonised with active JSLE serum. Although the decreases did not reach statistical significance, these

results suggest that serum from JSLE patients is more likely to decrease bacterial opsonisation leading to impaired phagocytic ability and decreased ROS production. These findings were also observed when apoptotic neutrophils were used instead of freshly-isolated neutrophils. Apoptotic neutrophils were incubated with the bacteria opsonised with JSLE serum and both phagocytic ability and ROS production were significantly decreased, compared with freshly-isolated neutrophils incubated with the bacteria opsonised with healthy control serum. Taken together, these findings suggest that both phagocytic ability and ROS production by neutrophils in JSLE patients is decreased, probably because JSLE serum induces neutrophil apoptosis and is less effective in opsonisation. Clinically, this could lead to increased susceptibility to infections in JSLE patients. These findings indicate that neutrophil apoptosis is an important factor involved in both the pathogenesis of JSLE (i.e. a major source of autoantigens), and is related with the disease etiology of susceptibility to infections (i.e. less effective in bacterial opsonisation) in JSLE patients. The next experiments, therefore, focused on characterising JSLE serum, as it is likely to be a major factor that induces neutrophil apoptosis in JSLE patients.

It has been reported that the levels and types of cytokines in serum regulate neutrophil survival *in vitro* (238). In previous studies, an imbalance of cytokine levels has been reported in the serum of patients

with JSLE (e.g. GM-CSF, IL-1, IL-6, IL-17, type I IFNs and TNF-α) (83, 146, 219). Therefore, it is important to investigate the effect of these cytokines on neutrophil apoptosis and to determine their levels in serum from patients with JSLE. The results showed that only three of the cytokines tested (GM-CSF, IL-1 and low concentrations of TNF-α) significantly saved neutrophil apoptosis, whereas the others tested showed no significant effects. Unfortunately, the Luminex assay did not show decreased levels of GM-CSF, IL-1 and TNF-α in serum, which could have offered an explanation for the increased neutrophil apoptosis observed in JSLE patients. Moreover, most cytokines in JSLE serum that have been previously reported as increased, particularly IL-6, IL-17, type I IFNs and TNF-α, and that their levels correlated with the degree of disease activity (83, 146, 219), were undetectable in the assay used in my studies. However, these cytokines were also undetectable in serum from healthy juvenile controls. These findings probably either reflect that (a) the levels of these cytokines in serum are essentially very low in both JSLE patients and healthy juvenile controls, (b) the detection limitations of the assay used in this study are too low (i.e GM-CSF < 26.34 pg/mL, IL-1β < 11.25 pg/mL and IL-6 < 7.06 pg/mL) or (c) serum protein can somehow interfere with the detection assay.

There were four cytokines in serum from both JSLE patients and healthy juvenile controls detectable in my study, one of which was IL-8

and it was significantly higher in JSLE patients with active disease, compared to levels in serum from JSLE patients with inactive disease. IL-8, a potent neutrophil chemoattractant, has been reported as a cytokine that can save/delay neutrophil apoptosis (288, 289). For this reason, IL-8 is unlikely to be a factor that induces neutrophil apoptosis in JSLE patients. Moreover, the median levels of IL-8 detected in this study was 21.34 pg/mL (IQ range 11.30-58.35), which are low and unable to affect on neutrophil apoptosis (288) (also shown in Appendix; Figure 8.2). Nevertheless, there are other factor(s) in serum that induce neutrophil apoptosis (e.g. Fas, TRAIL) and these many be elevated in serum from patients with JSLE. The future plan, therefore, is to measure these factor(s) in patient serum. The relatively small sample size of my study is another issue that needs to be considered in future studies. For this, serum samples from both JSLE patients and healthy juvenile controls are necessary to be recruited, and the disease status more rigorously defined (see above). Even though the levels of IL-8 detected in active JSLE serum are unlikely to be a factor that can regulate neutrophil apoptosis, their levels could be useful as a biomarker of disease activity in JSLE patients. The levels of this chemokine could predict disease flare in inactive JSLE patients, as has been proposed in adult-onset SLE with disease flare (264).

There are at least three cytokines (e.g. GM-CSF, IL-1 and TNF-α) that significantly delayed neutrophil apoptosis in my study, one of which was GM-CSF. The results demonstrated that GM-CSF was the most potent cytokine that could save/delay neutrophil apoptosis. As JSLE serum induced neutrophil apoptosis, this study therefore focused upon the effect of GM-CSF on neutrophil apoptosis and the next aim was to determine whether GM-CSF could overcome the pro-apoptotic effect of JSLE serum and protect neutrophils against apoptosis. The results showed that GM-CSF not only overcame the pro-apoptotic effect of JSLE serum and saved neutrophil apoptosis, but also prevented caspase activation (-3, -7, -8 and -9) and increased/maintained the levels of the anti-apoptotic protein Mcl-1 in neutrophils incubated with JSLE serum. This finding suggests that GM-CSF could be considered as an alternative treatment in patients with JSLE, in order to decrease number of apoptotic neutrophils, leading to decreased autoantigen production, and subsequent decreased T- and B- cell activation.

GM-CSF is a common therapeutic agent used for treatment of several diseases, particularly hematological malignancy (e.g. leukemia and lymphoma) (290, 291). GM-CSF is usually administered in patients with leukemia following a complete course of chemotherapy. The purpose of GM-CSF administration is to increase number of normal granulocytes and monocytes, in order to protect the patients from

opportunistic infections, as chemotherapeutic agents have a common side effect on bone marrow suppression (290). As the adverse effects of GM-CSF are minimal (292, 293), the concept of GM-CSF administration in patients with JSLE, as an alternative treatment in order to decrease neutrophil apoptosis, and thereby decrease autoantigen production, is feasible. Future plans should identify the autoantigens on the apoptotic neutrophil surface induced by JSLE serum, and determine whether the expression of autoantigens on the cell surface of apoptotic neutrophils is decreased following GM-CSF treatment.

Although IL-1 and low concentrations of TNF-α also showed an anti-apoptotic effect on neutrophils in this study, the application of both cytokines for treatment in patients with JSLE would be problematic. It has been reported that both IL-1 and TNF-α are involved in the pathogenesis of lupus nephritis, and renal histopathology from these patients reveals high levels of these cytokines surrounding the inflamed tissue, suggesting that both cytokines play an inflammatory role in patients with JSLE. Furthermore, the effect of TNF-α on neutrophil survival/apoptosis is biphasic. Low concentrations (~10 ng/mL) protect against neutrophil apoptosis whereas higher concentrations (>30 ng/mL) promote apoptosis (248). Consequently, the application of TNF-α as a therapeutic treatment is seemingly inappropriate, and obtaining the

appropriate dosage of TNF-α will be extremely challenging in JSLE patients.

The precise role of TNF-α in the pathogenesis of SLE is still controversial. Although TNF-α appears to be an important pro-inflammatory cytokine involved in the pathogenesis of JSLE and adult-onset SLE (195), anti-TNF-α therapy in treatment of patients with SLE is unsuccessful. Therefore, understanding the role of TNF-α in the pathogenesis of JSLE (and other inflammatory conditions) in which this cytokine may play a role in pathology is crucial, particularly in terms of neutrophil responses to TNF-α under conditions that mimic acute and chronic inflammation (e.g. in JSLE). Here, my results showed that neutrophils down-regulated the expression of proteins involved in death receptor signaling (i.e. FADD, TRADD, TNFR1, TNFR2, caspase 8 and caspase 10) following TNF-α exposure, in order to protect themselves against apoptosis, and in parallel, up-regulated the expression of anti-apoptotic genes (BCL2A1, CFLAR and TNFAIP3). This phenomenon allows neutrophils to survive and function in TNF-α-rich environments, and enables them to perform their role in inflammation. These findings suggest a tissue-specific role of TNF-α in JSLE, as high concentrations (e.g. in serum) probably induce apoptosis resulting in autoantigen exposure, whereas low concentrations (e.g. in kidneys) delay apoptosis leading to tissue inflammation and organ damage. An interesting

observation in this study is elevated transcripts for TNF-α in neutrophils following TNF-α exposure. This autocrine production probably provides a positive feedback loop for neutrophils and subsequently explains their resistance to TNF-α induced cell death.

Apart from the effects of JSLE serum and recombinant-cytokines on neutrophil function, this study also investigated the function of neutrophils isolated from JSLE patients. First, the apoptotic rate of JSLE neutrophils was measured. The results showed that the levels of neutrophil apoptosis in JSLE neutrophils at time 0 were slightly higher than that of healthy juvenile control neutrophils, whereas comparable levels were observed in both groups after 2 h incubation in human AB serum. Although this higher rate of JSLE neutrophil apoptosis at time 0 did not reach statistical significance, it suggests that JSLE neutrophils might have been activated by JSLE serum *in vivo* and the pro-apoptotic effects of JSLE serum were reversed after neutrophils were incubated with human AB serum *in vitro*.

Previous studies have shown that the transcript for the anti-apoptotic protein, Mcl-1 can exist in two forms, a normal transcript (Mcl-1_L) and its splicing variance (Mcl-1_S). These proteins, encoded by these two splice variants, have opposing effects on apoptosis. I therefore determined if there were variations in the levels of these two transcripts in neutrophils from JSLE patients and healthy juvenile controls.

However, no differences in the levels of these two transcripts were detected. Taken together, JSLE serum might affect JSLE neutrophils at the levels of Mcl-1 protein but not the level of Mcl-1 splicing. Nevertheless, longer incubation times and incubations with healthy juvenile control serum are required to confirm these findings.

Next, my study measured the phagocytic ability and chemotaxis activity of JSLE neutrophils, as these properties have been reported to be impaired in adult-onset SLE patients, leading to opportunistic infections (97, 112, 123). The results showed that both phagocytic ability and chemotaxis are not significantly altered in JSLE patients, reflecting that the susceptibility to infections in JSLE patients is probably caused by defects in JSLE serum that can induce neutrophil apoptosis and decrease the efficiency of bacterial opsonisation.

This study finally investigated the effects of a common anti-malarial drug (hydroxychloroquine) used in patients with JSLE, on neutrophil functions. Corticosteroids and anti-malarial drugs are usually an initial treatment for patients with JSLE. It has been reported that corticosteroids have several inhibitory effects on neutrophil functions such as phagocytosis, ROS production, chemotaxis and cell apoptosis (294–297). However, the effects of anti-malarial drugs, particularly hydroxychloroquine have never been reported. Here, my study demonstrated that hydroxychloroquine at concentrations found in patient

serum (0-23 μM) have no effect on neutrophil functions, in terms of cell survival, phagocytosis, ROS production and chemotaxis. The administration of hydroxychloroquine in JSLE patients at normal doses is therefore unlikely to lead to neutrophil dysfunction and increase the risk of bacterial infection. However, had time permitted, it would have been useful to have repeated these studies on the effects of hydroxychloroquine in the presence of corticosteroids, as these two drugs are usually used in combination.

In conclusion, my study showed several abnormalities of neutrophil function in JSLE patients. The main findings are that JSLE serum induced neutrophil apoptosis and resulted in decreased bacterial opsonisation, leading to defective neutrophil phagocytosis and ROS production. Factor(s) in JSLE serum (i.e. cytokines) regulated neutrophil survival/death, but only IL-8 levels were detectable and significantly increased in JSLE serum. Although IL-8 did not induce neutrophil apoptosis, it could probably be used as an indicator of disease activity in JSLE patients. GM-CSF was the most protective cytokine and overcame the pro-apoptotic effect of JSLE serum. GM-CSF could potentially be used as an alternative treatment in JSLE patients. The effects of TNF-α are probably tissue-specific and concentration-dependent and so the clinical application of TNF-α/ant-TNF-α treatment in JSLE patients is unlikely to be of benefit. The functions of neutrophils from JSLE and

neutrophils treated with hydroxychloroquine (a common anti-malarial drug used for treatment of JSLE) still function normally. Taken together, this study demonstrated that factor(s) in JSLE serum play important roles in JSLE neutrophils.

Future directions

Further studies should recruit both JSLE patients and healthy juvenile controls in order to increase the sample size to confirm the validity of some of the key experiments presented in this thesis. Particular areas of focus would be serum analysis particularly to confirm whether IL-8 and other pro-apoptotic proteins (e.g. FAS and TRAIL) are markedly increased in JSLE patients. This could explain the mechanism by which JSLE neutrophils become apoptotic *in vivo*. In addition, disease activity scores (i.e. SLEDAI scores) should be recorded and then used to search for a correlation between disease activity and the levels of neutrophil apoptosis induced by JSLE serum, and to determine whether the serum levels of IL-8 are significantly increased in patients with active disease. As GM-CSF is a potent cytokine for delaying neutrophil apoptosis, it is important to determine whether the production of autoantigen from apoptotic neutrophils induced by JSLE serum would be decreased after GM-CSF treatment. This finding would support the idea that GM-CSF could probably used as an alternative treatment in patients with JSLE. Neutrophils isolated from JSLE patients, particularly those

with active disease, would be used to investigate their functions in terms of apoptosis, phagocytosis, ROS production and chemotaxis and confirm that JSLE neutrophils still function normally in host defense and that serum from JSLE patients is a major factor involved in dysregulation of neutrophil function.

Chapter 8: Appendix

Table 8.1 Demographic data of healthy juvenile controls (n=9) and the patients diagnosed with JSLE and fulfilled ≥ 4 of the ACR criteria for SLE; inactive JSLE patients (n=12) and active JSLE patients (n=7).

Demographic data	Healthy juvenile controls	Inactive JSLE	Active JSLE
Number of patients	9	12	7
Age	13.4 (± 1.3)	15.3 (± 1.9)	14 (± 1.4)
Gender: female, male	5,4	8,4	4,3
ANA positive	N/A	12	7
Medications			
• Corticosteroids	0	12	5
• Hydroxychloroquine	0	8	3
• Immunosuppressive drugs (e.g. cyclophosphamide, methotrexate, AZA, MMF)	0	1	7

Table 8.2 Neutrophil gene expression is independent of the concentration of TNF-α. Neutrophils were incubated exactly as described in the legend to Figure 4.6. After quantitation of transcript levels by real-time PCR, expression levels after incubation for 1 h with 40 ng/mL TNF-α (high concentrations) or 10 ng/mL TNF-α (low concentrations). Data represent mean fold changes (± SEM) of three separate experiments.

Genes	Low dose TNF-α (10 ng/mL)	High dose TNF-α (40 ng/mL)
APAF1	-2.06 (± 0.53)	-0.38 (± 1.24)
BAX	1.4 (± 0.1)	-0.22 (± 1.61)
BCL2A1	4.31 (± 0.41)	4.95 (± 0.91)
BCL3	3.36 (± 0.32)	4.72 (± 1.82)
CASP10	-2.22 (± 0.45)	-2.31 (± 0.53)
CASP8	-3.54 (± 0.81)	-3.78 (± 1.3)
CD40	2.75 (± 0.44)	27.23 (± 19.6)
CFLAR	1.9 (± 0.07)	2.96 (± 0.53)
FADD	-5.69 (± 1.97)	-6.51 (± 0.91)
FAS	2.58 (± 0.35)	3.59 (± 0.24)
ICAM1	13.65 (± 1.3)	16.63 (± 5.5)
IKBKE	3.08 (± 0.24)	3.51 (± 0.99)
NFKB1	2.44 (± 0.65)	4.26 (± 0.76)
NFKB1A	16.46 (± 3.23)	16.82 (± 4.39)
NFKB2	5.38 (± 1.15)	8.07 (± 0.87)
REL	3.54 (± 0.43)	4.8 (± 0.8)
REL A	2.35 (± 0.04)	4.15 (± 0.2)
REL B	2.39 (± 0.13)	2.28 (± 0.25)
TNF	23.38 (± 5.01)	80.85 (± 42.28)
TNFAIP3	19.95 (± 1.93)	24.59 (± 8.75)
TNFRSF1A	-2.23 (± 0.43)	-2.44 (± 0.82)
TNFRSF1B	-1.46 (± 0.3)	-1.55 (± 1.6)
TRADD	1.32 (± 0.22)	-1.68 (± 0.34)

Table 8.3 Neutrophils were incubated for up to 6 h in the absence or presence of TNF-α (10 ng/mL) (as described in Figure 4.9). Gene expression was analyzed by real-time PCR (normalised to GAPDH). Samples were collected 1 h, 3 h and 6 h after addition of TNF-α. Data shown are mean fold changes (± SEM) of three separate experiments.

Genes	1h	3h	6h
TNF	13.37 (± 4.77)	4.82 (± 2.64)	7.32 (± 8.46)
FADD	-3.34 (± 1.14)	-3.13 (± 2.14)	-1.54 (± 0.61)
CASP8	-1.71 (± 0.36)	-2.25 (± 1.03)	-1.72 (± 1.38)
CASP10	-1.41 (± 0.16)	-1.44 (± 1.01)	-1.01 (± 0.63)
TNFRSF1A	-1.7 (± 0.28)	-1.28 (± 0.65)	-0.86 (± 0.54)

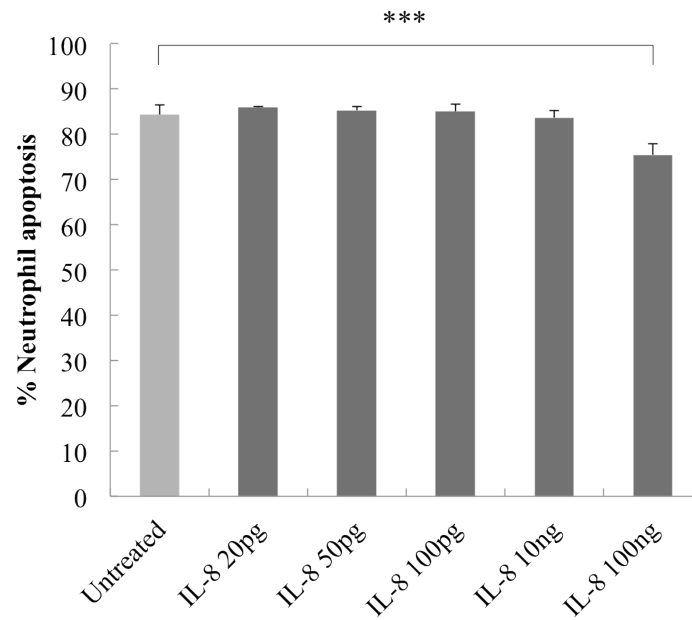


Figure 8.2 Effects of IL-8 on neutrophil apoptosis. The graph shows the percent of neutrophil apoptosis after incubation with 10% human AB serum with or without IL-8 at 22 h (3 replicates). The different concentrations of IL-8 (pg/mL or ng/mL) were shown. Data represent mean ± SEM (*** $p<0.005$, Student's t-test).

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