Investigating the role of

Neutrophil Extracellular Traps in the pathogenesis of

Juvenile-onset Systemic Lupus

Erythematosus (JSLE)

Institute of Child Health, University of Liverpool

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

Kanchani Kumari Makuloluwa

September 2014

Acknowledgement

First and foremost, I would like to offer my sincerest gratitude to my supervisors; Professor Beresford for giving me the opportunity to do this project and his continual support throughout and Dr. Angela Midgley for her patient guidance, encouragement and help every step of the way.

I would like extend my acknowledgement to all members of the Lupus Research team. It has been a pleasure to work with you and I am grateful for your support throughout. Everyone whom I've worked with in the lab, thank you for your lovely company and providing an enjoyable atmosphere to work in. I am extremely thankful to all those within the research department who so willingly donated their blood throughout the year.

I would like to thank my parents, sisters and friends for their unwavering support through everything.

Last, but not least, I am extremely grateful to all the study participants, without whom this research would not have been possible.

Abstract

Investigating the Role of Neutrophil Extracellular Traps (NETs) in the pathogenesis of Juvenile-onset Systemic Lupus Erythematosus (JSLE)

Kanchani Kumari Makuloluwa

Background: JSLE is a multisystem autoimmune disorder characterised by the production of autoantibodies against nuclear self-antigens. Activated neutrophils may undergo cell death by NETosis, forming NETs comprising of DNA. Dysregulated NETosis is increasingly being investigated for their role in Lupus pathogenesis through auto-antigen exposure. Endosomally localized Toll-like receptors (TLRs) 3, 7 and 9, expressed highly in JSLE, detect nuclear antigens to induce immune responses. Our hypothesis is that NETs offer a source of nuclear autoantigens that are being detected via TLR3, TLR7 and/or TLR9, resulting in the activation of the immune system.

Aim: To assess whether NETs originating from JSLE neutrophils are providing a source of nuclear autoantigens that are being detected through endosomal TLRs resulting in the activation of the immune system.

Methods: Purified JSLE and paediatric control neutrophils were incubated with 10ng/ml Interferon alpha (IFNα) or 10% JSLE sera to induce NETs. NETs were dismantled using micrococcal nuclease and quantified. Quantified NETs were incubated with healthy adult peripheral blood mononuclear cells (PBMCs) pre-treated +/- 100μM Hydroxychloroquine (HCQ) to block TLR activation. Protein levels of phosphorylated Interleukin-1 Receptor-associated Kinase 1 (pIRAK1) and Interferon Regulatory Factor 3 (pIRF3) (TLR7/9 and TLR3 signalling proteins) was determined by Western blotting. The supernatant from NET-stimulated PBMCs was incubated with either control PBMCs or JSLE neutrophils pre-treated +/- 10μM Janus Kinase (JAK) inhibitor to block IFNα signalling. Protein levels of phosphorylated IFNα-signalling protein, STAT1, was measured in the control PBMCs using Western Blotting; JSLE neutrophils were visualised using confocal microscopy for NETs.

Results: PBMCs incubated with IFNα or JSLE sera-induced NETs showed increased pIRAK1 and pIRF3 protein levels in both JSLE and paediatric control neutrophils-derived NETs, as compared to unstimulated PBMCs. The increase in pIRAK1 protein levels was not influenced by the source of the NETs. Incubation of NETs with PBMCs pre-treated with HCQ significantly reduced pIRAK1 protein levels ($p=0.028$; $n=6$). In control PBMCs incubated with supernatant from NET stimulated PBMCs there was increased pSTAT1 protein levels ($p=0.068$; $n=4$) compared to the control condition. pSTAT1 protein levels were reduced ($p=0.068$; $n=4$) in the control PBMCs that were pretreated with a JAK inhibitor. JSLE neutrophils released NETs following incubation with supernatant from NET stimulated PBMCs. NETs were absent in JSLE neutrophils pre-treated with a JAK inhibitor.

Conclusion: We have demonstrated that IFNα and JSLE sera-induced NETs may be an important source of autoantigens in JSLE that are being detected through TLR3, TLR7 and/or TLR9, leading to activation of these receptors. HCQ was effective in inhibiting the NET-induced activation of these receptors. Supernatant of NET-stimulated PBMCs induced the activation of JAK-STAT signalling pathways of control PBMCs and stimulated JSLE neutrophils to release NETs. A JAK inhibitor was successful blocking the induction of both these responses.

Table of Contents

List of Figures

List of Tables

Abbreviations

ACR – American College of Rheumatology

ANA – Anti Nuclear Antibodies

ANCA – Anti-Neutrophil Cytoplasmic Antibody

Anti-HNP – Anti-Human Neutrophil Peptide

Anti-RNP – Anti-Ribonucleoprotein

Anti-Sm – Anti-Smith

Anti- SSA – Anti-Smith Surface Antigens

Anti- SSB – Anti-Specific Soluble Ribonucleic Acid

APCs **–** Antigen Presenting Cells

BCR – B-cell Receptor

BILAG – British Isles Lupus Assessment Group

CO_2 – Carbon dioxide

CpG – Cytidine-Phosphate-Guanosine

DAPI – 2-(4-Amidinophenyl)-6-indolecarbamidine dihydrochloride

DCs – Dendritic Cells

DNA – Deoxyribonucleic Acid

DNase – Deoxyribonuclease

dsRNA – Double-stranded RNA

DTT – Dithiothreitol

ER – Endoplasmic Reticulum

FCS – Foetal Calf Serum

FITC – Fluorescein Isothiocyanate

HBSS – Hanks' Balanced Salt Solution

HCQ – Hydroxychloroquine

HOCl– Hypochlorous acid

HRP – Horseradish Peroxidase

H_2O_2 – Hydrogen Peroxide

IFNα – Interferon Alpha

IFNAR –Type 1 IFN Receptors

IL – Interleukin

IL-1R – Interleukin-1 Receptor

IRAK – Interleukin-1 Receptor-Associated Kinase

IRF – Interferon Regulatory Factor

IRS – Immunoregulatory DNA Sequences

JAK inhibitor – Janus Kinase Inhibitor

JAK-STAT – Janus Kinase-Signal Transduction and Activator of Transcription

JSLE – Juvenile-onset Systemic Lupus Erythematosus

MCET – Mast-cell Extracellular Trap

MHC – Major Histocompatibility Complex

ml – Millilitre

MMF – Mycophenolate mofetil

MPO – Myeloperoxidase

MyD88 – Myeloid Differentiation Primary-Response Protein 88

mDCs – Myeloid Dendritic Cells

NE – Neutrophil Elastase

NETs – Neutrophil Extracellular Traps

ng – Nanograms

nM – Nanomolar

NO – Nitric Oxide

ODN – Oligonucleotide

PAD4 – Peptidylarginine Deiminase 4

PAMP – Pathogen-associated Molecular Pattern

PBMC – Peripheral Blood Mononuclear Cell

PBS – Phosphate Buffered Saline

pDC – Plasmacytoid Dendritic Cells

pIRAK1– Phosphorylated IRAK1

pIRF3 – Phosphorylated IR3

PKC – Protein Kinase C

PMA – Phorbol Myristate Acetate

Poly I:C – Polyinosinic:polycytidylic acid

PRR – Pattern Recognition Receptor

PTM – Post Translational Modification

RBC – Red Blood Cells

RNA – Ribonucleic Acid

ROS – Reactive Oxygen Species

RPMI – Roswell Park Memorial Institute medium

SDS – sodium dodecyl sulfate

SLAM – Systemic Lupus Activity Measure

SLEDAI – SLE Disease Activity Index

ssRNA – Single-stranded RNA

STAT1 – signal transduction and activator of transcription

TANK – TRAF Family Member–Associated Nuclear Factor - Kappa β Activator

TBK1 – TANK Binding Kinase 1

TBS – Tris-Buffered Saline

TCR – T-cell Receptor

TIR –Toll/IL-1 receptor

TLR – Toll like Receptor

TNF – Tumor Necrosis Factor

TRAF – Tumor Necrosis Factor Receptor-Associated Factor

TRIF – TIR domain-containing Adaptor Inducing Interferon- β

Type 1 IFNs – Type 1 Interferons

µl - Microlitre

1 Introduction

1.1 Overview of the Immune system

The atmosphere we live in is abundant in an array of both non-pathogenic and pathogenic microbes ranging from bacteria to viruses, fungi and parasites [1]. The human body is constantly exposed to these organisms that have the capacity to access and disrupt internal homeostasis [1]. The immune system encompasses complex physiological mechanisms mediated by a network of specialized cells, tissues and organs that act in concert to generate a highly functional defence system, for the immediate recognition and disposal of these foreign, non-self-agents [1]. The absence of a fully functional immune system paves the way for the entry of these disease-causing microbes with potentially detrimental consequences [2]. Thus, the ability to mount effective immune responses is crucial to human survival [2]. Over time, the human immune system has become increasing complex and more diverse to meet the demands of an ever-increasing pool of pathogens that has the ability to rapidly evolve and reproduce, often faster than the human host [2]. The immune system is comprised of two distinct sub-divisions, the innate or non-specific immune system and the adaptive or specific immune system [1].

The innate immune system is the first line of defence, and provides immediate, relatively non-specific host protection against any invading pathogens [3]. Upon breach of these innate defences by pathogens, the adaptive immune system comes into play as the second line of defence, providing a more pathogen-specific response [3]. Despite their distinctive immunological features, in practice, there is a great deal of interaction between these systems, with the cellular components of the innate system playing a pivotal role in the initiation and subsequent propagation of the adaptive responses [1]. Each system is

dependent on or strengthened by the other, in order to provide optimal response against pathogens [1].

The immune system has the remarkable ability to distinguish and harness its immunological machinery against any non-self-entities, or antigens, which are capable of eliciting an immunological response. Both the innate and adaptive immune systems execute distinct mechanisms that permit the recognition of microbial agents, which is crucial for self/non-self-discrimination [4].

Leukocytes, or white blood cells, are the chief mediators of the innate and adaptive immune responses.[5, 6] Leukocytes originate from pluripotent hematopoietic stem cells in the bone marrow, and undergo their developmental and maturation stages within the bone marrow itself [5].

1.2 Innate Immune System

The innate immune system is composed of external and internal defence mechanisms that are readily available to react to any form of invasion [3]. The external defences provided by epidermis, ciliated respiratory epithelium, body secretions and mucous membranes are in place to prevent the penetration of the pathogen and gaining access to the internal environment of the host [3, 7]. However, if these pathogenic entities breech the external defences and gain entry into host tissue or circulation, then the internal innate defences come into play [3]. These are comprised of physiological barriers, phagocytosis and inflammatory responses [3]. One of the fundamental aspects of the innate immune defence involves the initial recognition of the foreign entity through pattern recognition receptors (PRRs)[4, 5].

PRRs are germ-line encoded receptors, present in limited numbers, with the ability to recognise highly conserved molecular motifs common to large groups of microorganisms

(both pathogenic and non-pathogenic), but not found in the human host [6]. These microbial molecular motifs are referred to as pathogen associated molecular patterns (PAMPs) [6, 8]. Common PAMPs include bacterial lipopolysaccharides, double-stranded RNA (dsRNA) and peptidoglycans [6]. Several properties of PAMPs make them ideal for recognition by PRRs. PAMPs are indispensable for the survival of microbes, thus, mutation or absence of these PAMPs, is unfavourable to their ability to thrive and function [8]. Secondly, as PAMPs are unique to microbial agents, it allows the distinction between self (host tissue) and non-self (microbial) entities by PRRs, and rapid identification of these non-self-agents precipitates in a host immune response to eliminate them [8].

The innate immune mediators, namely dendritic cells, macrophages and neutrophils all express PRRs [9]. Several classes of known PRRs exist. These include Toll-like receptors (TLRs), nucleotide-binding oligomerization domain-like receptors, retinoic acid-inducible gene 1- like receptors and lastly, Deoxyribonucleic acid (DNA) receptors (cytosolic DNA sensors) [9]. PRRs are expressed on cell surfaces, intracellular compartments, as well as being released into the circulation and tissue fluids [8].

Upon recognition of PAMPs by PRRs, these receptors signal to the host the presence of invading pathogens, culminating in an anti-microbial response mediated through the activation of complex intracellular signalling cascades, including kinases, adaptor molecules and transcription factors [7]. PRR-induced activation of intracellular signalling mechanisms leads to the stimulation of gene protein levels and generation of a wide range of molecules, including pro-inflammatory cytokines that play an instrumental role in mediating the initial innate immune response as well as the ultimate involvement of the adaptive immune response [7].

1.2.1 Toll like receptors

TLRs are the most comprehensively studied class of PRRs [9]. The Toll receptor of the fruit fly *Drosophila melanogaster* was the first member of the Toll family to be discovered [8]. This receptor, identified as a maternal-gene effect was originally known for its crucial role in the dorso-ventral axis formation during fly embryogenesis [8]. Toll receptors are composed of an extracellular domain and a cytoplasmic domain that possesses similarity to the mammalian Interleukin-1 Receptor (IL-1R) cytoplasmic domain [10, 11]. Human IL-1R signalling pathway is responsible for a variety of immune and inflammatory responses in vertebrates [12]. In addition to their structural homogeneity, the Toll-Dorsal and human IL-1R signalling transduction pathways are homologous [10, 13]. These similarities between Toll and human 1L-1R suggested a potential role for the Toll receptor in the adult fruit fly's immunity [8]. Toll is reported as a potent anti-fungal as mutations in the Toll signalling pathway drastically impairs the survival of the fly following fungal infection [14].

The identification of *Drosophilia* Toll and its role in the innate immune defence lead to the discovery of the first human homologue of Toll by Medzhitov et al, which was later renamed TLR4 [10, 15]. Subsequent studies lead to the characterisation of several other TLRs in humans all structurally related to Toll [10, 12, 16-18]. To date, 10 human and 12 murine functional TLRs have been identified [19].

Human TLRs are essential for early pathogen recognition and mediation of complex host defence mechanisms [20]. They are type 1 trans membrane receptors, encompassing an extracellular or luminal ligand binding domain of leucine-rich repeats that mediate pathogen recognition and an intracellular domain of Toll/Interleukin-1 receptor (TIR) that activates downstream signalling pathways [7, 8]. TLRs can be classified on the basis of their respective PAMP ligands and their cellular localisation [19, 21]. Each individual TLR has the ability to identify distinct, structurally unrelated PAMPs originating from several microbial

agents, namely bacteria, viruses, fungi and protozoa [7]. They are either located on the cell surface or localised within endo-lysosomal compartments within the cell (**Figure 1**) [22]. TLR location is reflective of the type of PAMP they recognise and accordingly the type of pathogen they come across. TLRs expressed on the cell surface recognise PAMPs in present in the extracellular environment (bacteria and fungi) whereas those that are expressed on intracellular endosomal membranes recognise PAMPs that been taken up into the cell via endocytosis (viruses and some bacterial) [23].

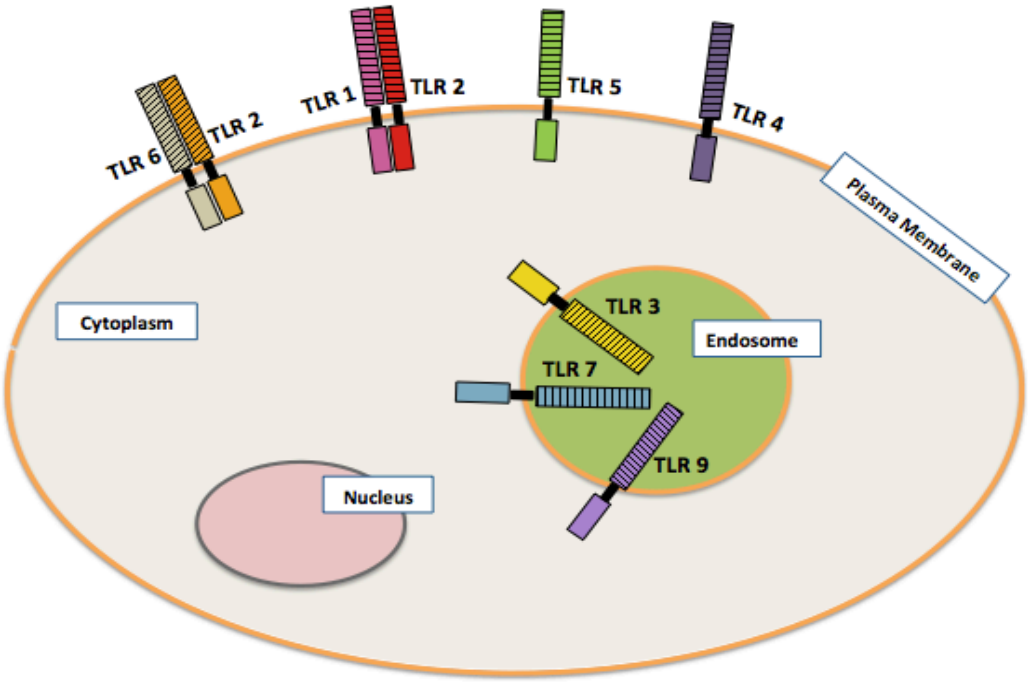


Figure 1: Cellular localisation of TLRs in TLR-expressing cells. Figure adapted from Takeda et al [22].

Distribution of TLRs in TLR-expressing cells. TLR 1, TLR 2, TLR 4, TLR 5, and TLR 6 are expressed on the cell surface. TLR 3, TLR 7, TLR 8 and TLR 9 are expressed intracellularly, at the luminal aspect of endo-lysosomal membranes.

1.2.1.1 Nucleic Acid-sensing TLRs: TLR3, TLR7 and TLR9

TLR3, TLR7 and TLR9, found within dendritic cells and other immune cells, are localized to intracellular vesicles, namely, endoplasmic reticulum (ER), endosomes, lysosomes and endolysosomes [24, 25]. Their intracellular protein levels permits the recognition of nucleic acids or derivatives, only when delivered to intracellular endosomal compartments

following the uptake of viruses, bacteria or other microbial agents [24, 25]. These nucleic-acid sensing receptors are only activated within acidified endo-lysosomal compartments [26]. TLR3, TLR7 and TLR9 are predominantly localized in the ER and traffic to the endolysosome upon ligand binding for the purpose of ligand interactions [19].

TLR3 recognizes viral dsRNA produced by many viruses during replication within infected cells, as well as a synthetic analogue dsRNA, polyinosinic-polycytidylic acid (poly (I: C)) [8, 19, 27]. Poly (I: C) imitates a viral infection, activating an antiviral response through the generation of inflammatory cytokines [19]. TLR3 signaling is crucial for anti-viral response due to its ability to induce a Type 1 interferon response [19]. TLR7 recognize ssRNA originating from RNA viruses as well as synthetic stimuli including derivatives of imidazoquinoline (imiquimod) [8, 19, 25, 28]. TLR9 recognizes unmethylated 2'- deoxyribo (cytidine-phosphate-guanosine) (CpG) dinucleotides commonly found in viral and bacterial DNA and as well as synthetic TLR9 ligand, CpG oligodeoxynucleotides.[19, 28]

The TLR signalling cascade is initiated by ligand binding, which induces the receptor to dimerize and undergo changes to their conformation, that is essential for recruitment of downstream signalling protein [20]. One of the initial steps involves the interaction between cytoplasmic TIR-domain of the receptor with TIR-domain containing adaptor proteins [29]. These adaptor proteins include myeloid differentiation primary-response protein 88 (MyD88) and TIR domain-containing adaptor inducing Interferon- β (TRIF) [22]. Different TLRs utilise different adaptor proteins, and the specific adaptor employed will be the key factor in determining the signalling pathways and responses that are initiated [30].

Accordingly, endosomal TLRs mediate their effects through two distinctive pathways: MyD88-dependent (**Figure 2.A**) and TRIF-dependent (**Figure 2.B**) pathways [29]. Activation of endosomal TLRs through these pathways results in the generation of Type 1 interferon molecules (Type 1 IFNs) including Interferon alpha (IFNα) [31].

1.2.1.2 MyD88-dependent Signalling Pathway

All TLRs employ MyD88, with the exception of TLR3[24]. Following ligand induced-receptor dimerization MyD88 is recruited to receptor where the cytoplasmic TIR domain of TLR interacts with TIR domain of MyD88 [20, 32]. MyD88 then recruits Interleukin-1 Receptor-Associated Kinase (IRAK4) to the receptor complex [22, 33, 34]. The binding of IRAK4 to MyD88 facilitates the IRAK4-induced phosphorylation of IRAK1 [20, 22]. Phosphorylation of IRAK1 involves the sequential phosphorylation of two of its amino acids; IRAK1 is initially phosphorylated at Threonine 209, followed by Threonine 387 [35]. This phosphorylation permits the attachment of Tumor necrosis factor (TNF) receptor-associated factor 6 (TRAF6) to IRAK1 [20, 36]. Upon activation of IRAK1, TRAF6 is recruited to the receptor complex, and IRAK1 binds to and stimulates TRAF6 [20, 22]. TRAF6 attaches to and stimulates transcription factor Interferon regulatory factor 5 and 7 (IRF5 and IRF7), for which, TRAF6 ubiquitin ligase activity is required [37] Upon activation, IRF5 and IRF7 translocate to the nucleus to activate transcription of IFNα regulated genes leading to robust generation of Type 1 IFNs in plasmacytoid dendritic cells (pDCs) in a TLR7 and TLR9 dependent manner (**Figure 2.A**) [25, 38, 39].

1.2.1.3 TRIF-Dependent Signalling pathway

TLR3 signaling utilises the adaptor molecule TRIF [25, 28, 39]. TRIF-mediated signalling gives rise to IRF3 activation and transcription of Type 1 IFNs (IFNα) [19]. Upon ligand binding, TLR3 recruits and interacts with TRIF via TIR-TIR connections [19, 40, 41]. TRIF recruits and associates with a complex of TRAF Family Member–Associated Nuclear Factor - Kappa β Activator (TANK)-Binding Kinase 1 (TBK1) and IkappaB Kinase epsilon (IKKε) resulting in the phosphorylation of interferon regulatory factor 3 (IRF3) [19, 41]. TRAF3 is also recruited and necessary for TRIF-induced activation of TBK1 and IKKε complex [19, 39].

Subsequent to phosphorylation, IRF3 translocates to the nucleus inducing production of IFNα [31]. (**Figure 2.B**)

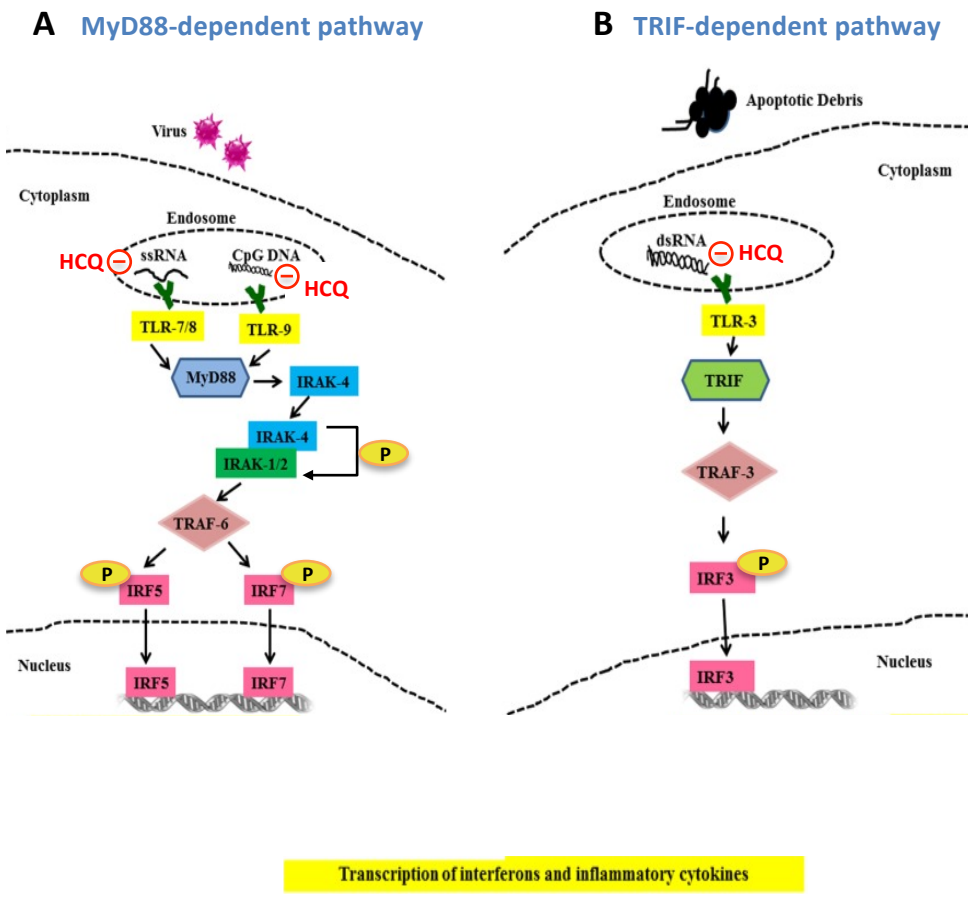


Figure 2: Endosomal TLR 3, 7 and 9 signalling pathways. Figure adapted from Shrivastav et al [31].

Nucleic acids are initially trafficked to endosomes where these receptors are located; following which, TLR signalling is activated. (**A**) MyD88-Depdentent signalling pathway for TLR7 and TLR9. Binding of nucleic acid to receptor induces recruitment of adaptor protein MyD88, which in turn recruits and activates IRAK4. IRAK4 activates IRAK1 via phosphorylation. This results in the recruitment and activation of Tumor necrosis factor (TNF) receptor-associated factor 6 (TRAF6), which stimulates transcription factors interferon regulatory factors 5 and 7 (IRF5 and 7). IRF5 and 7 translocates to the nucleus where they initiate transcription of Type1 IFNs. (**B**) TRIF-dependent pathway for TLR3. Ligand binding induces recruitment of adaptor protein TRIF, which in turn recruits TRAF3 and other signalling intermediates including TANK-Binding Kinase 1. These events eventually lead to phosphorylation of IRF3, which translocates to the nucleus to initiate transcription of Type1 IFNs. Yellow circles labelled with a "P" represents phosphorylation of molecules. HCQ = Hydroxychloroquine; HCQ is a drug used for the treatment of SLE and blocks the activation of endosomal TLRs (3, 7, and 9) by preventing Ligand-TLR interactions.

1.2.2 Neutrophils

As the most abundant form of leukocyte, accounting for 40-65% of the total white blood cell population, neutrophils (also known as polymorphonuclear neutrophils) play an essential role in the first line of defense of the innate immune response, as first responders to invading microbes. [42-44]. Neutrophils are produced in the bone marrow from hematopoietic stem cells by a process known as granulopoiesis, which occurs over a 7-14 day period.[45] Upon maturation these terminally differentiated cells are released into the circulation in a controlled manner in order to maintain homeostatic levels of mature neutrophils in the blood [46]. With an approximate diameter of 12-15 μm, neutrophils encompass characteristic morphological features of multi-lobed, segmented nuclei amid an array of granules containing host defense molecules [47]. Neutrophils typically exist at concentrations of 3-5× 10^6 cells/ml of blood, with a significant increase in their numbers seen in the presence of an infection [48]. Despite their vast numbers, neutrophils have a relatively restricted lifespan of 12-15 hours in circulation, and undergo spontaneous apoptosis in the absence of any inflammatory stimuli [42]. However, in an inflammatory setting, cytokine activation can extend neutrophil lifespan to several days [42]. Due to their remarkably short life span, neutrophils are constantly being synthesized in the bone marrow at a rate of 5-10 × 10^{10} cells, daily [49]. The need for constant production of neutrophils is a clear indication of their importance in host defense as first responders for any invasion. During developmental stages, secretory vesicles and distinct granules are formed [45]. These include azurophilic (primary), specific (secondary) and gelatinase (tertiary) granules (**Table 1**). These granules employ an arsenal of highly charged anti-microbial proteins and peptides to combat pathogens, as summarized in **Table 1** [50].

Azurophilic (primary)	Specific (secondary)	Gelatinase (tertiary granules)	Secretory vesicles
Myeloperoxidase (MPO)	Lactoferrin	Gelatinase	Alkaline phosphatase
Neutrophil elastase (NE)	Cathelicidins	Lysozyme	
Cathepsin G	Lysozymes		
Proteinase 3			
Defensins			
Bactericidal permeability- increasing factor			

Table 1: Contents of Neutrophil granules and secretory vesicles [45, 51, 52].

The constituents of primary, secondary, tertiary granules and secretory vesicles of neutrophils.

1.2.2.1 Neutrophil Anti-microbial Strategies

Neutrophils are crucial mediators of innate immunity as reflected by the severe diseases that are associated with dysfunctional neutrophil activity [53]. Neutrophils in circulation are rapidly recruited to the site of insult via host and/or microbial-derived products [54]. Neutrophils target their extensive weaponry to eliminate microbes through the execution of three distinct pathways, which include phagocytosis, degranulation and formation of Neutrophil Extracellular Traps (NETs) (**Figure 3**) [52]. Upon initial encounter of microbes, neutrophils bind to and ingest the organisms through phagocytosis [54, 55]. Ingested microbes are killed through the use of anti-microbial peptides derived from neutrophil granules or reactive oxygen species (ROS) [54, 55]. These anti-microbial peptides can also be released into the extracellular environment to destroy pathogens via degranulation [55]. Lastly, neutrophils can eliminate extracellular pathogens via release of NETs [55].

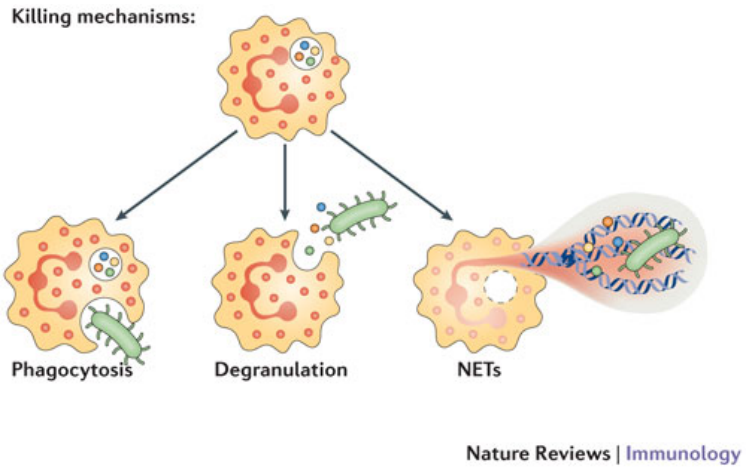


Figure 3: Neutrophil anti-microbial strategies [55].

Neutrophils exert their anti-microbial effects via three distinct mechanisms. These include phagocytosis, degranulation and NET formation.

1.2.2.2 Neutrophil Extracellular traps

In 2004, Brinkmann et al discovered a novel form of anti-microbial strategy employed by neutrophils [56]. Upon activation by stimuli including interleukin-8 (IL-8), Phorbol Myristate Acetate (PMA) and lipopolysaccharide, neutrophils extrude their entire chromatin into the extracellular space or tissue to form an extracellular fibrillary structure, termed NETs [56].

In their initial description, NETs were considered to be released from viable cells through an active process unrelated to cell death. [56]. However, further investigations showed that NETs are released at the moment of cell membrane rupture and death of the cell, demonstrating that they emerge from neutrophils undergoing cell death [44, 57]. This novel form of cell death, termed NETosis, specifically describes the mechanism of cell death resulting in the production of NETs, which is a process distinct from other forms of cellular death such as apoptosis and necrosis [57-59].

Extracellular Trap (ET) formation is not limited to neutrophils, and can be observed in other granular cell types, including mast-cells, macrophages and eosinophils but not basophils [60, 61]. As it appears to be a process shared by different cell types, a more generalized

term, ETosis, was coined by Wartha et al, which entails cell death with the expulsion of intracellular chromatin and associated proteins to form extracellular traps [62].

Since the discovery of NETs, they have been largely regarded to be part of the innate immune system; playing a crucial role in the host defense as an antimicrobial mechanism to ensnare and kill microorganisms [63]. However, these structures are increasingly being implicated at the center of many pathological conditions [64]. Thus, NETs may operate as a double-edged sword of immunity, mediating both protective and destructive responses [64].

The mesh-like NET scaffold is composed of a DNA backbone studded with histones and anti-microbial peptides derived from primary, secondary and tertiary granules, as well as some cytoplasmic proteins (**Figure 4.A**) [56, 65]. Accordingly, as their structural backbone is composed of chromatin, NET scaffold can be disintegrated with deoxyribonuclease (DNase) treatment [44, 56]. High-resolution Scanning Electron Microscopy analysis revealed that these structures contain linear DNA stretches with a diameter of 15 to 17nm and globular protein structures with ~25nm diameter that can aggregate into larger threads with up to ~50nm diameter (**Figure 4.B**) [56, 63, 66]. Only mature neutrophils are considered to form NETs as naïve neutrophils have not developed the molecular machinery required for their formation [56, 67].

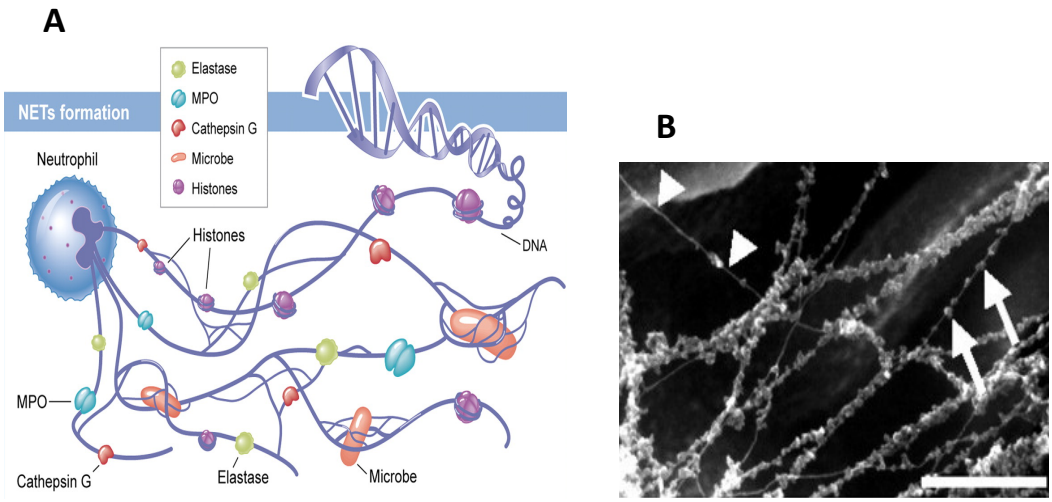


Figure 4: Structure of NETs. Figures adapted from Miyata et al [68] and Brinkmann et al [56].

(**A**) The structural components of NETs. The mesh-like NET structure is composed of a nuclear DNA backbone. This is decorated with nuclear histones and various granular anti-microbial peptides including but not limited to NE, MPO and Cathepsin G (**B**) High-resolution Scanning Electron Microscopy analysis of NETs demonstrating their structure of smooth linear fibers (arrowheads) and globular domains (arrow) (Scale Bar represents 500µm)

1.2.2.3 Biochemical and Morphological events leadings to generation of NETs

To date, extensive pools of NET-inducing stimuli have been identified and can be loosely categorized into synthetic and physiological stimuli, as well as microorganisms and their products [69]. PMA, a synthetic activator of the protein kinase C pathway (PKC) is one of the most potent inducers of NETosis, and generates NETs in approximately 30% of the neutrophil population within 2-3 hours [42, 50, 57, 64]. A wide range of bacteria including *Staphylococcus Aureus* [70] and *Mycobacterium Tuberculosis* [71] have been identified as NET inducers [72]. Furthermore, fungi including *Candida Albicans* and protozoan parasites such as *Leishmania Amazonensis* elicit NET formation [64, 73, 74].

NETosis can also be induced by physiologically relevant stimuli, namely, antibodies, antibody-antigen complexes, ROS, pro-inflammatory cytokines (TNF alpha (TNF-α) and IL-8), activated endothelial cells, platelets and nitric oxide (NO) [64, 66, 75-78]. These stimuli attach to a range of neutrophil receptors, including TLRs, cytokine, Fc and complement

receptors, all of which are implicated in the activation of a cascade leading to NET generation[64]. Although many aspects of NETosis remains unclear advances have been made towards understanding the molecular and morphological basis of NETs [59, 79].

NETosis is initiated by the binding of stimuli to aforementioned receptors on neutrophil surfaces [64]. Ligand binding stimulates the release of calcium from their intracellular stores (ER), and opens membrane calcium channels, thus mediating a rise in intracellular cytoplasmic calcium levels [64]. Rise in intracellular calcium levels triggers PKC activity which permits the membrane bound and cytosolic NADPH oxidase subunits to assemble to form a functional complex that is required for the production of ROS [79]. The NADPH oxidase complex mediates the oxidation of NADPH, liberating two electrons that are donated to oxygen molecules (O_2) to yield superoxide anions [44, 57]. The superoxide anions dismutate (spontaneously or catalytically by superoxide dismutase) to produce hydrogen peroxide (H_2O_2) molecules and dioxygen [44, 57]. The generated H_2O_2 is subsequently converted by myeloperoxidase (MPO) to produce Hypochlorous acid (HOCl) [44, 66]. Generation of these ROS is essential for NETosis as addition of catalase (degrades H_2O_2) or NADPH oxidase inhibitor interestingly inhibits the generation of NETs and neutrophils from patients with chronic granulomatous disease, a condition characterized by a mutation in one of the NADPH oxidase subunits, which failed to generate NETs when stimulated with PMA or bacteria [42, 44, 57]

Chromatin decondensation is essential for NET formation and mediated by two key neutrophil-specific enzymes stored in azurophilic granules, namely NE and MPO (**Figure 5**) [80, 81]. Neutrophil activation and subsequent generation of ROS, mediates the release of NE and subsequently MPO from their storage compartment (azurophilic granules) [82]. NE translocates to the nucleus early on during NETosis and induces degradation of nucleosomal histones (proteolytic cleavage), initiating the process of extensive chromatin

decondensation [82]. MPO, released after NE during the latter stages of the process, binds to chromatin to facilitate the decondensation process initiated by NE. [82, 83]. These two enzymes work synergistically to enhance the decondensation process [82].

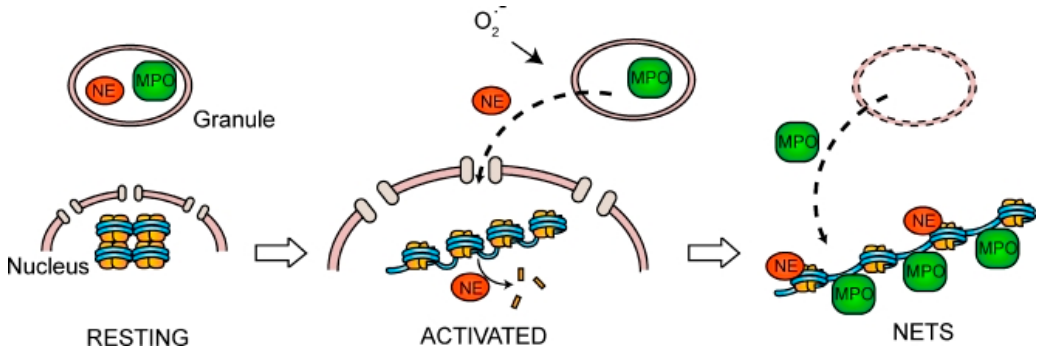


Figure 5: Role of NE and MPO in chromatin Decondensation [82].
This is a diagrammatic representation of the mechanism by which NE and MPO mediates chromatin decondensation. Normally, NE and MPO are contained within azurophilic granules of neutrophils. However, following activation and subsequent generation of ROS, NE leaves their storage granules and translocates to the nucleus where they cleave histones to induce chromatin decondensation. Subsequently, MPO is also released which, binds to chromatin further facilitating decondensation. Synergistically, NE and MPO enhance the decondensation process eventually leading to plasma membrane rupture and release of NETs.

Post-translational modifications (PTM) of histones play a pivotal role in the regulation of chromatin function and structure including chromatin decondensation [84, 85]. Histone citrullination is a PTM integral for chromatin decondensation, which involves the conversion of positively charged arginine residues to uncharged citrulline resides, catalyzed by the action of the enzyme Peptidyl Arginine Deiminase 4 (PAD4), in a calcium dependent-manner [59, 81, 85, 86]. The loss of the positive charge of histones may cause weakening of the DNA-histone interactions facilitating the unraveling of the compact nature of chromatin [64]. These histone modifications have been shown to be crucial for the conversion of higher-order chromatin from a condensed to a decondensed state during NETosis [85]. Thus, PAD4 mediated hypercitrullination of histones promotes decondensation chromatin required for NET formation with PAD4 inhibition decreasing citrullination of histone H3 and the generation of NET-like structures [81, 85, 86].

Fuchs et al demonstrated the sequence of morphological transformations occurring during NET formation [57]. After stimulation, nuclei lose their characteristic lobular shape and expand to occupy the entirety of the cell and the segregation between eu- and heterochromatin is lost [57]. Additionally, decondensation of chromatin is set in motion, with maintenance of an intact nuclear membrane [57]. Concurrently, the space between the inner and outer nuclear membrane begins to expand and the two nuclear membranes begin to separate [57]. Thereafter, the nuclear envelope fragments into a chain of individual small vesicles, by which point chromatin present is decondensed [44, 57]. The final stages involve the loss of the nuclear and granular membranes allowing the intracellular mixing of decondensed chromatin with cytoplasmic and granular constituents [44, 57]. The integrity of the plasma membrane remains intact throughout these stages up until the mixing of nuclear, granular and cytoplasmic components [57]. Following inter mixing of intracellular components the plasma membrane is permeabilized and ruptures to extrude NETs into the extracellular milieu, with concurrent death of the cell [44, 57].

NETosis has been demonstrated as a unique form of cell death, not associated with apoptosis or necrosis, exhibiting distinctive morphological and structural features [57]. Apoptosis is a caspase-dependent process and neutrophil stimulation with apoptosis-inducing stimuli results in features characteristic of apoptosis including chromatin condensation, nuclear fragmentation, cytoplasmic vacuolization and maintenance of intact nuclear membranes [57]. Contrastingly, in the presence of necrosis-inducing stimuli there is loss of nuclear structure (fusion of nuclear lobules) and the nuclear and granular membranes remain intact, which is reflective of a necrotic morphology [57]. Neither process was reported to induce formation of NETs [57]. These features, characteristic of apoptosis and necrosis are absent during NETosis [57]. Furthermore, NETosis occurs faster than apoptosis [87].

1.2.2.4 Function of NETs

The antimicrobial mechanism of NETs have been theorised to be a two-step process. NETs initially trap microbes to facilitate subsequent NET-mediated killing via antimicrobial peptides [67]. The NET scaffold serves several purposes. It functions as a physical barrier aimed at immobilising pathogens to prevent their dispersion, therefore limiting the spread of infection [50, 88]. It facilitates the effective concentration and synergy between NET-localized proteins, thereby amplifying their antimicrobial effects [50]. Lastly, the NET scaffold also anchors the antimicrobial agents to their DNA scaffold, limiting the ability of these potentially destructive agents from exerting insult to host tissue [50]. The structural integrity of NET must remain intact in order for the effective execution of their antimicrobial function through microbial containment and delivery of high local concentration of anti-microbial peptides, as evidenced by the drastic reduction in their bactericidal activity when the scaffold was dismantled using DNase treatment [56].The ability of NETs to bind to these pathogens is likely due to ionic force-mediated interactions between the anionic microbial surfaces and cationic NETs constituents [44, 74]. Although NET-mediated trapping is an established feature, their ability to exert microbicidal effects remains unclear.

1.3 Adaptive immunity

Upon breach of the innate immune defences by pathogens, the adaptive immune system comes into play as the second line of defence, providing means of a more versatile response. This response is capable of overcoming the limitation of target-specificity faced by the innate immune system, enabling an antigen-specific response against the pathogens encountered [1]. Adaptive immunity is broadly categorized into two classes of responses: Humoral responses (mediated by B-cells and antibodies) and cell-mediated responses

(effected by T-cells) [89]. Central to these processes is the recognition of antigens by T and B-cells, mediated through the expression of T-cell receptors (TCR) and B-cell receptors (BCR) in these cell subsets [3].

Dendritic cells (DCs) bridge the innate and adaptive immune systems [90]. DCs (Antigen Presenting Cells (APCs)) capture antigens and process them into peptides fragments, which are then presented on major histocompatibility complex (MHC) molecules for the recognition by T-cells and occurs in the presence of co-stimulation which results in T-cell activation [91]. This activation induces the naïve T-cells to proliferate and differentiate (clonal expansion) into effector T-cells and memory cells, including CD4+ T helper cells (T helper 1 and T helper 2) and CD8+ cytotoxic T-cells that have identical antigen specificity [89]. T helper cells produce cytokines that cause antigen-activated B-cells to differentiate into antibody producing plasma cells and memory cells [3]. Once these antibodies come into contact with their specific antigen, they target it for destruction by the immune system[3]. Memory B and T-cells confer protection against subsequent re-exposure to the same antigen [91]. Regulatory T-cells are a type of T lymphocytes that function to inhibit the proliferation of potentially self-reactive lymphocytes [91].

1.4 Autoimmunity

The defensive mechanisms employed by the immune system are used to eliminate microbial cells and their toxic material in an aggressive manner. It is therefore paramount that self-tolerance is maintained and these defensive mechanisms are not elicited against the host's own cells [4].

As previously mentioned, the immune system is capable of distinguishing between self-and non-self-entities and this ability is crucial to maintain non-responsiveness to self. However if self-tolerance is lost, it can result in antigen-driven immune responses against self-

antigens (i.e. autoantigens), characterizing autoimmunity [3, 4]. Autoimmune response is often elicited by the activation of auto reactive T and B-cells [3] triggered by infection or specific antigen exposure.

Normally auto reactive T and B-cells are eliminated (clonal deletion by apoptosis), re-programmed (receptor editing to a non-self-version) or inactivated (clonal anergy) by primary and secondary lymphoid organs (central tolerance) [92]. If these autoreactive cells escape central tolerance and enter the periphery, extrinsically regulated mechanism exists to limit the danger associated with these auto reactive cells (Peripheral tolerance) [1, 92]. These include physical elimination via apoptosis, limitation of co-stimulation required for their activation (clonal anergy) and active suppression by regulatory T-cells (regulated inhibition) [3]. Thus, these checkpoints are responsible for the maintenance of immunological self-tolerance [92]. However, if a situation arises where self-reactive cells bypass these checkpoints, autoimmunity can occur [4].

1.4.1 Autoimmunity and NETosis

Physiological mechanisms of cell death have been devised such that they exert no harmful effects to the host, and ensured through their rapid clearance to prevent the accumulation of this potentially dangerous cellular debris [93]. However in many autoimmune conditions, molecules associated with dying cells have been prime autoantibody targets [93]. The initial evidence linking NETosis to autoimmunity was in the context of small vessel vasculitis (SVV), a disease normally associated with the presence of anti-neutrophil cytoplasmic autoantibodies (ANCAs), including MPO ANCAs [94]. In SVV patients NET components have been found to be raised and SVV-derived ANCAs have been reported to induce neutrophils to produce NETs which contain target autoantigen MPO [94]. Furthermore, detection of NETs and MPO-DNA complexes in inflamed kidney biopsies in SVV was suggestive of a pro-

autoimmune capacity of these structure via autoantigen exposure and induction of vascular damage [94].

Notably, development or exacerbation of autoimmune responses has been shown to frequently follow an infection by pathogens involving NETosis [95]. Moreover, disruption of clearance mechanisms of NETs at both a genetic and cellular level may also be associated with autoimmunity through increased exposure of these structures to immune cells leading to autoantigen presentation [50].

1.5 Juvenile onset Systemic Lupus Erythematosus

Systemic Lupus Erythematosus (SLE) is considered a prototypical autoimmune condition characterized by the generation of autoantibodies against nuclear self-antigens [96]. SLE is diagnosed in people of all ages with female to male ratio of approximately 10:1 in adult onset disease.[97, 98]. Predominantly prevalent amongst women of childbearing age, 15-20% of all SLE patients are diagnosed during childhood [97, 99].

Although there is no strict definition of Juvenile-onset SLE (JSLE), it is generally classified as onset or diagnosis prior to the age of 17 [98]. JSLE has an incidence of 0.36-0.9 per 100,000 children per annum [96]. The incidence varies in accordance to racial background, with a higher occurrence rate reported amongst non-Caucasian children, mainly Hispanic, African Americans and Asians [96, 100, 101]. Onset of JSLE is rare in children under the age of 5 [97, 98]. In childhood onset disease there is a less pronounced female predilection with a gender ratio of 4:3 and 4:1 in pre- and post-pubertal young individuals, respectively [102].

Although the overall presentation, clinical features and immunological profile of childhood-onset disease resembles those in adult SLE, it has been established that the age of disease-onset is a defining predictor of the clinical course and disease outcome [103, 104]. In comparison to adult-onset SLE, JSLE has a more severe disease presentation and clinical

course with a high rate of organ involvement and requirement for high-dose corticosteroid and immunosuppressive treatment [102, 103, 105].

Although there has been considerable improvement in the life expectancy of JSLE patients, childhood-onset disease poses higher mortality rates with lower life expectancy than in the general population [96, 101, 103, 106-108]. JSLE has a tendency to present with non-specific constitutional symptoms namely, fever, weight loss, lymphadenopathy making diagnosis a difficult task [100, 101, 109]. Furthermore, the rarity of childhood-disease has led to a low awareness of the JSLE amongst physicians leading to the delayed diagnosis [110]. However, timely diagnosis and prompt treatment is central for the prevention of chronic, debilitating long term outcomes [110].

1.5.1 Clinical and Immunological Characteristics

JSLE is a complex multi-organ disease with highly variable heterogeneity of clinical manifestations that range from non-specific symptoms to more severe life-threatening organ-failure and runs an unpredictable course [111]. The dynamic nature of the disease makes diagnosis of SLE very challenging [100].

Constitutional manifestations are frequent in JSLE and include fatigue, fever, anorexia, weight loss, alopecia and arthralgias that can present during disease onset as well as during disease flares [102]. Fatigue is more pronounced in childhood and found to be disabling and challenging to treat [112]. Characteristic malar, or butterfly rash is a common manifestation of JSLE affecting 60-85% of patients [100, 112, 113]. Childhood-onset disease is associated with more frequent and critical renal and neurological manifestations compared to adult disease [114, 115]. Renal disease affects up to 50-70% of patients with Lupus nephritis being one of the main presenting features of childhood disease and a determinant of disease outcome [103, 116]. Increasing evidence associates cardiovascular disease in JSLE with significant morbidity and mortality [100, 117].

The revised American College of Rheumatology (ACR) classification criteria of SLE are adapted for the diagnosis of both adult and juvenile onset SLE (**Table 2**) [103]. The classification contains 11 criteria, of which, at least 4 or more have to be met before diagnosis is formally made [100, 118]. Several systems have been devised and validated for assessment and monitoring of disease activity in JSLE, including SLE disease activity index (SLEDAI), British Isles Lupus Activity Group Index (BILAG) and Systemic Lupus Activity Measure (SLAM) that play a significant role in guiding disease management [112, 119].

The serological hallmark of SLE is the production of autoantibodies against a range of autoantigens [100, 102, 113]. Antinuclear Antibodies (ANA) can be found in more than 95% of JSLE patient sera, with ANA absent disease being a rare occurrence seen in 2-5% of the cases [102, 113]. Positive ANA is not specific for SLE as they are also present in other conditions including infection and other autoimmune conditions [102, 120]. ANAs are categorized into two groups, namely, autoantibodies to DNA and histones (anti-dsDNA and anti-histone antibodies) and autoantibodies to other nuclear antigens (anti-Smith (Sm), anti-Smith Surface antigens (SSA), anti-specific soluble ribonucleic acid (SSB) and anti-Ribonucleoprotein (RNP) antibodies) [121]. Anti-dsDNA antibodies are found in 61-93% of JSLE patients with active disease and have high specificity for SLE [102]. Anti-Sm antibodies, which are also highly specific for Lupus, can be detected in about 51% of JSLE patients [102, 122]. Anti-RNP antibodies are present in around 37%, anti-SSB in about 15% and anti-SSA antibodies in around 33% of JSLE patients [122]. Other autoantibodies detected in JSLE patients include anti-ribosomal P antibodies [122, 123]. Anti-dsDNA, anti-histone and anti-ribosomal P antibodies are more common in JSLE compared to adult disease [122-124].

Revised ACR 1997 Criteria for the Diagnosis of SLE
Malar Rash
Discoid Rash
Photosensitivity
Oral or nasal ulcerations
Non-erosive Arthritis
Nephritis
Persistent proteinuria > 0.5g/day cellular casts
Encephalopathy
Seizure
Psychosis
Pleuritis or Pericarditis
Cytopenia
Positive immunoserology
Antibodies to dsDNA
Antibodies to Sm nuclear antigen
Positive finding of antiphospholipid antibodies based on:
IgG or IgM anti-cardiolpin antibodies
Lupus anti-coagulant or
False-positive serological test for syphilis for at least 6 months*
Positive Anti-nuclear Antibody test

Table 2: Classification Criteria for SLE. Adapted from Tucker et al [110].
The table represents the Revised American College of Rheumatology Classification Criteria for SLE. Four out of the eleven criteria generally indicate a diagnosis of SLE.

1.5.2 Immuno-pathogenesis

Although the exact mechanism by which SLE arises is still largely unknown, significant progress has been made in the past decades, generating compelling theories into the pathogenesis of this disease. It is considered to be a multifactorial disease, caused by a complex interplay between genetic and environmental factors including infections and drugs [31, 125]. Dysfunctional innate and adaptive immune responses govern disease pathogenesis in Lupus [97].

B-cell hyperactivity, abnormal T-cell responses and T-cell cytokine production coupled with reduced regulatory T-cells activity are implicated in the development of autoimmunity in Lupus despite overall lymphopenia of T and B-cells observed in SLE [126, 127]. SLE is characterized by the loss of B-cell and T-cell tolerance against autoantigens [97]. The cause behind this loss of tolerance remain largely unknown; however, defects in several checkpoints of central and peripheral tolerance mechanisms of B-cells have been suggested to allow the accumulation of autoreactive mature naïve B-cells in peripheral compartments [128]. B-cells contribute largely to the pathology through autoantibody production against a range of self-antigens that characterizes the disease [129]. The improvement of clinical disease observed in Lupus patients receiving B-cell depletion therapies is a reflection of their pathological role [127]. SLE patients display constantly elevated levels of blood plasma cell precursors which indicates that Lupus B-cells are in constant state of stimulation to differentiate into autoantibody- producing plasma cells [103]. Autoreactive B-cells have also been speculated to induce disease phenotypes via antibody-independent mechanisms, by either serving as APCs to activate pathological T-cells or by directly facilitating local inflammation as SLE mice lacking ability to produce autoantibodies went on to develop Lupus nephritis, which was not observed in B-cell deficient Lupus mice [130]. Similarly to B-cells, there are evident abnormalities in SLE T-cells including enhanced signaling responses following activation of these cells, likely allowing for rapid responses to antigenic stimuli [103]. Type 17 T helper cells are a T-cell subset that is responsible for the generation of IL-17, a cytokine with strong inflammatory response-inducing capacity [131]. These cells are found in excess in SLE and observed in diseased kidneys of Lupus patients [131]. Furthermore, IL-17 levels are raised Lupus sera and these cytokines are reported to promote autoantibody formation [131].

Regulatory T-cells are crucial in preventing the breakdown of self-tolerance that could lead to autoimmunity [131]. Not only is there a significant deficiency of these cells in Lupus patients [132] but their suppressive functions are largely impaired [133]. This could account for the cytokine disturbance characteristic of SLE, as IL-6, a pro-inflammatory cytokine produced in excess in Lupus [134] has inhibitory effects on regulatory T-cell functions [135]. Levels of cytotoxic CD8+ T-cells are observed to be raised in Lupus patients and found to be associated with renal disease [127, 131]. Notably, NETs, through direct contact were observed to reduce the activation threshold of CD4+ T-cells, suggesting a pathological role for these structures [136]. Once the autoimmune state is triggered, the responses can expand beyond the inciting autoantigens to other antigens, leading to the propagation of autoimmunity [137].

In SLE, autoantibodies form immune complexes with auto-antigens (self-nucleic acids) producing pathogenic moieties that mediate cytokine production and deposits in various parts of the body inducing inflammation and tissue damage [78, 129, 138]. As self-nucleic acid containing immune complexes are major inducers of Lupus pathology, the presence of self-DNA in the extracellular milieu to form part of these disease mediators is a key step in SLE pathogenesis [138]. Loss of tolerance to self-DNA is closely associated with the onset of clinically overt disease[103].

In SLE, disruption of normal physiological cell death pathways results in self-DNA readily being made available to detection by nucleic acid endosomal TLRs, which results in immune activation [139]. Under normal physiological conditions, mechanisms have been set in place to ensure that host DNA is non-immunogenic and not accessible to intracellular innate nucleic acid sensors. Self-DNA is normally restricted to the nucleus or the mitochondria, away from the access of the immune system [25, 31]. Furthermore, DNases

and RNases readily degrade extracellular self-nuclear material before they can gain access into intracellular TLR compartments [25].

The intracellular localisation of these nucleic acid receptors is likely to contribute towards preventing recognition of self-DNA present extracellularly, whilst facilitating recognition of viral/microbial DNA that is released into intracellular TLR compartments during infection [140, 141]. However, even if self-nucleic acids gain access to these nucleic acid receptors, self-DNA is a poor TLR ligand with poor immuno-stimulatory activity [142]. Endosomal TLRs normally distinguish between self and microbial DNA thorough the level of methylation of cytosine-phosphate-guanine (CpG) dinucleotides, although for RNA, theses discriminatory features are less obvious [143]. Unmethylated DNA constitutes the ideal TLR9 Ligand [144]. Viral/bacterial DNA is largely unmethylated, containing high number of unmethylated CpG motifs rendering them potent TLR ligands [144]. In contrast, mammalian DNA contains low levels of CpG motifs and of those present, the majority is methylated [142]. In addition mammalian DNA contains a high number of TLR9 inhibitory DNA sequence elements [142]. Accordingly these features provide tolerance to self-DNA, and if a situation arose where self-DNA were hypomethylated, it could lead to recognition of the unmethylated CpG motifs by TLR9 [142]. Thus bypassing these protective mechanisms can allow self-DNA to access endosomal TLRs, thereby inducing an immune activation.

Until recently aberrant apoptosis coupled with defective clearance of apoptotic debris resulting in increased load of apoptotic material was considered the primary source of self-nucleic autoantigens [145-147]. It was recently observed that the presence of extracellular DNA is not always pathological and a feature of the physiological process NETosis [64, 147]. Similarly to apoptosis, evidence suggests that this process is largely dysregulated in Lupus, and provides an additional source of autoantigens contributing to the autoimmune state in

SLE [147]. Therefore dysregulation of these two cell death pathways, are likely to act in concert as initiators of autoantigen presentation in SLE [139].

1.5.2.1 NETs and SLE

The original observation of NET production placed these structures in the setting of host defence, serving a role in innate immunity in the fight against infections [56]. However, despite their evident protective role, an increasing pool of evidence implicates NETs at the centre of many pathological conditions, labelling this novel paradigm as a doubled edged sword of immunity, serving both protective and destructive roles [64].

Since their discovery, extensive research has been carried out to investigate the exact nature of their contribution towards immune dysregulation seen in Lupus. The involvement of NETs in SLE pathogenesis follows from the presence of autoantibodies against NET-localised molecules including dsDNA, histones and numerous NET-associated proteins (e.g. MPO) [148]. Several NET localised proteins are also present in high levels in Lupus patients [139]. Pro-inflammatory cytokines including TNF-α, IL-17A and IL-8 can stimulate NET formation, indicating that these structures are likely being produced excessively in highly inflammatory conditions, including Lupus [64, 149]. Elevated levels of anti-RNP, anti-LL37 and anti- (human neutrophil peptide) HNP antibodies is a common occurrence in SLE and these autoantibodies have been reported to elicit NETosis in a FCγRIIa dependent manner [147, 150, 151]. IFNα is found in excess in Lupus patients and reported to prime neutrophils to produce NETs and therefore it can be expected that in highly interferogenic environments, including Lupus sera, there is increased formation of NETs [78, 151-155]. A recently discovered neutrophil subset, termed low density granulocytes, (described further in section 4.3.2) has an increased capacity to produce NETs owing to their enhanced ability to produce IFNα [156]. Taken together, aberrant NETosis occurring in Lupus patients [157] is likely to result in an increased load of NET autoantigens.

Under normally physiological conditions, NETs are rapidly cleared to prevent the prolonged exposure of these potentially immunogenic structures to the immune system [158]. Calcium-dependent Deoxyribonuclease I (DNase) is a serum endonuclease that can hydrolyse DNA [159, 160]. Accordingly, the DNA backbone of NETs can be dismantled using DNase1 [159, 161]. Once released, the NET DNA scaffold is degraded by extracellular DNase I which facilitates the subsequent clearance from the circulation by macrophages [158]. Impaired clearance of NETs is increasingly becoming evident in Lupus patients [78, 147]. In SLE, low NET degradation is associated with more severe disease and found to correlate with anti-NETs and anti-dsDNA autoantibodies [162]. This may signify the importance of timely removal of NETs by DNase, to prevent counter-productive role of NETs, as a source of autoantigens resulting in autoantibody production.

Hence, it can be expected that an imbalance between NET formation and clearance (aberrant formation and impaired clearance) may lead to persistence of these structures in a non-degradable state, harboring numerous autoantigens, allowing this antimicrobial mechanism to become pathological, resulting in autoantigen presentation [157].

Even in dysregulated NETosis, the intracellular localisation of nucleic acid receptors prevents recognition of extracellular self-DNA externalised by NETs [141]. However, self-DNA when contained within immune complexes (nucleic-acids complexed with autoantibodies) has been shown to activate pDCs and this is considered to be the primary mechanism through which self-DNA activates the immune system in SLE [163]. This has been attributed to the ability of autoantibodies in these immune complexes to internalise self-DNA complexes into pDCs through FCγ surface receptor II (FcγRIIa)- mediated endocytosis which results in the activation of TLR9 signalling pathways of cytokine production [163, 164]. In Psoriasis, self-DNA was found to activate pDCs by forming complexes with cationic peptide LL37 (self-DNA-LL37 complexes) that transported the DNA

via receptor-independent lipid-raft mediated endocytosis [165, 166]. Since, a similar role for these cationic peptides has become evident in Lupus, but in the context of NETs. Lande et al demonstrated that DNA immune complexes containing neutrophil antimicrobial peptides LL73 and HNP could activate pDC to produce IFNα in SLE patients [78]. These antimicrobial peptides were found to be essential for the capacity of autoantibodies in immune complexes to induce the uptake of self-DNA into pDCs via FcγRII mediated internalization (receptor mediated endocytosis) as they protect DNA from nuclease degradation (likely through the promotion of DNA aggregation to produce insoluble particles) [78]. These self-DNA-antimicrobial complexes, forming the immunogenic core of immune complexes were found to be released by NETs [78].

Notably, anti-DNA and anti-LL37 antibodies were reported in SLE patients suggesting that these NET-derived complexes are functioning as autoreactive B-cell autoantigens (**Figure 7**). These anti-LL37 autoantibodies not only induced neutrophils to release NETs, but also promoted receptor-mediated uptake of self-DNA-antimicrobial complexes from NETs into pDCs resulting in IFNα production [78]. LL37, produced by neutrophils, is upregulated in the blood of SLE patients [155]. With regards Psoriasis, self-RNA was reported to activate pDCs in a TLR7-dependent manner resulting in IFN production, and although this is not in the context of SLE, it could be a likely mechanism occurring in SLE [140]. Anti-RNP antibodies from Lupus sera induced NET formation in a – FcγRIIa and TLR7– dependent manner, and these NETs activated FcγRIIa-blocked pDCs indicating that immune complex carryover was not responsible for pDC activation, but rather the NET components themselves [151]. These results suggest mechanisms by which NETs activate the immune system via autoantigen exposure in SLE.

However, dysregulated NETosis on its own is not enough to break immunological tolerance, and considering the negligible immuno-stimulatory activity of self-DNA, additional events

are likely to occur to convert NET material into pro-auto immunogenic material, [144, 167]. This apparent discrepancy may potentially be explained by the ability of NETs to affect immunogenicity of autoantigens converting them into potent activators of endosomal TLRs [168].

Therefore not only are these structures being produced in large quantities and ineffectively cleared but they also manage to bypass all protective mechanism set in place to prevent self-antigen exposure, thereby gaining access to intracellular nucleic acid sensors and leading to activation of the immune system.

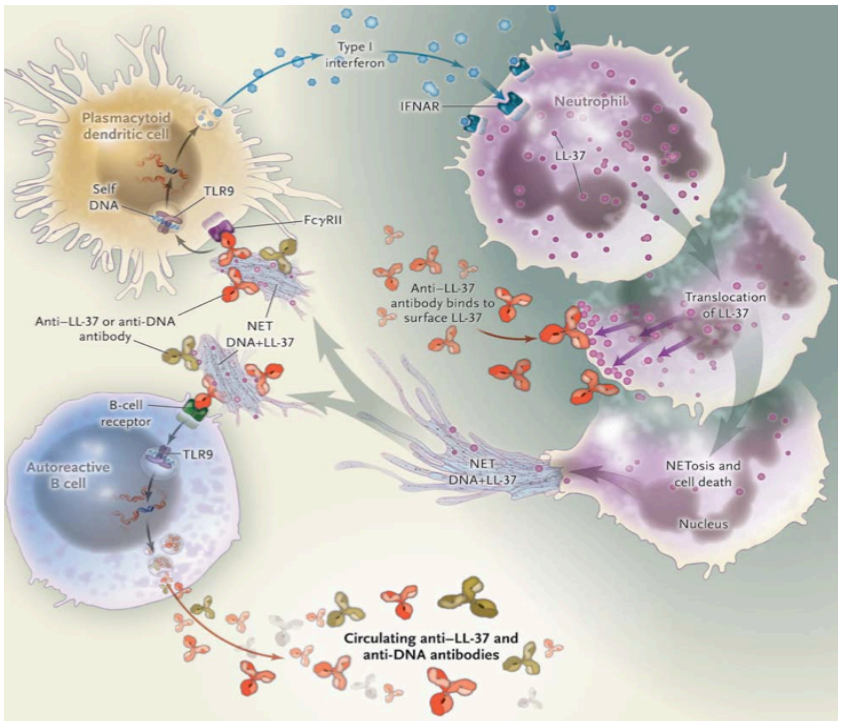


Figure 6: NETs in the activation of the immune system in Lupus [150].
This is a schematic representation of the self-amplifying effects that occur as a result of autoantigen presentation by NETs. IFNα can prime neutrophils to release NETs. NETosing neutrophils release NETs that get taken up by pDCs as NET-associated DNA-LL37 immune complexes, along with anti-DNA and and-LL37 autoantibodies which activate pDCs via endosomal TLR9 leading to robust IFNα production. In turn, IFNα primes neutrophils for further NET release. Furthermore, these NET-derived antimicrobial peptides and DNA can serve as autoantigens activating autoreactive B-cells. NETs activate these autoreactive B-cells likely through their ability to co-engage both B-cell receptor and TLR9 in B-cells, resulting in the production of anti-LL37 and anti-DNA autoantibodies. These autoantibodies can also in turn induce further NETosis. Accordingly, these autoantibodies induce persistent activation of pDC by promoting the uptake of self-DNA complexes into pDCs (via receptor mediated endocytosis) as well as promoting further NET production. Additionally, anti-NET antibodies are reported to impair NET degradation. These events set into motion a viscous cycle of chronic pDC activation, IFN production, NET release; all of which likely contributes to the initiation and propagation of autoimmune process in SLE.

1.5.2.2 IFNα and SLE

pDCs represent a subset of immune cells unique in their ability to potently generate copious amounts of IFNα in response to a wide range of stimuli including bacteria, viruses and protozoa [94, 169, 170]. These cells normally detect viral and microbial nuclear DNA via endosomally localised TLRs in order to induce rapid and potent generation of Type1 IFNs as an anti-viral defence strategy [166]. Under normal physiological conditions these cells do not react to self-DNA [166]. However, in SLE self-DNA gains access to intracellular compartments leading to a TLR-mediated chronic IFNα production [166]. In SLE, the presence of chronically activated pDCs leading to an unabated production of IFNα occurring early on in disease is a key event contributing to the development of an autoimmune state [78].

Type 1 IFNs are cytokines with immuno-modulatory effects crucial for the host defence against viral infections [171, 172]. They signal through the Janus Kinase (JAK)-signal transduction and activator of transcription (STAT) signalling pathway (JAK-STAT)(**Figure 7**) [173]. IFNα is a subtype of type 1 IFN molecules [173]. Although a wide range of immune cells are able to produce IFNα molecule, pDCs (also known as IFN-producing dendritic cells) are conceived as the primary source of type 1 IFNs [94]. Initially referred to as 'natural interferon-producing cells', one pDC has the capacity to produce approximately 10^9 molecules of IFNα within a time span of 12 hours which is 100-1000 fold higher than the ability of any other IFN-generating cell [174, 175]. This can partly be attributed to the TLR7 and TLR9 expression as well as high expression of IRF3, IRF5 and IRF7 in pDCs [176, 177]. pDCs are usually present in low levels in blood of SLE patients as they tend to migrate to areas of inflammation including skin and kidney [178-180].

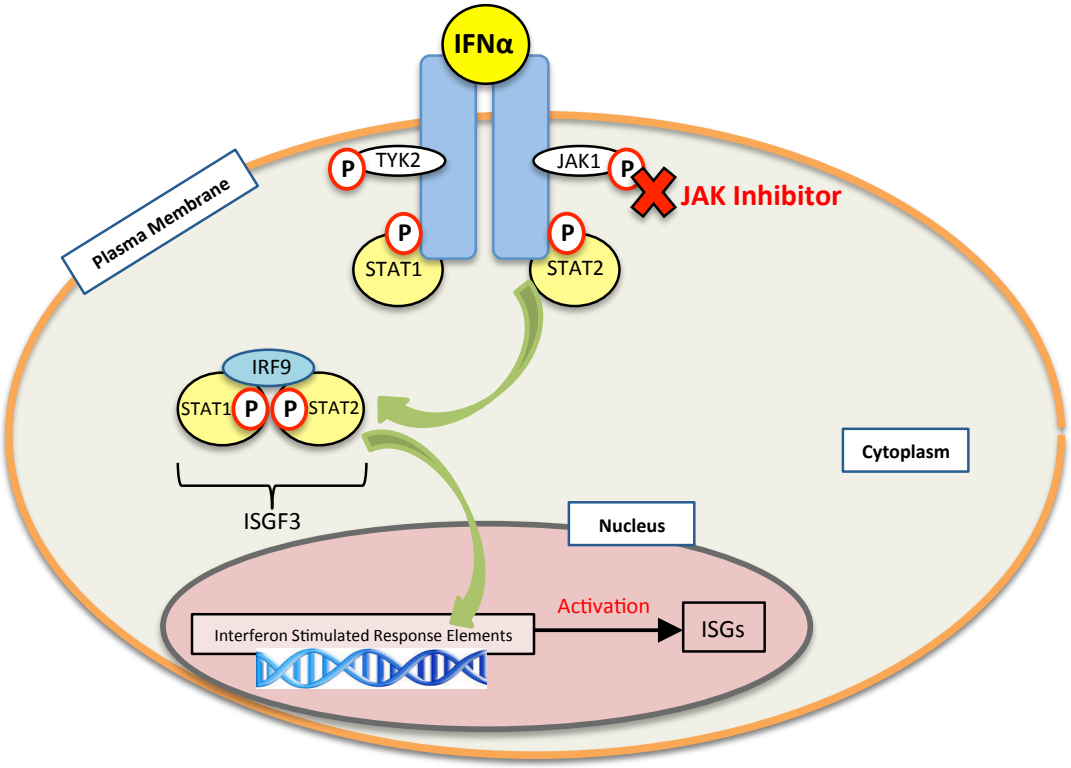


Figure 7: JAK-STAT signalling pathway induced by Type 1 IFNs. Figure adapted from Aghemo et al [181] and Clark et al [182].

Type1 IFNs signal through JAK-STAT signaling pathway to activate gene expression. Interaction of IFNα with Type 1 IFN receptors (IFNAR) located on the cell surface leads to activation of receptor-associated kinases, which include JAK1 and Tyrosine kinase 2 (TYK2). These activated kinases phosphorylate transcription factors STAT1 and STAT2, which in turn interacts with IRF9 to form a complex termed IFN-stimulated gene factor 3 (ISGF3). This translocates to nucleus and binds to IFN-stimulated response elements (ISRE) that is present in the promoter region of some Interferon stimulated genes (ISGs), and thereby activates their transcription. Janus Kinase (JAK) inhibitors prevent the activation JAK-STAT signaling pathway of IFNα. Circles with a red outline labelled with a "P" represents phosphorylation of molecules.

Despite their evident protective role, prolonged exposure to these cytokine can contribute towards development of autoimmune states through breakdown of tolerance [171]. IFNα is considered a key mediator of SLE pathogenesis contributing significantly to the cytokine disturbance seen in the disease [152, 183]. Raised serum levels of type 1 IFNs are a common finding in Lupus patients, correlating with disease activity and severity [94, 152, 184]. Moreover, the "interferon alpha signature", a hallmark feature of both adult and childhood SLE alike, represents the raised expression of type 1 IFN-inducible genes found in most Lupus patients [174, 176].

Although the exact pro-autoimmune effects of Type 1 IFNs are still largely unclear, several mechanisms have been proposed (**Figure 8.A and 8.B**). Normally, self-reactive T-cells managing to bypass central tolerance in thymus will be dealt with by peripheral tolerance mechanisms, resulting in the elimination of these autoreactive cells [174]. Immature myeloid DCs facilitate maintenance of peripheral tolerance by promoting regulatory T-cell induction as well as capturing autoantigens and presenting them to autoreactive T-cells without co-stimulation, leading to deletion or anergy of these autoreactive cells [127, 174]. Accordingly, any inappropriate stimulation of these immature DCs could disrupt peripheral tolerance [127]. IFNα can promote the maturation of myeloid DCs, and these matured DCs potently induce T-cell activation and effector differentiation thereby disturbing the delicate balance controlling peripheral tolerance [127, 174, 185]. Monocytes have been reported to differentiate into antigen presenting DCs following exposure to Lupus sera in an IFNα dependent manner [178]. These matured DC were capable of presenting autoantigens to CD4+T-cells [178]. Therefore the highly interferogenic SLE sera represent a chronic DC inducing environment, likely to mediate the autoimmune process in Lupus.

Type 1 IFNs also promote costimulatory molecule upregulation (CD80 and CD86) thereby facilitating autoreactive T-cell survival and expansion [173]. Furthermore, SLE sera-induced DCs have been found to trigger cytotoxic CD8+ cell differentiation that in turn promotes formation of nucleosomes and granzyme B-dependent autoantigens with potential pro-autoimmune effects [127].

Furthermore, IFNα demonstrates the capacity to upregulate TLR7 and IRF7 expression in pDCs, myeloid dendritic cells (mDCs), and monocytes [174]. As a result, the sensitivity of these cells to immune complexes containing nucleic acids are increased, amplifying further generation of Type 1 IFNs [174].

Type 1 IFNs also have the capacity to affect B-cell functions through various mechanisms. IFNα released by activated pDCs can induce activated B-cells to differentiate into plasmablasts and, along with pDC-derived IL-6, cause these plasmablasts to become plasma cells that can produce antibodies [186]. This can lead to a viscous cycle as these autoreactive B-cells can produce autoantibodies that form immune complexes with self-antigens which in turn serve as chronic activators of pDCs in a TLR-dependent manner pDCs and therefore leading to chronic IFN production. Furthermore, IFNα lowers activation threshold of B-cells via BCR, thereby facilitating rapid antibody responses [187]. Moreover, in DCs, IFNα induces the upregulation of B-lymphocyte stimulator protein (BLyS) and proliferation inducing Ligand (APRIL) thereby promoting B-cells survival [173].

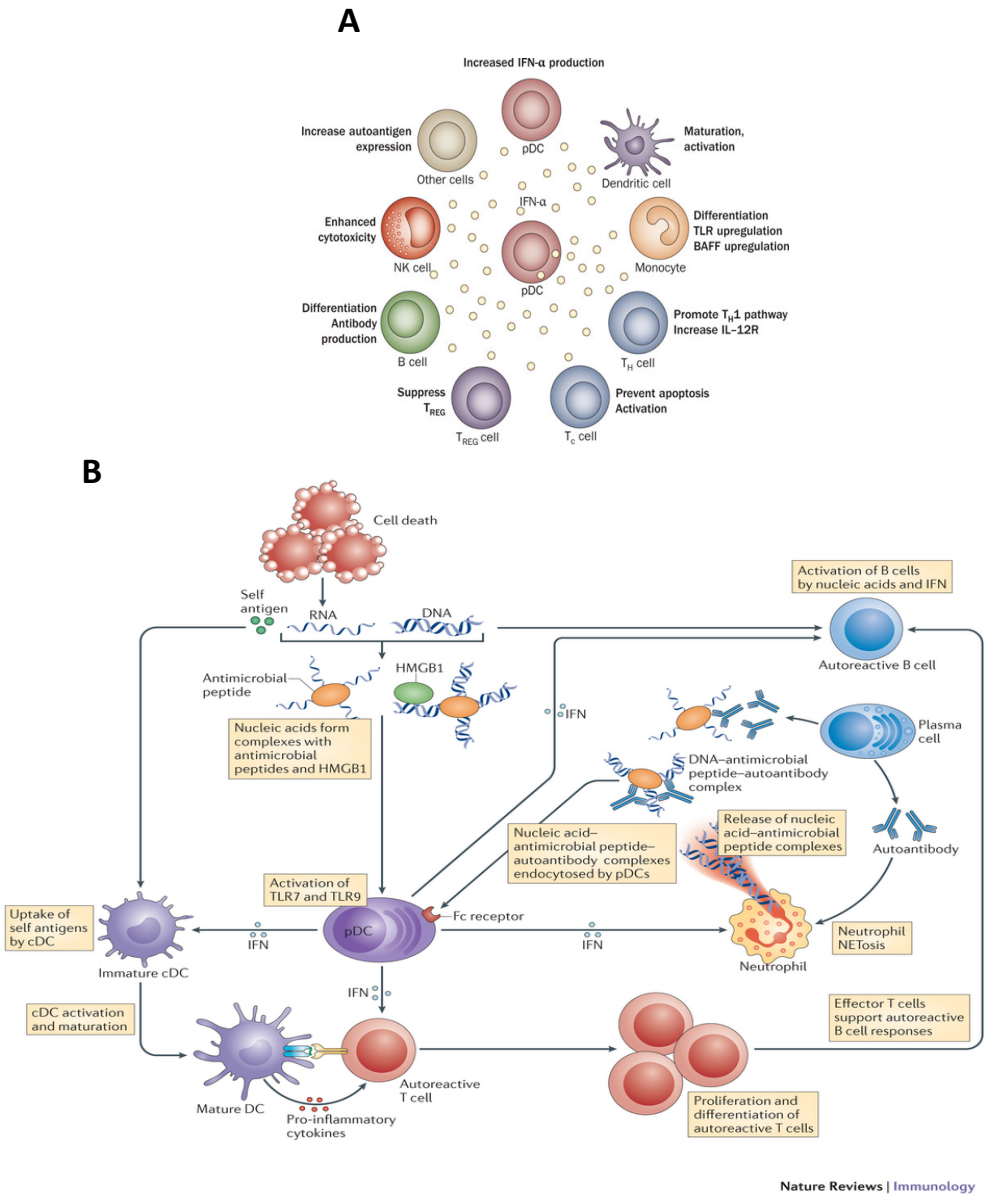


Figure 8: IFNα in the activation of autoimmune responses in Lupus [188, 189].

(**A**) This is a schematic representation of the effect of chronic IFNα production on specific cell types, which results in promotion of pro-autoimmune responses in Lupus. Chronic activation of pDCs results in unrestricted IFNα production. (**B**) Pathogenesis of Lupus with regards to deregulated cell death pathways and IFNα. Dying cells (dysregulated apoptosis) release nucleic acid containing complexes that activate pDCs in a TLR7/9 dependent-manner to produce IFNα. In turn, IFNα acts on range of immune cells to bring about Lupus pathology including promotion of T-cells expansion and differentiation as well as maturation of dendritic cells. Furthermore, IFNα, together with pDC-derived IL-6 induces autoantibody formation by autoreactive B-cells (likely to have escaped negative selection) and also stimulates release of NETs containing DNA-antimicrobial complexes. Autoantibodies form immune complexes with these nucleic acid-antimicrobial complexes, that get taken up into pDCs through receptor (FcγRIIa) mediated endocytosis, which in turn, activate pDCs to release more IFNα, putting into motion a viscous cycle of increased IFNα production and eventual autoimmune disease.

1.5.3 Management

As with any autoimmune condition, disease management poses a significant challenge to health care providers owing to the unpredictable nature of clinical manifestations and disease course. With childhood onset disease regarded to be more severe in terms of disease presentation, clinical course and organ involvement and the considerable challenges of diagnosing disease at an early age makes management of JSLE a very difficult task [190].

Disease management of JSLE patients must take into account the impact of drug therapy on the physical, psycho-social, and cognitive developmental during their formative years, as drug-induced side effects can have a profound impact on drug compliance [190]. Poor therapeutic outcomes are often related to non-compliance, prominent in patients entering their adolescent years where body image is a prime concern, especially with regards to corticosteroid-related side effects [191]. With proper education of the patient early on in the disease, regarding the severity of disease and the risks associated with failure to comply with treatment regimens, compliance can be improved [191]. Thus a multi-disciplinary approach tailored to each individual patient much be executed to avoid such problems [191]. Furthermore, it is important to give special attention to the impact of the disease on their growth and development, both in terms of the physical and mental wellbeing [192].

Pharmacological therapies are tailored to the individual patient on the basis of the disease activity and severity, organ involvement and occurrence of disease flares [191, 193]. Unfortunately, disease management is challenged by the paucity of published clinical trials involving treatment regimens for JSLE. Thus, most pharmacological interventions used in the paediatric setting have been extrapolated from clinical trial data from adult-onset SLE together with the clinician's experience and best-practice guidelines [101, 194]. To achieve

an effective treatment of JSLE, the risk associated with the disease must be balanced against those associated with pharmacological interventions. JSLE management focuses on several key aspects, which include control and or elimination of the inflammation, maintenance of quality of life throughout their development, reduction of long-term complications and lastly, minimization of risk of drug-related side effects [194]. Accordingly, the therapeutic strategy involves initial induction therapy followed by maintenance therapy [193].

Induction therapy is aimed at inducing remission of the disease flare to control disease activity as well as aggregative treatment of any potentially life threatening manifestations, including severe organ involvement [193]. Corticosteroids remain the first line of treatment for JSLE, with majority of patients requiring them at some stage of their disease [195]. In induction therapy, the primary aim is to control any ongoing inflammation and the use corticosteroids has greatly improved the overall outcome of disease for paediatric patients [193]. The dose and duration of the drug is governed by the severity of the disease and organs involved [195]. Mild forms of the disease are treatable with oral corticosteroids [193]. Moderate and severe disease will require the use of intravenous methylprednisolone, followed by high dose oral prednisolone [193]. Induction regimes also involve other immunosuppressive drugs to decrease the to dose of corticosteroids required, with the use of intravenous cyclophosphamide for cases with major organ damage, and for the less severe cases, azathioprine or methotrexate, although recently, mycophenolate mofetil has come into use as a potential alternative is specific cases [101]. Use of antimalarials, Hydroxychloroquine (HCQ) in particular, is recommended for every patient and their efficacy is described in detail in the discussion (Section 4.3.3).

HCQ, a drug initially developed for the treatment of Malaria, is widely being used for the treatment of RA and SLE following reports of the efficacy of this drug in treating these

rheumatic conditions [196]. Although there are no clinical trials validating the use of HCQ in a paediatric setting, it is widely used for the treatment of JSLE owing to the beneficial effects seen in adult disease [194, 197]. Despite the obvious benefits of this drug, the exact mechanism of action underlying its effect in individual diseases are poorly understood. The therapeutic effect of HCQ in Lupus has been largely attributed to the ability of these weak bases to inhibit endosomal TLR acidification and maturation by raising the intracellular PH [198]. Acidic environment if required for the binding of nucleic acids to intracellular TLRs, thus HCQ effectively blocks ligand-receptor interactions thereby preventing TLR activation [198-200]. Further to pH alterations, antimalarial agents have been suggested to exert TLR antagonism by directly binding to nucleic acids masking their TLR-binding epitopes [201]. The precise therapeutic plasma concentration of HCQ used in the treatment of JSLE has not been fully established, however, in a study conducted to investigate plasma HCQ concentration and efficacy in RA, although no therapeutic concentration was concluded from study, plasma concentrations of HCQ were found to vary in a range of 69-518 ng/ml in the patients with RA [202].

The aim of maintenance therapy is to prevent occurrence of relapses following induction of remission [193]. Although the length and choice of administrating a specific drug is normally on an individual patient basis, generally at least 2-3 years of therapy is advised to maintain ongoing remission, especially in severe cases with renal involvement [101, 193]. Typically, mycophenolate mofetil (MMF) and azathioprine are the preferred choices of maintenance therapy and usually given together with oral corticosteroids [101].

For use as second line treatment option, new biological agents that target B-cells have come into play including Rituximab and Belimumab [102]. Rituximab is a monoclonal antibody that functions as a B-cell-depletion therapy by targeting the protein CD20 expressed on the surface of B cells [102]. Rituximab, in combination with

cyclophosphamide has been found to be effective in the treatment of Lupus nephritis in paediatric patients, although the safety of this drug and potential long term risk is still a topic of concern [193]. Belimumab is a monoclonal antibody that functions by inhibiting BLyS, which is involved in the induction of B-cell survival and differentiation [203]. It was recently approved for the use in adult disease, although not investigated in paediatric setting [102].

In recent years, advancements made regarding the overall biology of the disease have enabled early diagnosis and prompt individualised treatment of the disease. This is reflective of the improved life expectancy observed over the last few decades with a 10 year survival rate of more than 90% [102].

However, even with these recent advancements, many of these drugs have very adverse side effects leading to poor long term prognosis making treatment even more of a challenge. Currently one of the most common causes of mortality in Lupus is owing to complications of drug-related side effects including infections and cardiovascular disease [204].

Cyclophosphamide use is associated with occurrence of severe infections, compared to other drugs including MMF and azathioprine thus validating the use of these drugs for induction and maintenance agents as opposed to cyclophosphamide [193]. Other risks of cyclophosphamide include malignancy, neutropenia and infertility [193]. Corticosteroid related side effects, a common cause of non-compliance in patients, are associated with increased risk of infection, depression, confusion/psychosis, weight gain, sleep disturbances, hyperglycaemia, osteoporosis and accelerated atherogenesis [193, 205]. As such, assessment of long-term risk-benefit of corticosteroids is particularly challenging and the increased need for aggressive corticosteroid use in patients further burdens the need for more targeted treatments with minimal adverse effects. Accordingly, there is still a

great need for the development of novel drugs that are both effective and have fewer side effects in order to achieve optimal treatment outcomes for patients in the future.

1.6 Summary of introduction

JSLE is an incurable multisystem rheumatic disease characterised by loss of tolerance to nuclear antigens [96]. JSLE manifests prior to age 17 with a female predilection and diagnosis is formally made when 4 of 11 clinical and laboratory criteria are met in childhood [98, 100, 102, 118]. Childhood onset disease is normally associated with more severe presentation, clinical course and higher mortality rates [102, 103, 105]. Current drug therapies including corticosteroids, HCQ and immunosuppressive agents have contributed significantly to the overall improvement in disease outcomes over the past few decades [102]. Unfortunately, currently no curative treatments exist, owing to unpredictable disease courses and limited understanding of the underlying disease mechanism making it particularly challenging task for the development of targeted treatments. The disease pathogenesis involves widespread immune dysregulation and loss of tolerance towards nuclear self-antigens [96, 97]. Until recently the availability of these autoantigens were largely attributed to dysregulated apoptosis [145-147].

As part of a novel antimicrobial mechanism neutrophils were found to undergo cell death through NETosis to release structures known as NETs [94]. However, dysregulated NETosis, involving aberrant formation and defective clearance of these structures is evident in Lupus patients and considered to contribute towards development of autoimmunity by providing an additional source of autoantigens which may get detected by nucleic acid receptors (TLRs 3, 7 and 9) of the immune system [94]. With existing data regarding the role of NETs in Lupus pathology being largely related to adult disease, investigating these structures in the setting of JSLE will be greatly beneficial particularly with regards to future prospects of developing NET- targeted treatments to help improve disease outcomes.

1.7 Hypothesis

NETosis is a novel antimicrobial mechanism, and its dysregulation, observed in Lupus patients, may result in auto-inflammatory responses. Accordingly, the study hypothesis is that NETs provide a novel source of autoantigens detected through endosomal TLRs leading to immune activation in JSLE. Moreover these NET autoantigens can stimulate the immune system to produce IFNα, which can in turn stimulate neutrophils to release more NETs, setting into motion a self-perpetuating cycle with regards to production of NETs and IFNα.

1.8 Aim

The aim of this project was to assess whether NETs from JSLE neutrophils are providing a source of auto-antigens detected through endosomal TLR 3, TLR7 and TLR9, if the source of the neutrophils, generating the NETs is a determining factor in immune activation and furthermore, if NET-induced activation of the immune systems stimulates IFNα production and should this occur, if this response can be inhibited.

2 Methods

2.1 Patients

All patients recruited for this study were participating in the UK JSLE Registry and Repository: "Clinical characteristics and immunopathology of JSLE" for which full approval by the Liverpool Paediatric Research Ethics Committee was already granted (Research Ethics Committee number: 06/Q1502/77). All participating JSLE patients fulfilled the revised American College of Rheumatology (ACR) criteria for the diagnosis of SLE prior to the age of 17. Written, informed consent from all participating patients or their parent/guardian was obtained. Healthy paediatric controls were patients attending day case procedures, being investigated for non-inflammatory musculoskeletal symptoms or attending routine elective surgery where no inter-current infection or history of autoimmunity was present. All patients were recruited from outpatients clinics and inpatients wards at Alder Hey Children's NHS Foundation Trust in Liverpool. All patient samples were obtained during routine blood sampling required for disease monitoring. Around 5-10ml of blood was collected and transferred to the laboratory for immediate processing. Following collection all study samples were anonymized. Healthy adult control samples obtained from laboratory staff was used for optimization stages before patient samples were used.

2.2 Cell Preparation

All blood samples from JSLE, pediatric and adult healthy control patients were collected in heparinized blood bottles and processed within one hour of phlebotomy to isolate neutrophils and peripheral blood mononuclear cells (PBMCs) using either Polymorph Prep (Axis-Shield, Norway; Catalogue Number: NYC-1114683) or Histopaque 1077 (Sigma-Aldrich, UK; Catalogue Number: 10771). Processing of samples was carried out in sterile fields of tissue culture hoods. All plastics and reagents were obtained from Fisher Scientific, UK unless otherwise stated.

2.2.1 Neutrophil Isolation

5 ml of Polymorph Prep was decanted into a 13ml universal tube. Each 5 ml of heparinized whole blood was carefully layered on top of an equal volume of polymorph prep and centrifuged (Thermo Electron, UK) at 550*g* for 30 minutes with break setting=0 producing distinct layers of plasma, PBMCs, neutrophils and red blood cells (**Figure 9**). The plasma and PBMCs were carefully removed and using Pasteur pipette. The remaining clear band of neutrophils was transferred into a separate sterile 13ml universal tube, to which 10 ml of Roswell Park Memorial Institute media (RPMI) (Lonza, UK) was added. This mixture was then centrifuged at 690*g* for 10 minutes with break setting=5. A pellet was generated at the bottom of the tube, containing Red Blood Cells (RBC) red blood cells and PMN. The supernatant was discarded taking care not to disturb the pellet. The pellet was re-suspended in the remaining RPMI residue. To lyse the contaminating RBC residue, 1 ml of RPMI media and 9ml of ammonium chloride lysis buffer was added for 3 minutes and centrifuged at 690*g* for 5 minutes with break setting=5. The supernatant was discarded as before, and the RBC-free pellet was re-suspended in residual media. The cells were re-suspended in 1ml of RPMI media containing 2% Fetal Calf Serum (FCS) (Sigma Aldrich, UK), and the concentration was determined using a Haemocytometer.

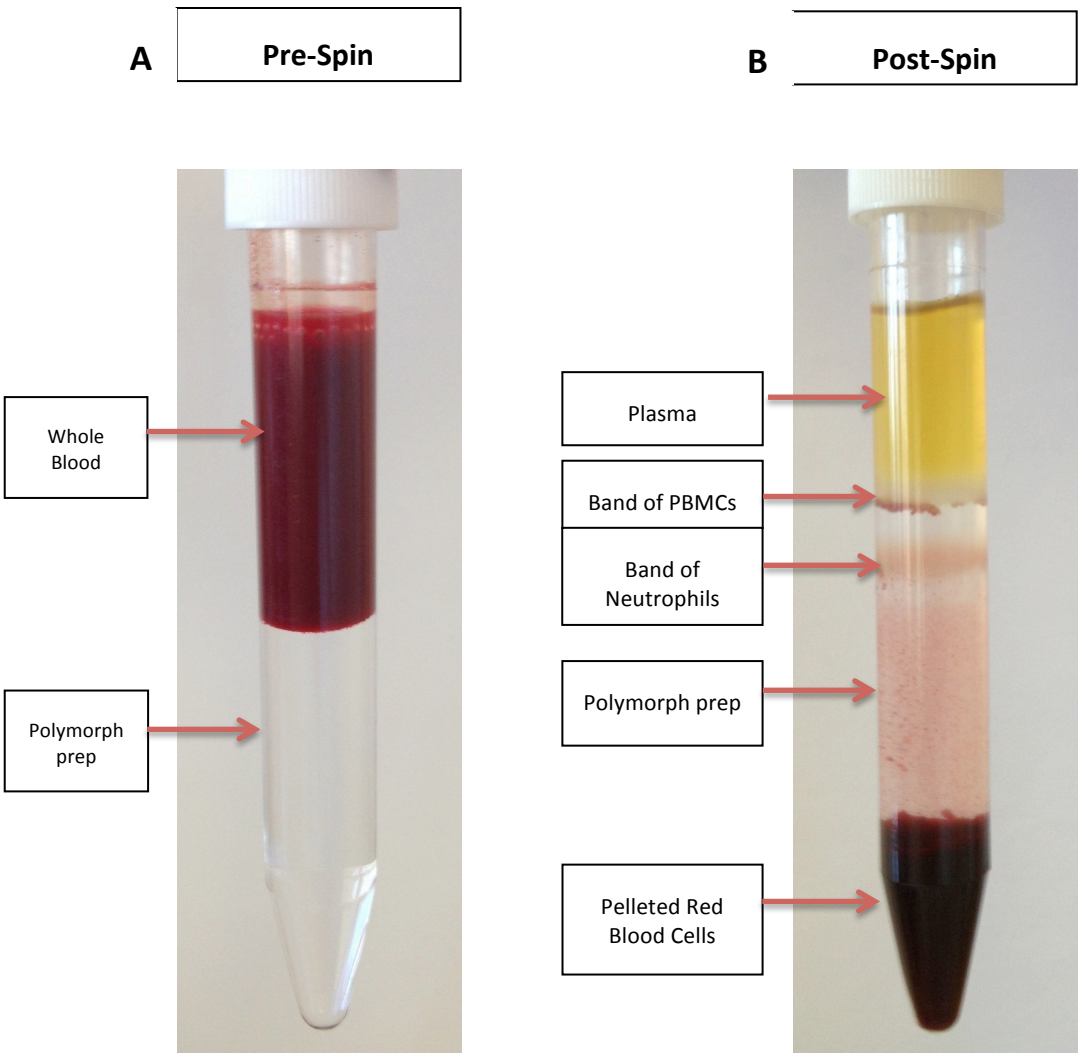


Figure 9: Isolation of neutrophils from whole blood.

In equal proportions whole blood was layered on top of Polymorph prep in a 13ml universal tube (**A**) centrifuged at 550*g* for 30 minutes with break setting=0 (**B**) to produce a clear separation of blood components including distinct layers of plasma, Peripheral Blood Mononuclear Cells (PBMCs), neutrophils and red blood cells.

2.2.2 Peripheral blood mononuclear cell Isolation

5ml of heparinized whole blood was decanted into a sterile 13ml universal tube and topped up with an equal volume of RPMI media. 3ml of Histopaque 1077 was added to a separate 13ml universal tube. The diluted blood was carefully layered on the Histopaque 1077, and centrifuged at 690*g* for 20 minutes with break setting=0 producing a clear band of PBMCs at the interface between the medium and the Histopaque 1077 (**Figure 10**). The top layer of medium and plasma were removed and discarded. The band of PBMCs was carefully harvested into a sterile 13ml universal tube to which 10ml of RPMI media was added. The cells were then centrifuged at 690*g* for 15 minutes with break setting=5, producing a pellet. The supernatant was discarded taking care not to disturb the pellet. The pellet was re-suspended in the residual media 10ml of RPMI is added, and centrifuged at 390*g* for 10 minutes with break setting=5. The supernatant was discarded as before, and the pellet re-suspended in residual media. Cells were re-suspended in 1ml of RPMI and counted using a Haemocytometer chamber.

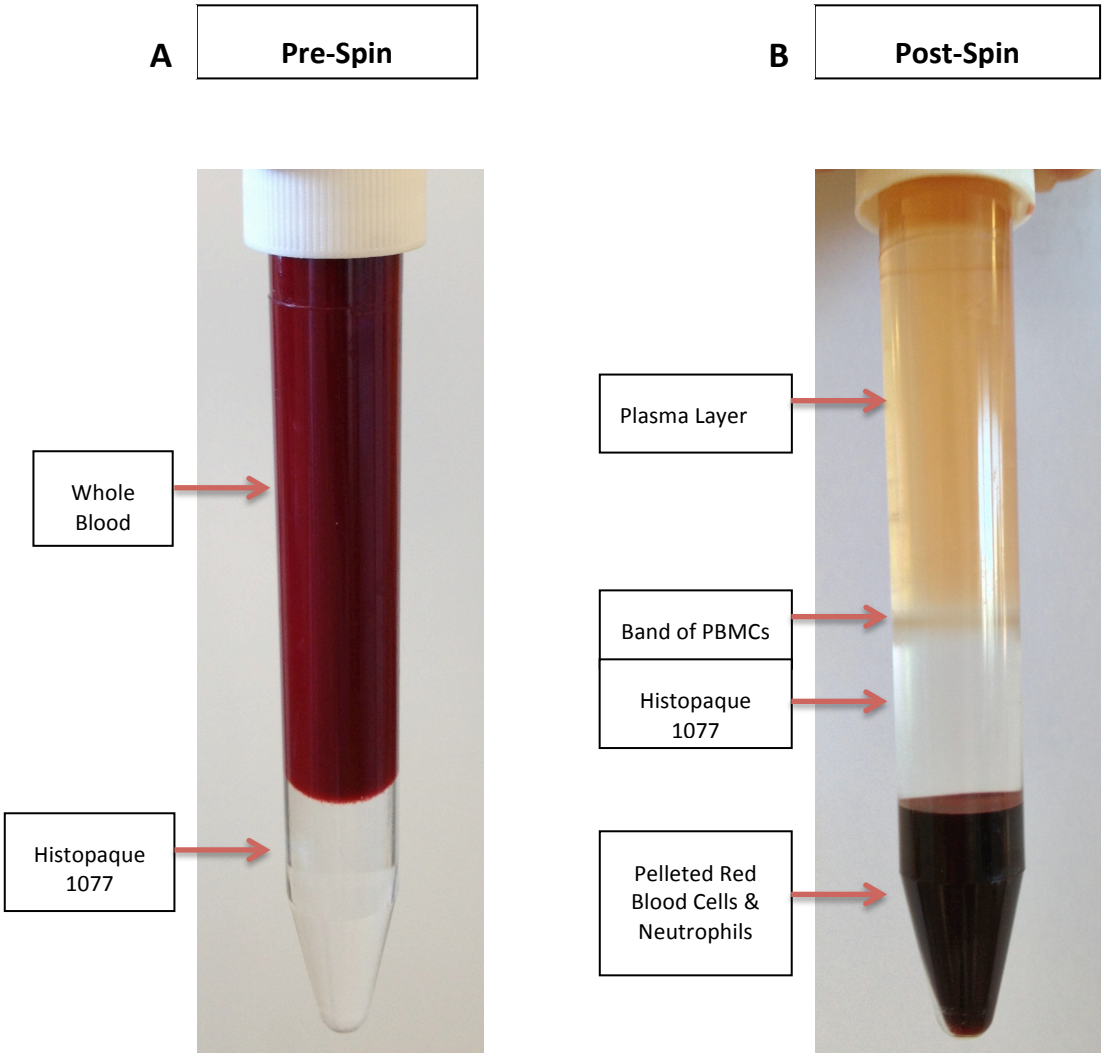


Figure 10: Isolation of PBMCs from whole blood.

Whole blood diluted in RPMI media was layered on top of Histopaque 1077 in a 13ml universal tube (**A**) and centrifuged at 690*g* for 20 minutes with break setting=0 (**B**) to produce a clear separation of blood components including distinct layers of plasma, Peripheral Blood Mononuclear Cells (PBMCs) and pelleted red blood cell and neutrophils.

2.3 Induction of NET synthesis

Neutrophils isolated from peripheral blood of pediatric control and SLE donors (Section 2.2.1) were stimulated to produce NETs. Neutrophils were seeded at 2×10^5 cells in 500µl of 2% FCS in RPMI media per well of a 24 well plate, and incubated for 30 minutes at 37 degree Celsius (°C) with 5% Carbon dioxide (CO_2). After 30 minutes, the neutrophils were treated with either phorbol myristate acetate (PMA) (160nM) (Sigma-Aldrich, UK), Interferon alpha (IFNα) (10ng/ml) (Peprotech, UK), JSLE (10%) and control (10%) sera and incubated for a further two hours at 37° C with 5% CO_2. A control condition comprised of untreated neutrophils, where NET formation was not expected to occur. Subsequent to the two hour incubation, supernatant was carefully removed and discarded, and 450µl of sterile phosphate buffered saline (PBS) added to each well.

2.3.1 Visualization of NETs using Indirect Immunofluorescence

NETs were visualized using confocal microscopy. Sterile 13mm round glass coverslips were placed into each well of a 24-well culture plate. Neutrophils were seeded at a concentration of 2×10^5 cells in 500µl of 2% FCS in RPMI media per well of a 24 well plate and incubated for 30 minutes at 37° C with 5% CO_2. Following incubation, cells were treated with PMA (160nM)(Sigma-Aldrich, UK), JSLE sera (10%) and IFNα (10ng/ml) (Peprotech, UK) and incubated for 2 hours at 37° C with 5% carbon dioxide. Experimental control comprised of 2×10^5 cells unstimulated neutrophils. The cover slips were removed ready for staining. To visualize NE and DNA by immunofluorescence, the cells seeded on coverslips were fixed with 4% paraformaldehyde (Sigma Aldrich, UK) and permeabilised and blocked with 0.3% Triton X 1x Tris buffered saline (TBS) and 2% BSA, 0.3% Triton X 1x TBS respectively. Cells were then incubated for 30min with 0.05µg/ml of the primary antibody rabbit anti NE (Abcam, UK). Following incubation cells were washed with 1 x TBS

and incubated for 30mins with the secondary antibody Alexa Fluor 488 labelled goat anti-rabbit immunoglobulin G (Abcam, UK). Following incubation the cells were once more washed with 1xTBS. DNA was stained with 1µg/ml 2-(4-Amidinophenyl)-6-indolecarbamidine dihydrochloride (DAPI) (Sigma-Aldrich, UK), for three mins. Cells were washed with 1xTBS and viewed with a confocal laser-scanning microscope (Leica DM2500). Images were taken with a 40 x objective and detection settings were kept constant between conditions to compare fluorescence intensities.

2.3.2 Quantification of NET double-stranded DNA: Pico green Assay

Wells containing NETs were treated micrococcal nuclease (Sigma Aldrich, UK) to dismantle the NET scaffold leaving a supernant of NET material. Add 500 mUnits of micrococcal nuclease (dilute 1 in 100 i.e. add 5ul to 495ul PBS) and add 50ul of this to the culture and 5ul 0.1M CaCl and incubate for 10 min at 37°C. Enzymatic digestion was terminated by adding 5mM EDTA. The supernatant was removed and centrifuged at 200*g* for 8 minutes. This supernatant was stored at -80°C.

The release of NET-dsDNA in the cell- free supernatant collected after 2 hour stimulation was quantified using Picogreen Assay (Life Technologies, UK;Catalogue number: P7589).

Pico green is a dye, which intensely fluoresces upon binding to double-stranded DNA. It allows a highly sensitive means of dsDNA quantification. 1× TE buffer was prepared by diluting 20× TE buffer 20-fold in DNase/RNase free water. The total volume of 1× TE buffer needed was dependent upon sample number and volume of Pico green working solution required. Pico green fluorescent dye working solution was prepared by diluting Pico green dye stock 200 fold in 1× TE buffer to give 1:200 dilution. 2µg/ml stock solution of lambda DNA was prepared from 100 µg/ml by 1:50 dilution in 1× TE buffer which was then serially diluted to produce a standard. A standard curve was created in the same plate for each assay. One hundred microliters of the cell-free supernatant was transferred into a flat-

bottom 96-well plate for the quantification of double stranded DNA using the Quant-iT Picogreen assay (Invitrogen, Carlsbad, CA). One hundred microliters of Picogreen reagent was added to the samples, which were then incubated at room temperature in the dark for 4 min. Extracellular DNA was measured with a spectrofluorometer (Perkin Elmer, Massachusetts, USA) at 480-nm excitation and 520-nm emission. dsDNA concentration in each well was calculated using the standard curve obtained from each assay.

2.4 Stimulation of PBMCs

PBMCs were isolated from healthy adult controls (Section 2.2.2). Isolated PBMCs were counted using a haemocytometer and diluted in RPMI media to a concentration of 1×10^6cells/ml of media and 1ml of sample was added per well to a 24 well plate. Each sample was incubated with TLR 3 /7/9 Ligands **(Table 3**) or incubated with 160nM PMA (Sigma-Aldrich, UK) /10ng/ml IFNα (Peprotech, UK)/ 10% JSLE sera -induced NETs (Section 2.3). A control condition comprised of 1×10^6 unstimulated PBMCs. The conditions were incubated for 15 minutes at 37° C with 5% CO_2. 15 minutes proved to be the incubation period to generate an optimal signal, after testing of 5, 15, and 60-minute time intervals. Following this incubation period, proteins were extracted (Section 2.5) and stored ready for western blot analysis of phosphorylated IRAK1 and IRF3 protein levels (pIRAK1 and pIRF3).

TLR Ligand	Concentration	Company
TLR 3 (Poly I:C)	1μg/ml	Tocris Bioscience, UK
TLR 7 (Imiquimod)	0.5mM	Tocris Bioscience, UK
TLR 9 (ODN1668)	0.1μg/ml	Enzo Life Sciences, UK

Table 3: Concentrations of TLR Ligands.

2.5 Protein extraction

Using a sterile Pasteur pipette all samples were transferred from respective wells into labeled 1.5ml microcentrifuge tubes and centrifuged at 11,100*g*, for 3 minutes, generating a cell pellet. All supernatant was removed, leaving a dry pellet. The pellet was re-suspended and left on ice. Cell lysis solution was made up in accordance with cell number, to produce 2×10^5 cells per 10 μl. The mixture constituted 1% protease and phosphatase inhibitor (Fisher, Catalogue number: 11552261), 10% DL-Dithiothreitol (DTT)(Sigma Aldrich, UK) in 89% sodium dodecyl sulfate buffer (SDS) solution (10% glycerol (Sigma Aldrich, UK), 125mM TRIS buffer (PH 6.8), 3% SDS (Bio-Rad,UK), 0.2 % bromophenol blue (Sigma Aldrich, UK) and water). Micro centrifuge tubes were transferred to a heating block set at 100 ° C. 50 ul of cell Lysis mixture was added to each sample. Samples in lysis buffer were boiled for 3 minutes, with intermittent vortexing every 60 seconds. Samples were stored at -20° C ready for use at a later stage.

2.6 Western Blot analysis

Western blotting, also known as Immunoblotting is a widely used analytical technique for the study of proteins [206]. It allows identification of specific proteins from complex protein mixture extracted from cells [206]. The technique can be broadly categorized into three main steps: Separation of protein mixture by molecular size using Polyacrylamide Gel Electrophoresis, transfer of separated proteins to an immobilizing membrane whilst maintaining their relative position, and lastly, detection of target protein using antibodies specific to protein of interest [206].

2.6.1 Sample preparation

Samples for western blot analysis must be initially lysed by chemical disruption in order to extract the proteins. Specific antibodies recognize specific regions of target proteins

(epitopes) located within the protein 3D conformation [207]. In order for detecting antibody to access these specific regions, proteins must be adequate unfolded (denatured) to reveal the epitopes that would otherwise be obscured by secondary, tertiary and quaternary protein folding. Cell lysis is accomplished in the presence of an ionic denaturing detergent, SDS and reducing agent, DTT [208]. SDS is an anionic detergent that denatures and linearizes the proteins and subunits into linear chains [208] (**Figure 11**). It breaks hydrogen bonding within and between molecules disrupting secondary and tertiary structure facilitating protein unfolding [208]. The binding of SDS to linearized proteins imparts a uniform distribution of negative charge per unit mass, allowing for separation to be based solely on molecular size, not on shape or charge (**Figure 11**) [208]. DTT (a reducing agent) is used to reduce and prevent re-oxidation of intra- and inter- molecular disulphide bonds of the proteins (both within and between protein subunits) disrupting tertiary and quaternary structures [208] (**Figure 11**). The protease and phosphatase inhibitors block/inactivate endogenous proteolytic and phospholytic enzymes released during cell lysis from subcellular compartments that would otherwise degrade the protein of interest. Bromophenol blue, a small anionic indicator dye molecule, helps to visualize the migration of proteins during gel electrophoresis functioning as a tracking dye [206]. Glycerol increases sample density to help samples sink easily and maintain at the bottom of the well, avoiding overflow into neighboring wells [206]. The samples, once diluted in lysis buffer must be heated adequately to further facilitate the completion of denaturing and unfolding process and avoid formation of secondary structures upon cooling of the mixture [207, 208].

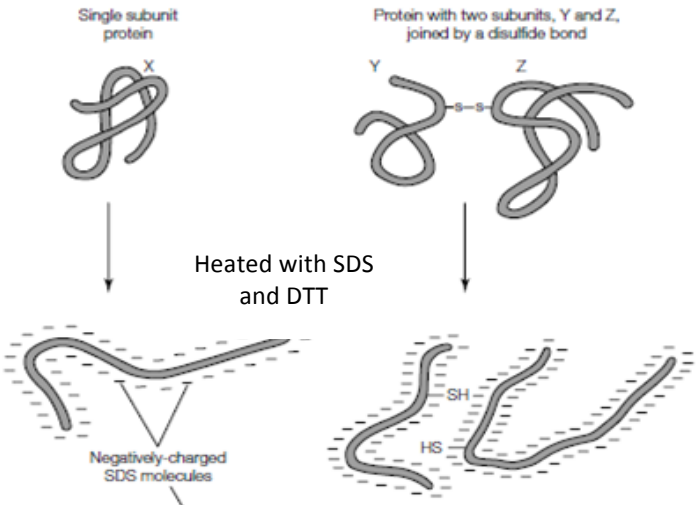


Figure 11: Denaturation and Reduction of proteins into linear chains by SDS and DTT treatment.

Heating of the protein mixture in the presence of reducing agent DTT and SDS denatures and linearizes all proteins to their primary structure (secondary, tertiary and quaternary structures are all disrupted during this process retaining only their primary amino acid structure). SDS denatures proteins into linear chains and imparts uniform negative charge to the proteins. DTT reduces inter and intra molecular disulphide bonds. This image was adapted from http://www.expertsmind.com/topic/electrophoresis-of-proteins/sds-page-94215.aspx

2.6.2 SDS- PAGE (Polyacrylamide gel electrophoresis)

The second step of a Western blot involves size-dependent separation of proteins using gel electrophoresis. During electrophoresis, upon application of a voltage the SDS-treated negatively charged proteins loaded migrate through porous polyacrylamide gel toward the positively charged electrode (anode)(**Figure 12.A**), separating into bands according to their molecular size (**Figure 12.B**) [209]. The polyacrylamide gel functions as a three-dimensional sieve, with smaller proteins migrating faster through the gel than larger ones.

The samples are accompanied by protein mixture of known molecular weights (molecular weight standard), which produces bands at varying molecular weights. These bands are used as a reference range for the confirmation of the identity of the target proteins of known molecular weights following separation by electrophoresis.

A B

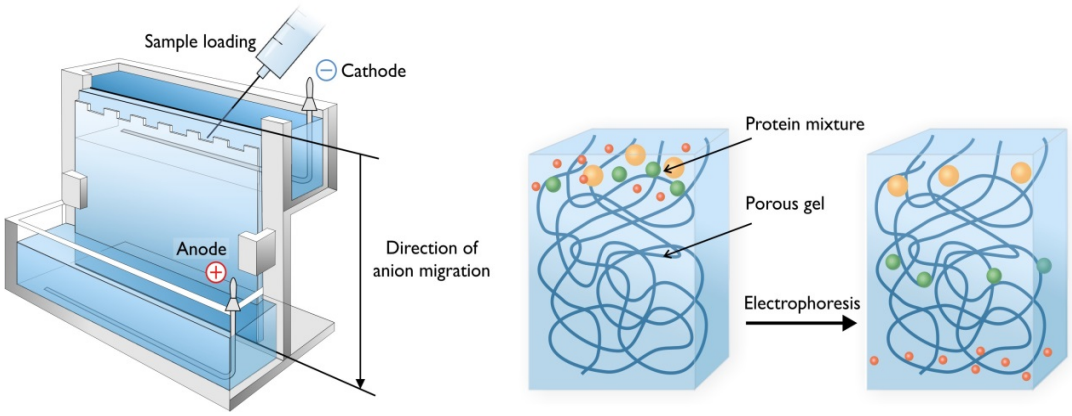


Figure 12: Schematic of the Gel Electrophoresis apparatus and the protein separation in the polyacrylamide gel.

(**A**) Protein samples and a molecular weight standard are loaded into the sample wells of the gel. A voltage is applied across the gel forcing the SDS-treated negatively charged proteins to migrate towards the positively charged anode (smaller proteins run faster) (**B**) SDS-PAGE gel after electrophoresis. The proteins get separating according to their specific molecular masses. This image was obtained from http://elte.prompt.hu/sites/default/files/tananyagok/practical_biochemistry/ch07s03.html

2.6.3 Electro transfer of Proteins onto membrane: Semi-dry blotting

Following separation, the proteins are transferred from the gel to an immobilizing synthetic PVDF membrane, in order to make the protein of interest more accessible to antibody detection [210]. PVDF membranes are hydrophobic and interact with proteins via hydrophobic interactions [207]. The transfer of proteins is achieved by orientation of an electric field perpendicular to the plane of the gel, enabling the proteins to transfer from the gel onto the immobilizing PVDF membrane [206]. The semi-dry blotting apparatus consists of the gel and the PVDF transfer membrane sandwiched between buffer- soaked ion reservoir stacks of filter paper, which are in direct contact with the top cassette electrode (cathode) and bottom cassette electrode (anode) (**Figure 13**). When a current is applied, the negatively charged proteins migrate from the gel onto the PVDF transfer membrane.

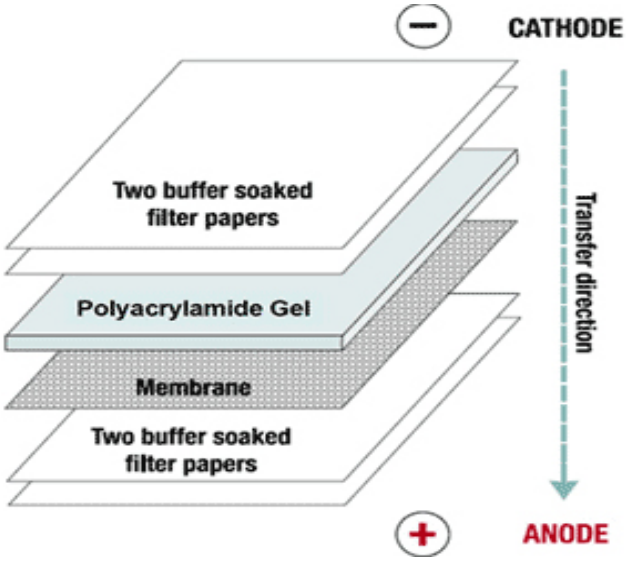


Figure 13: Semi-dry transfer stack assembly.
This is a diagrammatic representation of the assembly of the transfer stack in a semi-dry transfer apparatus. The polyacrylamide gel and transfer membranes are sandwiched between buffer soaked filter paper that are in direct with the two plate electrodes. When a current is applied, the proteins migrate from gel onto the immobilizing gel. This image was obtained from http://www.gibthai.com/services/technical_detail.php?ID=18&page=11

2.6.4 Blocking

Following electro transfer, the protein of interest can be detected on the immobilizing membrane using immunological detection. A crucial step prior to detection is the blocking of all additional unreacted binding sites on the membrane using a protein that is immunologically neutral. Blocking agents should bind to the surface of the membrane where protein is not already present, without interference with target protein. Blocking prevents non-specific binding of antibody to the membrane (**Figure 14**) [208].

2.6.5 Antibody incubation

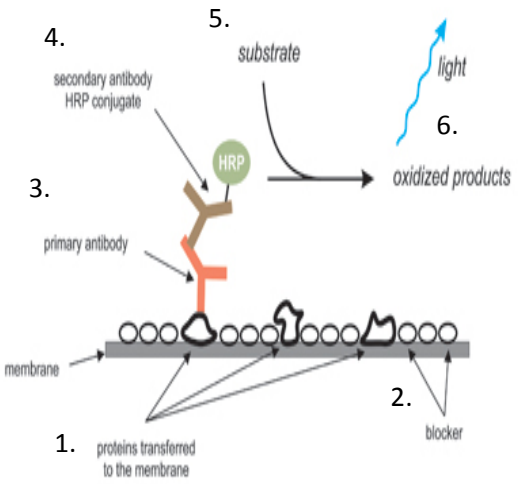
The desired protein can be visualized on the immobilized membrane through immunological detection (**Figure 14**) by the use of a primary antibody specific to the protein of interest. The primary antibody detects specific amino-acid sequences (epitopes) of the desired protein that is exposed following disruption of higher order protein structures under reducing and denaturing conditions [207]. A secondary antibody directed

against the species in which the primary antibody was raised is introduced to react with the primary antibody, already bound to protein of interest on the membrane [207]. The secondary antibody is typically conjugated with an enzyme, namely horseradish peroxidase (HRP) [207].

Incubation with primary and secondary antibodies must be done under agitation to allow adequate homogenous covering of the membrane and avoid uneven binding. Following each antibody incubation thorough washing must be carried out to remove any antibody that is unbound as well as to minimize the background [206].

2.6.6 Detection and Imaging

Detection requires the presence of a substrate that generates light emission upon reaction with enzyme (HRP) conjugated to the secondary antibody that can be detected to by film or digital imaging (**Figure 14**). The HRP enzyme reacts with chemiluminescent substrate to emit detectable light signal. The light generated can subsequently be detected by exposing the membrane to X-ray film or by using a digital imaging system for light capture. The light produced creates a band on the X-ray film or on the digital imaging screen, corresponding to the site where the HRP-labeled antibody has attached to target protein-bound primary antibody. Commercially available digital imaging systems include charge-couple device (CCD) camera-based imager or scanners. X-ray film development requires a subsequent digital imaging in order for further analysis to be made. As the substrate is catalyzed by the HRP conjugated to the secondary antibody, which is attached to the target protein-bound primary antibody, the intensity light signal emitted and subsequent detection corresponds to the quantity of the target protein present in the membrane.



1.Transfer of protein to the membrane

2. All unoccupied sites on the membrane is blocked

3. Incubated with Primary antibody specfic to the protein of interest

4. Incubated with Secondary antibody which binds to the primary antibody

5. Substrate added to the membrane

6. Enzyme catalyses the substrate, forming a dectectable light product at the site of the antigen-antibody complex

Figure 14: Immunological detection of the target protein.
Schematic of the steps involved in the immunological detection of the target protein. This image was obtained from http://www.western-blot.us/procedure-of-western-blot/western-blot-detection

2.6.7 Analysis

Digital images can be analyzed both qualitatively and quantitative with respect to the protein of interest. Qualitative protein analysis determines the presence of target protein on the western blot. Quantitative protein analysis can be used to determine the amount of target protein present. For accurate quantitative measurement of protein levels the target protein must be normalized to an internal control that will account for variations due to sample inconsistencies (varying protein concentrations) and handling errors (uneven protein loading). A 'housekeeping protein' which is essential for the basic functioning of a cell and has a relatively uniform rate of protein levels in cells is employed as the internal control (β-Actin). The internal control must also have a different molecular weight to the protein of interest. Target protein can be quantified once normalized through to the protein levels of the internal control.

2.7 Measuring phosphorylated proteins levels following TLR Ligand/ NET stimulation

Proteins extracted from samples (Section 2.5) were obtained from storage and analyzed for the protein levels of downstream signaling proteins by western blot analysis. A commercially bought pre-cast gel was used (Bio-Rad, UK). The pre-cast gel cassettes were placed in the gel support of the electrophoresis module, which was then placed in the buffer tank (Bio-Rad, UK). The tank was filled to appropriate level with running buffer solution(250mM Tris, 1% SDS, 1.92M glycine). SDS- treated samples were boiled for 3 minutes in a heating block at 96°C. The protein samples were carefully loaded into each well (320,000 cells per well). First well was loaded with an appropriate molecular weight standard (protein ladder) (Bio-Rad, UK). Once samples were loaded, tank was connected to a power supply and a voltage was applied across the gel. Voltage was set at 160 volts and run for 45 minutes. Upon completion of the run the gel cassettes were dismantled carefully to obtain the gels.

Following SDS-PAGE, the proteins were transferred from the gel to a PVDF membrane, using a semi-dry blotting system. Commercially bought pre-packaged transfer packs (Bio-Rad, UK) containing PVDF membranes and filter paper stacks pre-wetted with transfer buffer were used. The anode stack of filter paper was layered with the PVDF transfer membrane on the cassette base and a blot roller was used to remove any trapped air bubbles. The gel was carefully placed on the PVDF transfer membrane. The cathode stack of filter paper was placed on top of the gel and once again, using a blot roller, any trapped air bubbles were removed. The cassette was transferred into the semi-dry trans-blot machine (Bio-Rad, UK) and was run under mixed Molecular weight protocol for 7 minutes. Following transfer, the PVDF membrane was placed into a small tissue culture flask containing blocking buffer (1×TBST with 5% dry milk powder) and blocked for 1 hour at

room temperature on an orbital shaker. The membrane was washed briefly using a solution of 1×Tris-Buffered Saline with 0.1% Tween 20 (1× TBS-T) for 2×30 second time intervals. 1×TBS-T was prepared by a 1 in 10 dilution of 10×TBS solution (10mM Tris, 150mM NaCl, pH 8).

Primary antibody was diluted in blocking buffer in accordance with the required dilution (**Table 4**). The membrane was incubated with the appropriate primary antibody solution at 4° C and left overnight under gentle agitation.

The membrane was washed with 1×TBS-T at 2×30 second, 2×5 minute and 1×15 minute time intervals with agitation. Similarly to the primary antibody, the secondary antibody was diluted in blocking buffer to the required concentration (**Table 4**). The membrane was incubated with the secondary antibody solution at room temperature, for one hour, with agitation. Following incubation, membrane was washed again in 1×TBS-T at 2×30 second, 2×5 minute and 1× 15 minute time intervals with agitation.

Chemiluminescent substrate (LI-COR, UK; Catalogue number: 926-95000) was prepared and added onto the membrane ensuring homogenous covering of membrane and left for 5 minutes. The membrane was transferred to the C-DiGit Blot Scanner (Digital Imaging System) (LI-COR, UK) and images were captured. Membranes were probed for β-Actin in a similar manner for the purpose of normalization.

Primary Antibody	Secondary Antibody	Primary Antibody dilution	Secondary Antibody dilution	Observed Molecular weight	Catalogue Number
Anti-IRAK (Phospho T387) Antibody*	Anti-Rabbit	1-500	1/3000	74kDa	ab139739
Anti- IRF3 (phospho S386) antibody*	Anti-Rabbit	1/1000	1/1000	50kDa	ab76493
Phospho-Stat1 (Tyr701)(58D6) Rabbit mAb**	Anti-Rabbit	1/1000	1/1000	84kDa	9171
B-Actin*	Anti-Mouse	1/10,000	1/5000	42kDa	ab8226

*Abcam; ** Cell Signaling

Table 4: Antibodies used for western blot analysis.

2.8 Measuring phosphorylated protein levels in HCQ pre-treated PBMCs following stimulation with TLR Ligands or NETs

HCQ is an immune-modulatory agent that functions as a TLR3/7/9 antagonist both *in vitro* and *in vivo* by inhibiting the activation of these endosomal TLR signaling pathways [198]. PBMCs were purified from healthy adult controls (Section 2.2.2). Isolated PBMCs were counted using haemocytometer (Sigma Aldrich, UK) and diluted in RPMI media to a concentration of 1×10^6cells/ml of media and 1ml of sample was added per well to a 24-sample plate. Cells were either left unstimulated or incubated with varying concentrations of HCQ (10 μM, 50 μM and 100μM). Conditions were incubated for 30 minutes at 37° C with 5% CO_2. The HCQ-treated and untreated cells were then incubated with TLR7 Ligand or TLR9 ligand or NETs for 15 minutes at 37° C and 5% CO_2. A control condition comprised of 1×10^6 unstimulated PBMCs. Upon completion of incubation proteins were extracted (Section 2.5) and stored ready for western blot analysis.

Cell lysates were analyzed for the protein levels of pIRAK1 using western blot analysis and results subsequently normalized for β-Actin expression (Section 2.7).

Owing to time constraints, TLR3 experiments with regards to HCQ could not be pursued in this thesis.

2.8.1 Measuring cell viability of HCQ pre-treated PBMCs

An apoptotic assay was undertaken to ensure that suppression of TLR signaling is due to the direct inhibitory effects of HCQ on TLR signaling activation and not due to HCQ-mediated cytotoxicity on the control PBMCs leading to cell death. An early feature of apoptosis is the loss of plasma membrane asymmetry [211]. Under normal conditions, the membrane phospholipid phosphatidylserine (PS) is located in the inner leaflet of plasma membranes [212]. During apoptosis, PS translocate from the inner to the outer leaflet of plasma membrane, hence exposing the PS to the external environment [212] . Annexin V, a fluorescently labeled Ca^{2+} dependent phospholipid binding protein, with high affinity for PS, attaches to the exposed PS on apoptosis cell surfaces [211].

2.8.1.1 Annexin V Apoptotic Assay

PBMCs (1×10^6 cells) purified from healthy adult controls were either left unstimulated or treated with varying concentrations of HCQ (10 μM, 50 μM and 100μM) for 30 minutes at 37 and 5% CO_2. Following treatment, PBMCs were removed and re-suspended in 1ml of Hanks' Balanced Salt Solution (HBSS) (Invitrogen, UK) and centrifuged for 5 minutes at 690*g* to pellets the cells. The supernatant was discarded, and the cell pellet was re-suspended by repeated flicking motions. Fluorescein Isothiocyanate (FITC)-labeled Annexin V (Sigma Aldrich, UK) was added at a 1:100 dilution and cells were incubated on ice for 15 minutes, away from light. Cells were pelleted by centrifuging at 390*g* for 5 minutes. Following centrifugation, the supernatant was discarded and the cell pellet was

resuspended in 500ul of HBSS and analyzed by flow cytometry (Beckman Coulter FC 500 MPL Flow cytometer).

2.9 Exposure of control PBMCs to supernatant from NET-stimulated PBMCs

As mentioned previously in Section 1.5.2.2, Type 1 IFNs, such as IFNα signal through the JAK-STAT signaling pathway resulting the in the phosphorylation of STAT1 (**Figure 7**).

PBMCs were purified from healthy adult controls (Section 2.2.2). Isolated PBMCs were counted using a haemocytometer and diluted in RPMI media to a concentration of 1×10^6cells/ml of media and 1ml of sample was added per well to a 24-sample plate. Each sample was incubated with either 160nM PMA or 10ng/ml IFNα or 10% JSLE sera-induced NET material (Section 2.3) to stimulate production of IFNα. A control condition comprised of 1×10^6 unstimulated PBMCs. Each condition was incubated for 4 and/or 6 hours at 37° C with 5% CO_2. Following incubation at each time point supernatant was removed and stored ready for stimulation of control PBMCs. Cell-free supernatant was expected to contain IFNα molecules.

PBMCs were purified from healthy adult controls (Section 2.2.2). Purified PBMCs were counted and diluted in RPMI media to a concentration of 1×10^6cells/ml of media and 1 ml of sample was added to round-bottom tubes. Cells were either left unstimulated or incubated with a Janus Kinase (JAK) inhibitor (10μM) (Millipore, Catalogue number: 420097) for 30 minutes at 37° C with 5% CO_2. JAK inhibitors interfere with JAK-STAT signaling pathway of IFNα to inhibit IFNα signaling (**Figure 7**) [182]. Following incubation, cells were centrifuged at 390*g*, for 5 minutes, with break setting=5, to generate a cell pellet. All supernatant was removed, leaving a dry pellet of PBMCs, which was then re-suspended. Cell-free supernatant from NET-stimulated PBMCs and unstimulated PBMCs (control condition) was allowed to thaw and added to the control PBMCs pretreated with

and without a JAK inhibitor, and incubated for 15 minutes at 37° C with 5% CO_2. Following incubation, proteins were extracted (Section 2.5) for western blot analysis.

Cell lysates were analyzed for the protein levels of protein levels phosphorylated STAT1 (pSTAT1) (Phospho-Stat1 (Tyr701) (58D6) Rabbit mAb detects endogenous levels of Stat1 only when phosphorylated at tyrosine 701. The antibody detects phosphorylated tyrosine 701 of p91 Stat1 and also the p84 splice variant. It does not cross-react with the corresponding phospho-tyrosines of other Stat proteins) using western blot analysis and results subsequently normalized to β-Actin expression (Section 2.7).

2.10 Exposure of JSLE neutrophils to supernatant from NET-stimulated PBMCs

IFNα has been shown to prime neutrophils to release NETs [94]. To investigate whether IFNα produced through NET-induced immune activation could in turn stimulate neutrophils to release NETs, supernatant from NET-stimulated PBMCs was incubated with JSLE neutrophils and induction of NETs was visualized using confocal microscopy following staining for DAPI.

PBMCs isolated from healthy adult controls (Section 2.2.2) were either left unstimulated or incubated with JSLE sera induced NETs (Section 2.3 and 2.3.2) for 6 hours at 37° C with 5% CO_2 to induce IFNα production. Following incubation, cell-free supernatant was removed and stored ready for stimulation of JSLE neutrophils. Cell-free supernatant was expected to contain IFNα molecules.

Neutrophils were then isolated from JSLE patients (Section 2.2.1) and either left unstimulated or treated with a JAK inhibitor (10μM) for 30 minutes at 37° C with 5% CO_2 to inhibit IFNα signaling. JSLE neutrophils pretreated ± a JAK inhibitor (10μM) were left unstimulated or incubated with the supernatant from control PBMCs stimulated with JSLE

sera-induced NETs, for 2 hours 37° C with 5% CO_2. Positive control conditions comprised of JSLE neutrophils stimulated with either 10ng IFNα or 10% JSLE sera. Possible NET formation was subsequently visualized using confocal microscopy (Section 2.3.1).

2.11 Statistical Analysis

All statistical analysis was carried out using Statistics Package for Social Sciences version 21.0.0.1. All datasets analyzed were non-parametric. Mann-Whitney U test was utilized for analyzing the statistical significance between non-paired study samples and Wilcoxon Signed Rank Test was used for analyzing the statistical significant between paired study samples. In both cases Probability (*p*) values <0.05 were considered significant.

3 Results

3.1 Demographics

This study involved 12 JSLE and 13 healthy paediatric control patients; the mean age was 15.6 (range 9.5 -19.1) and 12.2 (range 6.6-17.9) years, respectively, at the time of sampling. Healthy paediatric control group contained male: female ratio of 3:10 (23% male and 77% female) and were entirely made up of white British patients. For JSLE patient group the male to female ratio of 1:3 (25% and 75%) and were primarily made up of white British patients with the exception of 2 patients (Indian and Pakistani). Majority of JSLE patients involved in study had well-controlled disease; the patients were also within normal ranges for CRP levels. **Table 5** summarises the age of diagnosis and the disease activity scores, clinical biomarker data and health status assessment scores of JSLE patients at the time of sampling. **Table 6** summarises current medication of JSLE patients at the time of sampling.

	Juvenile-Onset SLE patients* (n=12)
Mean Age of Diagnosis (years)	12.7 (3.4-16.9)
Disease Activity measures	
Mean BILAG-2004 Score	2.3 (0-11)
Mean SLEDAI Score	3.3 (0-8)
Clinical Biomarkers	
ESR (mm/hour)	4.4 (1-11)
CRP (mg/litre)	5.5 (1-17.2)
Complements (gm/litre)	
C3	1.2(0.95 -1.63)
C4	0.2 (0.11-0.32)
Health Status Measures	
C-HAQ Score	0.3 (0-1.13)
Global assessment by physician	17.9 (0-72)

Table 5: Disease activity, clinical biomarkers and health Status of JSLE patients

*Mean (range). This table summarizes the age of onset and the Disease activity scores and clinical biomarker data and health status assessment scores of the patient cohort at time of sampling; BILAG = British Isles Lupus Assessment Group; SLEDAI = Systemic Lupus Erythematosus Disease Activity Index; ESR = Erythrocyte Sedimentation Rate; CRP = C-reactive Protein; C-HAQ = Childhood Health Assessment Questionnaire.

Current Medications	Juvenile-onset SLE patients [No of patients (%)]
Hydroxychloroquine	7 (58)
Prednisolone	5 (42)
Methotrexate	0 (0)
Azathioprine	2 (17)
Mycophenolate Mofetil	8 (67)
Rituximab	2 (17)
Other	1 (8)

Table 6: Therapeutic profile of JSLE patients.

This table summarises the therapeutic profiles of JSLE patient cohort. All patient were on various combinations of drugs and none of them were comparable. Other = Cyclophosphamide, Intravenous Immunoglobin.

3.2 Visualization of NETs using Immunofluorescence

For Qualitative assessment of NET release, neutrophils purified from JSLE patients were seeded onto glass slides and either left unstimulated or treated with synthetic NET inducer, PMA (160nM). The neutrophils were fluorescently stained for structural components of NETs (DNA and NE) (Section 2.3.1). Extruded NETs were subsequently visualized using Confocal Microscopy (**Figure 15**). No NETs were observed in the unstimulated cells (**Figure 15.A**). However, when stimulated with 160nM of PMA for 2 hours, there was evident NET release from the neutrophils (**Figure 15.B and 15.C**), indicated by the presence of extracellular DNA and NE.

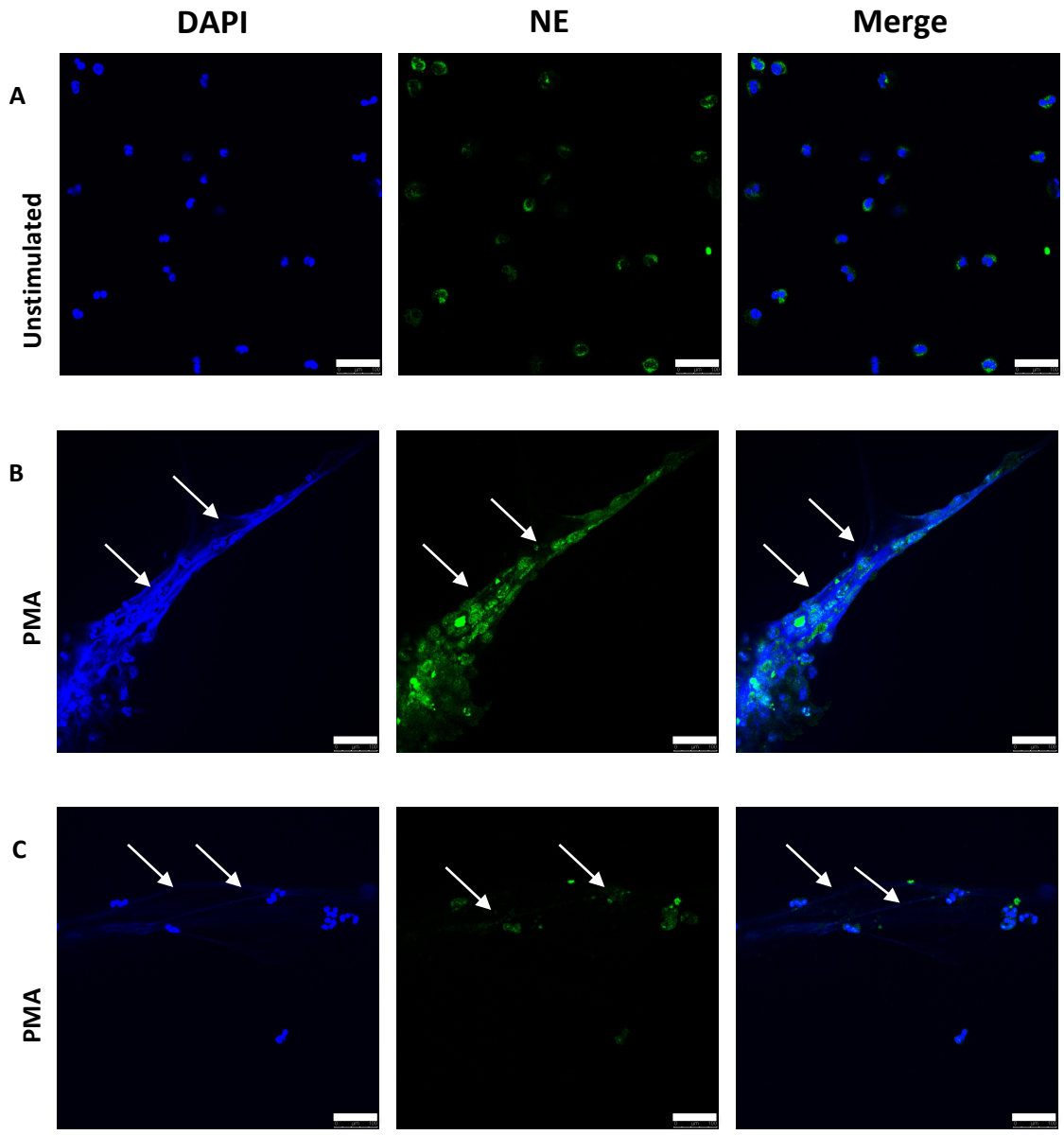


Figure 15: Analysis of NET production in PMA stimulated neutrophils using Immunofluorescence.

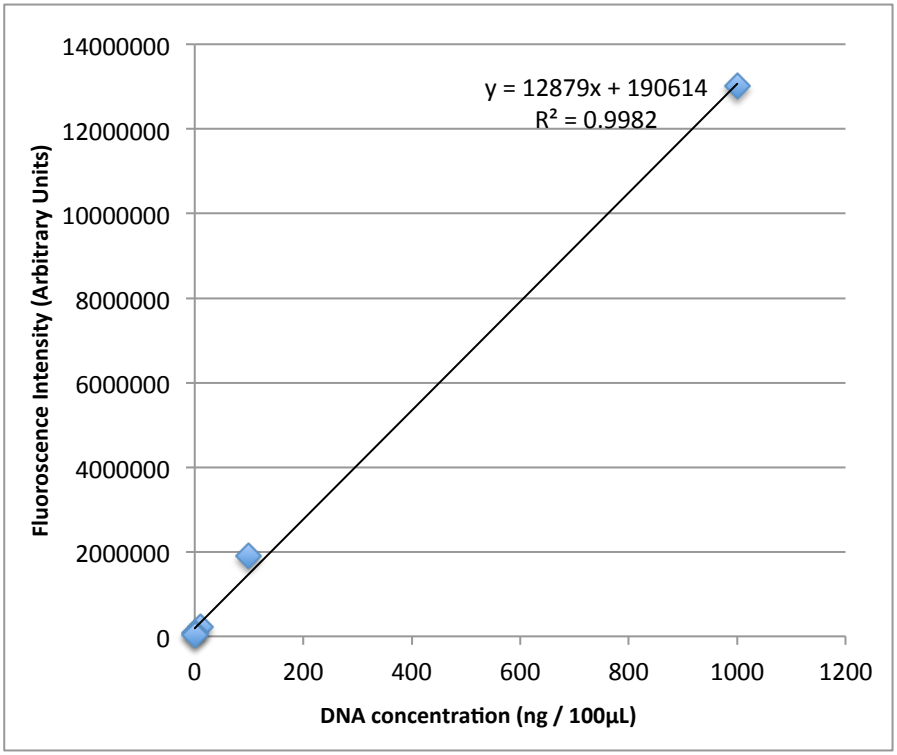
Neutrophils isolated from JSLE patients were either left unstimulated or incubated with PMA for 2 hours. NETs release was determined by co-staining with DAPI (blue) for nuclear DNA detection and anti-NE antibodies (green) for NE detection. Cells were subsequently visualized for extruded NETs by confocal microscopy. (**A**) Unstimulated neutrophils. (**B** and **C)** PMA-stimulated neutrophils. Scale Bar = 100µm; White arrows indicate NET release.

3.3 Quantification of NET release

Further to visualization of NETs, Quantitative assessment of NETs was performed using Spectrofluorometry to quantify the NETs. This quantification method relies on the fact that the structural backbone of NETs is composed of DNA. Neutrophils were isolated from peripheral blood of JSLE patients. Purified neutrophils were either left unstimulated or incubated with PMA (160nM), IFNα (10ng/ml), JSLE sera (10%) or pediatric control sera (10%). To quantify the NETs formed, micrococcal nuclease was added to the neutrophils to digest the NET scaffold and release the NET DNA into the supernatant. Subsequently, the cell-free supernatant with and without NET DNA was quantified using Quant-iT Picogreen assay (Section 2.3 and 2.3.2). The amount of extracellular DNA released by different NET inducing stimuli was assessed.

The concentration of extracellular dsDNA was increased in the supernatant following PMA (405 ng/ml, n=4), IFNα (69 ng/ml, n=4) and JSLE sera (73ng/ml, n=4) stimulation compared to the control conditions of pediatric control sera (17ng/ml, n=4) and unstimulated neutrophils (4ng/ml, n=4) (**Figure 16.B**).

A



B

Concentration of dsDNA ng / 100µL	NET stimulus
4	Unstimulated
405	PMA
69	IFNα
73	JSLE sera
17	Pediatric control Sera

Figure 16: Quantification of NET dsDNA using Quan-iT Picogreen Assay.
Neutrophils isolated from JSLE patients were either left unstimulated or incubated with PMA, IFNα, JSLE or Pediatric control Sera for 2 hours. Neutrophils were treated with micrococcal nuclease to digest and liberate NET DNA into the supernatant, which was quantified using Quan-iT Pico green assay **A.** Example of a Standard Curve from NET dsDNA Quantification Assay **B.** The concentration of extracellular dsDNA detected in the cell-free supernatant in cells left unstimulated and treated with PMA, IFNα, JSLE sera and pediatric control sera. Data represents mean values of experiments (n=4).

3.4 TLR Ligand/NET-mediated PBMC stimulation

Control PBMCs were either stimulated with TLR Ligands or NETs. Protein levels of downstream signaling proteins were subsequently measured using western blot analysis.

3.4.1 Measuring protein levels of pIRAK1 and pIRF3 in adult control PBMCs following TLRs 3 and 7/9 ligand stimulation

To establish Ligand binding-induced activation of TLRs, adult control PBMCs were incubated with known TLR ligands and investigated for activation of their signaling proteins. IRAK1 and IRF3 are downstream signaling proteins, which get phosphorylated following activation of TLR 7/9 and TLR 3, respectively.

PBMCs were isolated from whole blood of adult control patients and stimulated with respective TLR 3, 7 and 9 Ligands (Section 2.4). After 15 minutes of incubation, cell protein was extracted and protein levels of pIRAK1 and pIRF3 was determined by western blot analysis and normalized to β-Actin expression (Section 2.7). In cells stimulated with TLR3, 7 and 9 Ligands proteins levels of pIRAK1 and pIRF3 were increased compared to unstimulated PBMCs. (**Figure 17**).

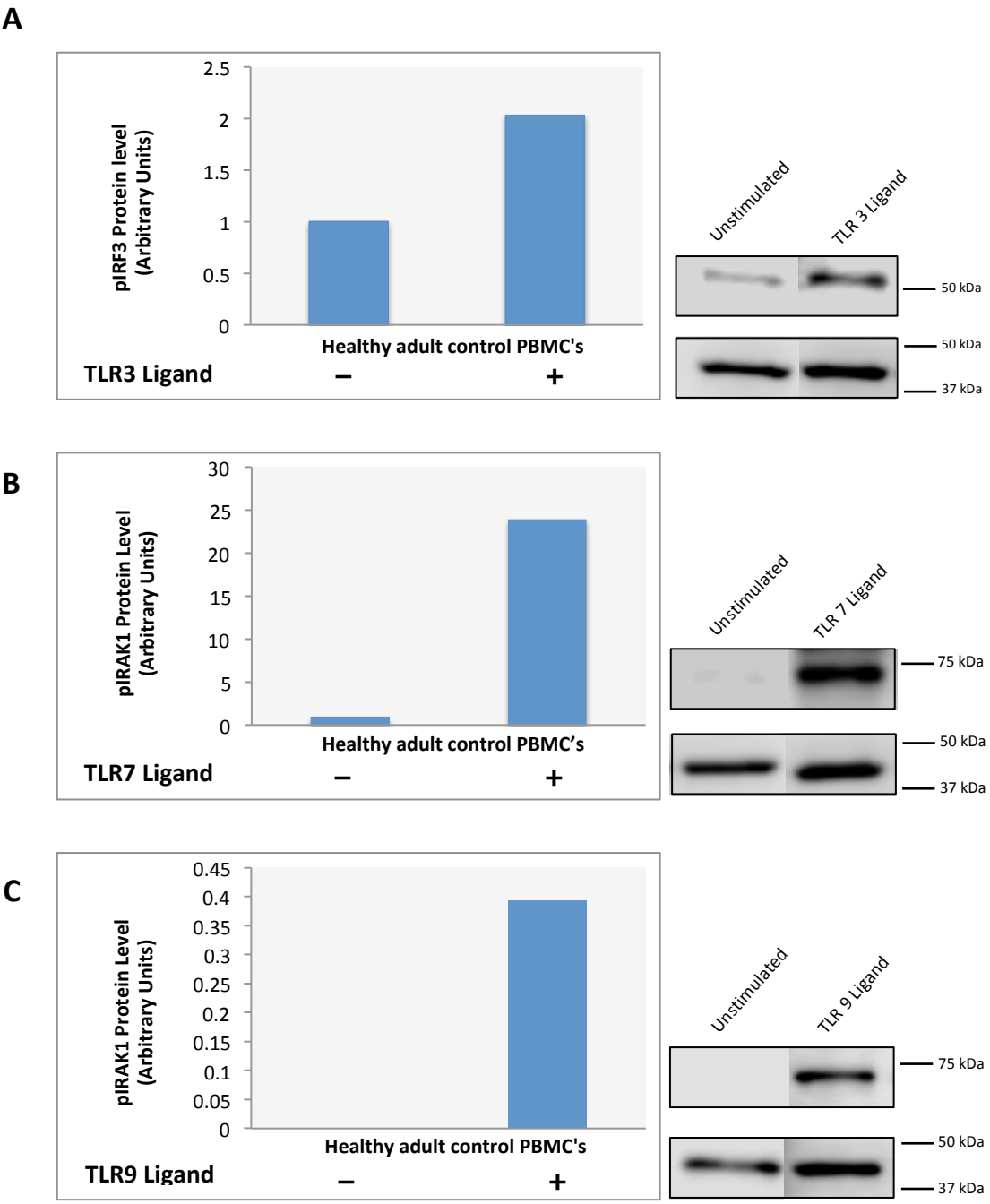


Figure 17: Protein levels of phosphorylated IRF3 and IRAK1 in adult control PBMCs stimulated with TLR 3, 7 and 9 Ligands.

PBMCs purified from healthy adult control blood were either left unstimulated or incubated with TLR 3/7/9 ligands. Subsequently, cell protein was extracted and analyzed for the protein levels of pIRF3 and pIRAK1 using western blot analysis. Results were normalized to β-Actin expression. (**A**) pIRF3 protein levels were higher in cells stimulated with TLR3 Ligand compared to unstimulated PBMCs. pIRAK1 protein levels were higher in cells stimulated with (**B**) TLR7 Ligand and (**C**) TLR9 Ligand compared to unstimulated PBMCs. n=1**.**

3.4.2 Measuring protein levels of pIRAK1 and pIRF3 in adult control PBMCs stimulated with IFNα and JSLE sera –induced NETS.

To investigate whether NETs signal through endosomal TLR 3, 7, 9, adult control PBMCs were stimulated with IFNα or JSLE sera-induced NETs and protein levels of pIRF3 and pIRAK1 was measured using western blot analysis and normalized for B-Actin protein levels.

PBMCs were isolated from whole blood of adult control patients (Section 2.2.2) and stimulated with IFNα/JSLE sera -induced NETs derived from JSLE (n=5) and pediatric control neutrophils (n=5) (Section 2.4). Cell protein was extracted and pIRAK1 and pIRF3 protein levels was determined by western blot analysis and normalized to B-Actin expression (Section 2.7).

Protein levels of both pIRAK1 (Figure 18.A) and pIRF3 (Figure 18.B) were higher in control PBMCs stimulated with IFNα - induced NETs from both JSLE and pediatric control neutrophils compared to the unstimulated control PBMCs (Figure 18). The increase in proteins levels was not influenced by the source of the NETs as there was no statistically significant difference in the increase in proteins levels of both pIRAK1 ($p=0.841$; $n=5$) and pIRF3 ($p=0.690$; $n=5$) between cells that were stimulated with JSLE and pediatric control neutrophil derived NETs.

Similarly, in control PMBCs stimulated with JSLE-sera induced NETs from both JSLE and pediatric control neutrophils the proteins levels of both pIRAK1 (Figure 19.A) and pIRF3 (Figure 19.B) were higher compared to the unstimulated condition (Figure 19). The increase in protein levels was not influenced by the source of the NETs as there was no statistically significant difference in the increase in protein levels of both pIRAK1 ($p=0.841$; $n=5$) and pIRF3 ($p=0.421$; $n=5$) between cells that were stimulated with JSLE and pediatric control neutrophil derived NETs.

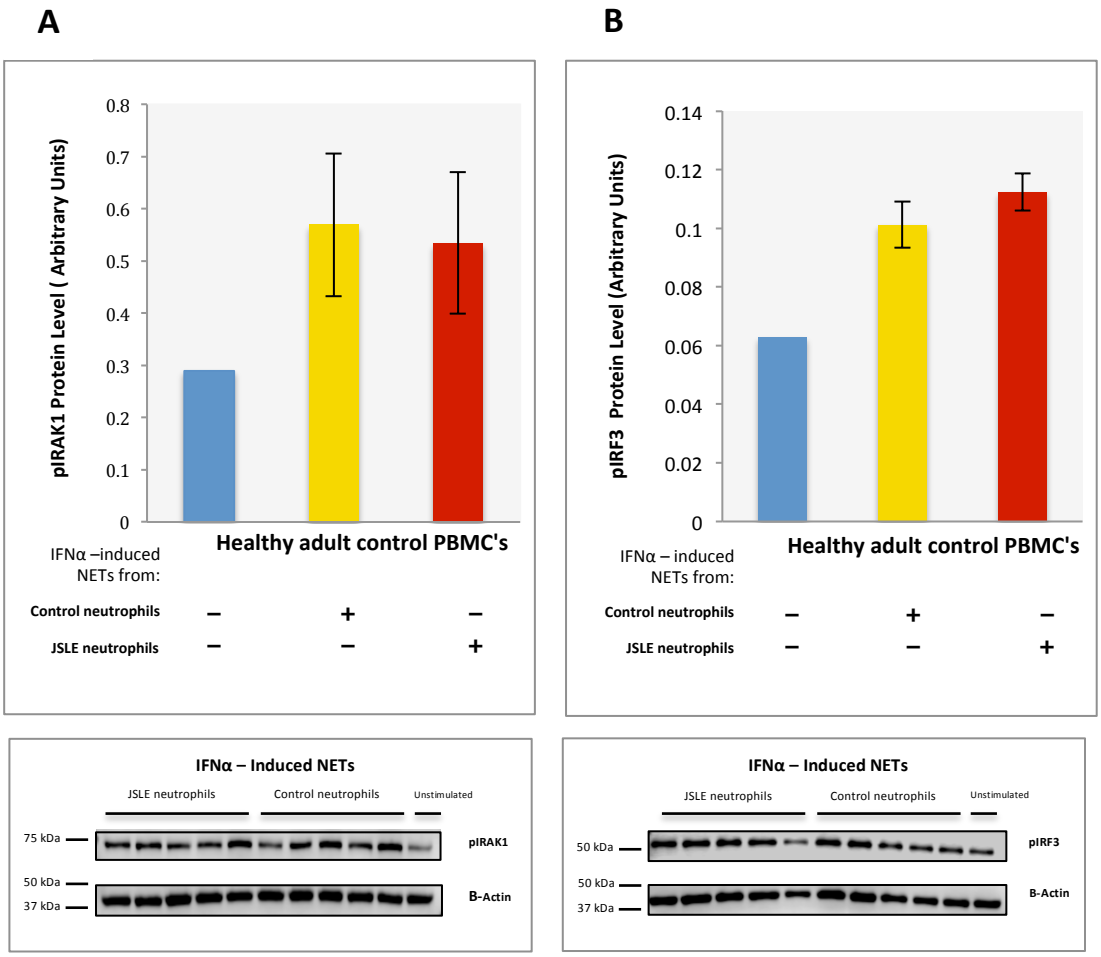


Figure 18: pIRAK1 and pIRF3 protein levels in adult control PBMCs stimulated with IFNα induced NETs.

PBMCs purified from healthy adult control blood were either left unstimulated or incubated with IFNα-induced NETs derived from pediatric control and JSLE neutrophils. Cell protein was extracted and analyzed for pIRAK1 and pIRF3 protein levels using western blot analysis and results were normalized to β-Actin expression. (**A**) pIRAK1 and (**B**) pIRF3 protein levels were higher in cells stimulated with IFNα-induced NETs from both JSLE and pediatric control neutrophils compared to unstimulated PBMCs. The increase in protein levels was not influenced by the source of the NETs, as there was no statistically significant difference in the increase in protein levels of both pIRAK1 ($p=0.841$; $n=5$) and pIRF3 ($p=0.690$; $n=5$) between cells that were stimulated with JSLE and pediatric control neutrophil derived NETs.

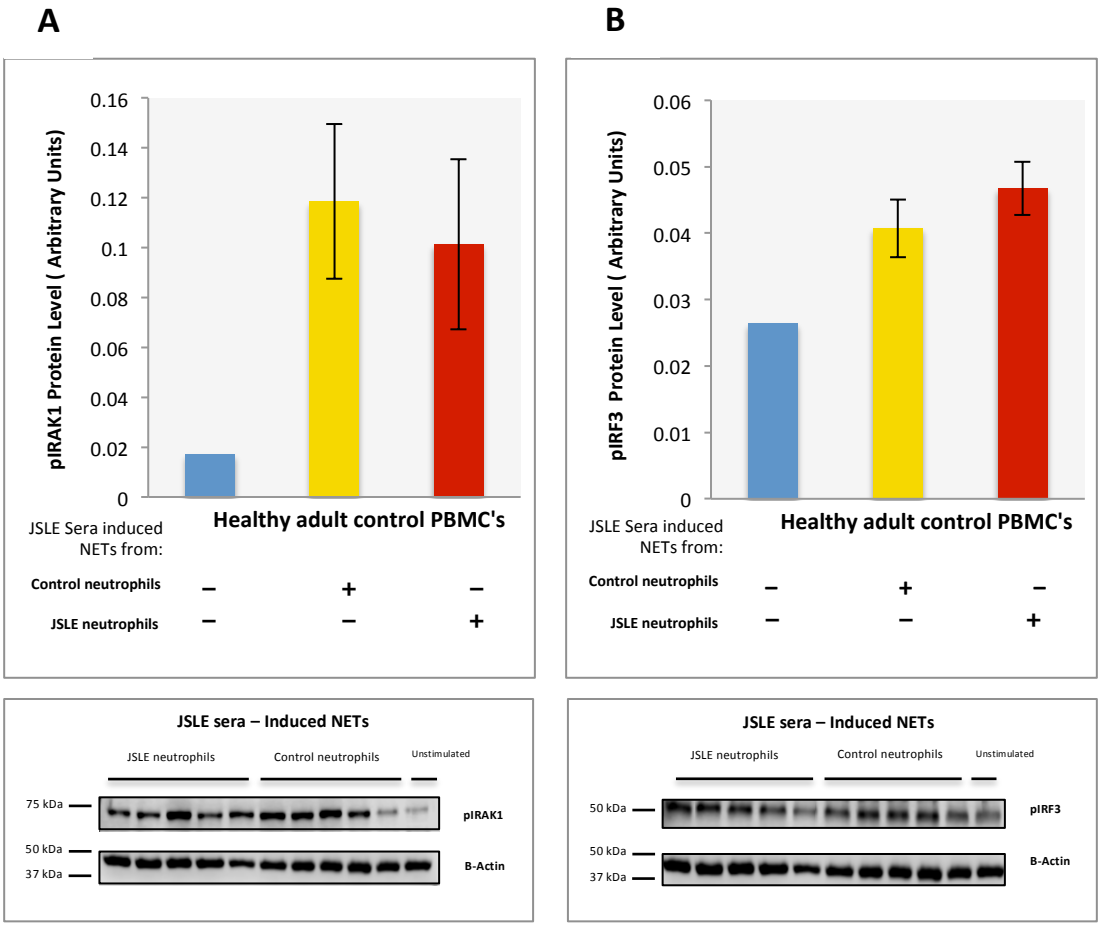


Figure 19: pIRAK1 and pIRF3 protein levels in adult control PBMCs stimulated with JSLE sera-induced NETs.

PBMCs purified from healthy adult control blood were either left unstimulated or incubated with a known concentration of JSLE sera-induced NETs derived from pediatric control and JSLE neutrophils. Cell protein was extracted and analyzed for pIRAK1 and pIRF3 protein levels using western blot analysis and results were normalized to β-Actin expression. (**A**) pIRAK1 and (**B**) pIRF3 protein levels were higher in cells stimulated with JSLE sera-induced NETs from both JSLE and pediatric control neutrophils compared to unstimulated PBMCs. The increase in protein levels was not influenced by the source of the NETs, as there was no statistically significant difference in the increase in protein levels of both pIRAK1 ($p=0.841$; $n=5$) and pIRF3 ($p=0.421$; $n=5$) between cells that were stimulated with JSLE and pediatric control neutrophil derived NETs.

3.5 Protein levels of pIRAK1 in HCQ-pre-treated adult control PBMCs following TLR Ligand or NET stimulation

As a proof-of-concept study to confirm that the NET-induced stimulation of PBMCs was TLR dependent, NETs and TLR ligands were incubated with control PBMCs that were pre-treated with HCQ to inhibit TLR activation. HCQ inhibits endosomal TLR signaling activation by interfering with endosomal acidification [199].

PBMCs were purified from healthy adult control and either left unstimulated or incubated with varying concentrations of HCQ (10 μM, 50 μM and μ100M) for 30 minutes. The HCQ-treated and untreated cells were then incubated with TLR7 Ligand, TLR9 ligand or PMA-induced NETs for 15 minutes. Cell protein was extracted and pIRAK1 protein levels were measured by western blot analysis (Section 2.8).

In a concentration dependent manner, HCQ pretreatment reduced TLR9 Ligand (**Figure 20.A**), TLR7 Ligand (**Figure 20.B**) and NET-induced (**Figure 20.C**) protein levels of pIRAK1 in TLR-expressing control PBMCs.

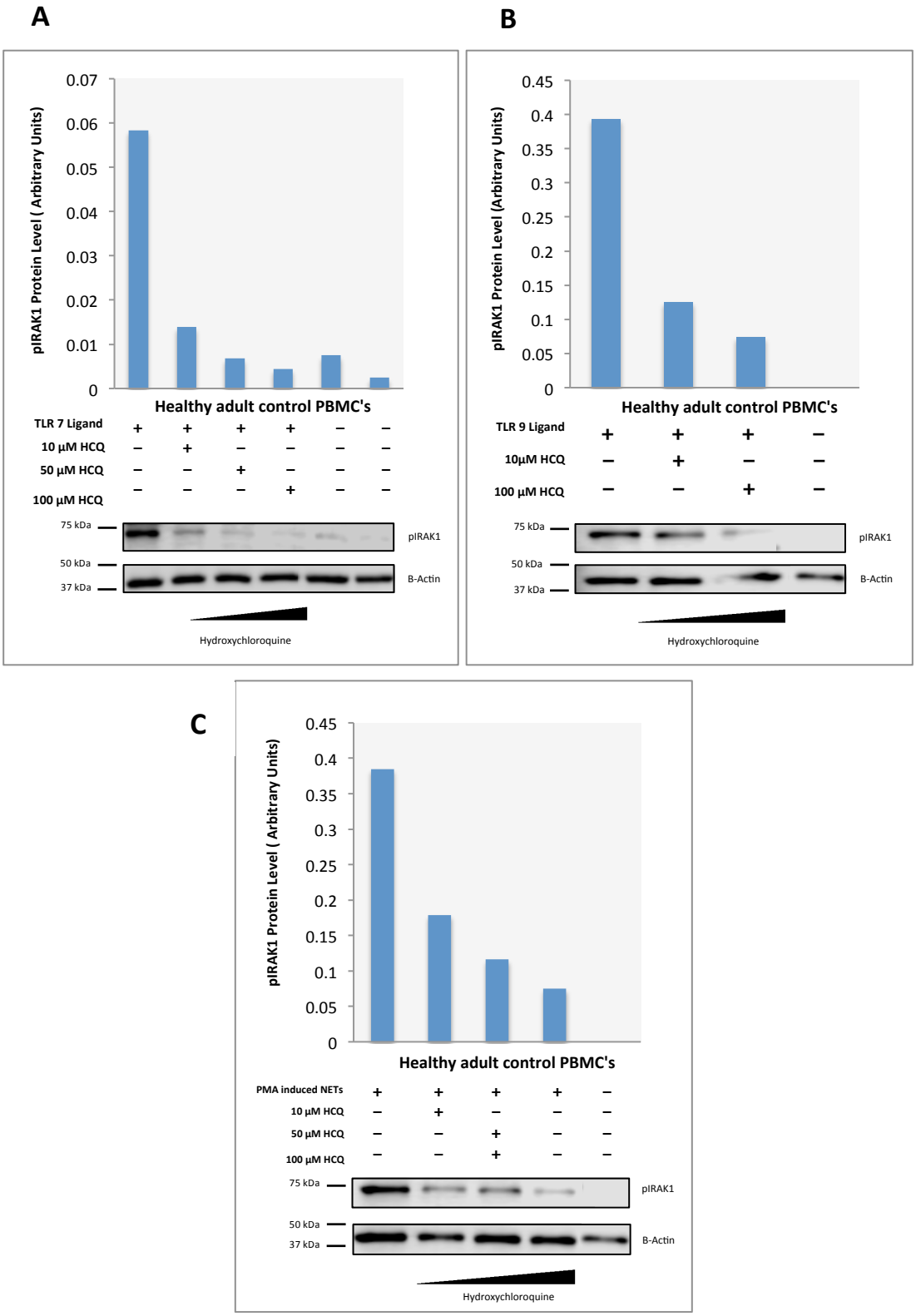


Figure 20: Effect of HCQ pretreatment of adult control PBMCs on TLR Ligand or NET induced protein level of pIRAK1 in the PBMCs.

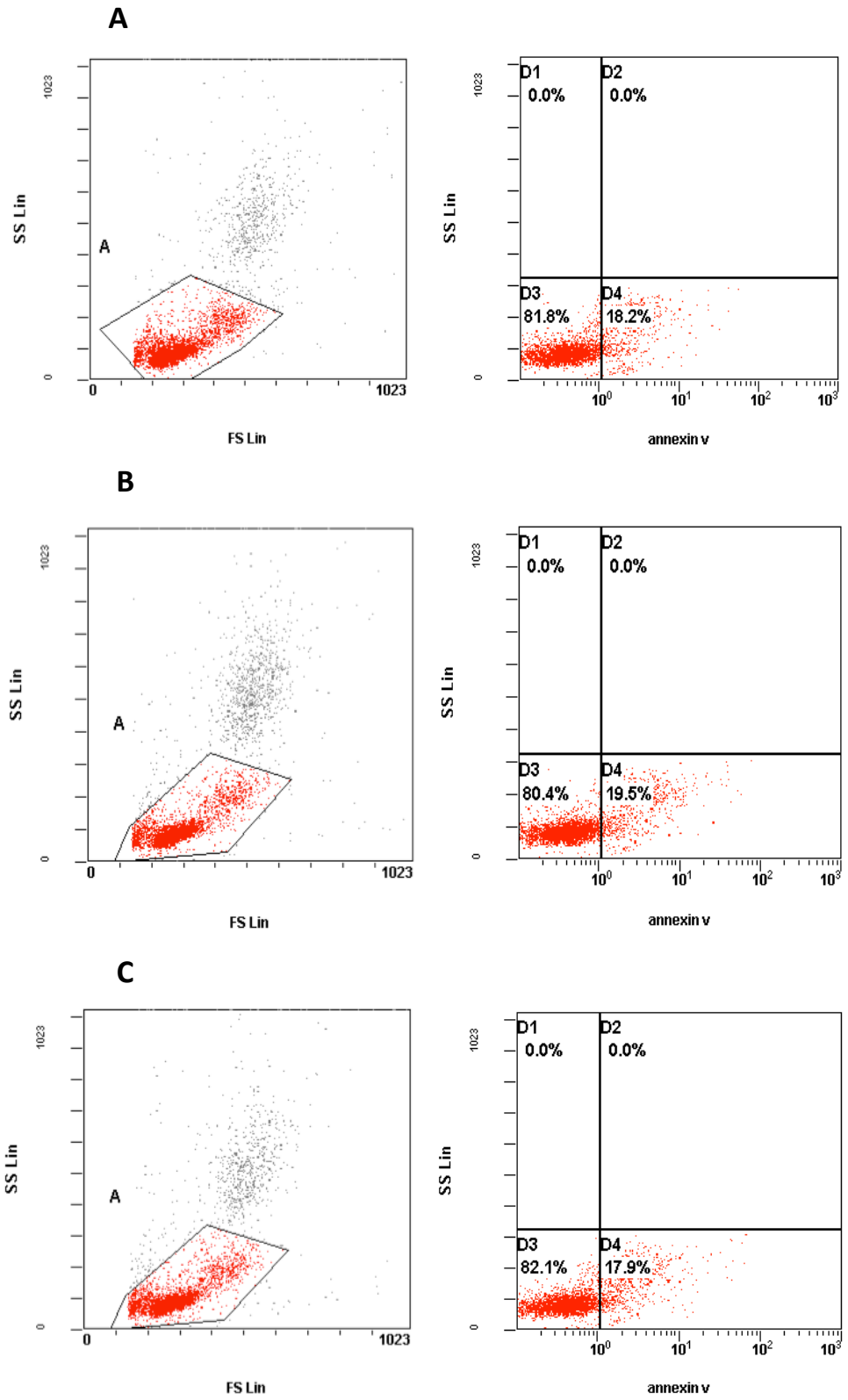
PBMCs purified from healthy adult control were either left unstimulated or incubated with varying concentrations of HCQ to inhibit TLR signaling. The HCQ-treated and untreated cells were stimulated with TLR7/9 Ligand or PMA-induced NETs. Cell protein was extracted and pIRAK1 protein levels were measured by western blot analysis. In a concentration-dependent manner, HCQ pretreatment reduced pIRAK1 protein levels in cells stimulated with (**A**) TLR7 Ligand, (**B**) TLR9 Ligand and (**C**) PMA-induced NETs. All experiments n=1.

3.5.1 Cell viability of adult control PBMCs treated with Hydroxychloroquine

To ensure that the inhibitory effect of HCQ on TLR7/9 Ligand and NET-induced activation of TLR7 and TLR9 in control PBMCs was not a result of HCQ-mediated cytotoxicity, an apoptotic assay was carried out to determine cell viability of control PBMCs following treatment of with HCQ.

PBMCs purified from healthy adult controls were either left unstimulated or treated with varying concentrations of HCQ (10µM, 50µM and 100µM) for 30 minutes. Percentage apoptosis was measured using flow cytometry (Section 2.8.1 and 2.8.1.1).

No difference in percentage apoptosis was detected between HCQ-treated (10µM HCQ=19.5% apoptotic cells; 50µM HCQ=17.9% apoptotic cells; 100µM HCQ =17.8% apoptotic cells) and untreated PMBC's (18.2% apoptotic cells) (**Figure 21).**


A
SS Lin
FS Lin
A
0
1023
D1
0.0%
D2
0.0%
D3
81.8%
D4
18.2%
annexin v
B
SS Lin
FS Lin
A
D1
0.0%
D2
0.0%
D3
80.4%
D4
19.5%
annexin v
C
SS Lin
FS Lin
A
D1
0.0%
D2
0.0%
D3
82.1%
D4
17.9%
annexin v

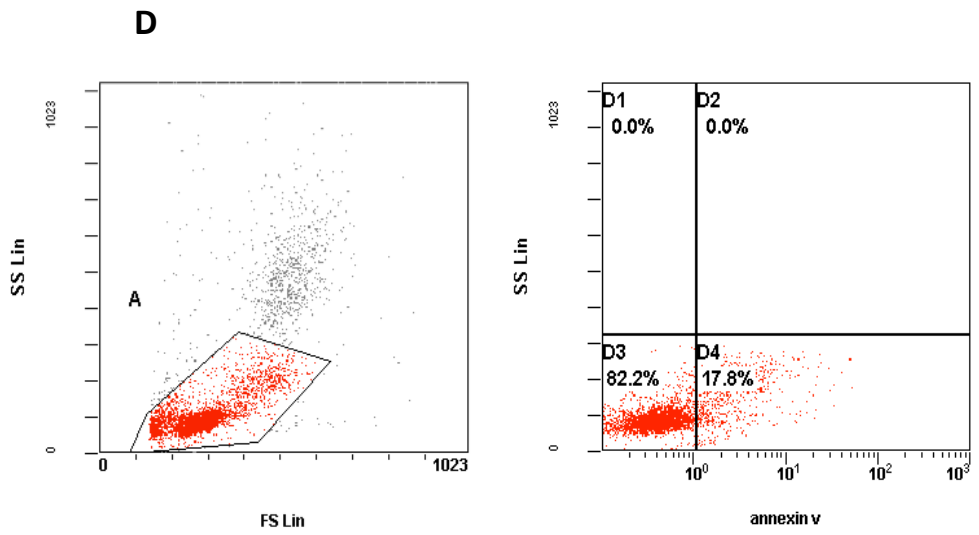


Figure 21: Flow Cytometric analysis of % apoptosis in HCQ-treated and untreated adult control PMBC's.

PBMCs purified from healthy adult controls were either left unstimulated or treated with varying concentrations of HCQ and stained with FITC-labelled Annexin V. Percentage apoptosis was measured using flow cytometry. Images shown represent FS (forward scatter) /SS (side scatter) plot and the corresponding Dot plot. Cells observed in the bottom right quadrant (i.e. positive for annexin v) are considered to be apoptotic. No difference in percentage apoptosis was detected on treatment with (**B**) 10µM (19.5% apoptotic cells), (**C**) 50µM (17.9% apoptotic cells) or (**D**) 100µM (17.8% apoptotic cells) of HCQ compared to (**A**) untreated PBMCs (18.2% apoptotic cell). Results shown are representative of one out of two experiment repeated with similar results.

3.6 Protein levels of pIRAK1 in HCQ pre-treated adult control PBMCs following stimulation with IFNα and JSLE sera induced NETs.

As HCQ appeared not to affect PBMC viability, even at high concentrations, it was investigated whether HCQ pretreatment of control PBMCs has the same inhibitory effects on IRAK1 phosphorylation following stimulation with IFNα and JSLE-induced NETs and thereby establish that IFNα and JSLE-induced NET stimulation of PBMCs was also TLR dependent. NETs were incubated with control PBMCs pre-treated with HCQ and subsequent protein levels of pIRAK1 were measured by western blot analysis.

PBMCs purified from healthy adult controls were either left unstimulated or incubated with 100 μM of HCQ for 30 minutes. The HCQ-treated and untreated cells were either left unstimulated or incubated IFNα or JSLE-induced NETs for 15 minutes. Cell protein was extracted and pIRAK1 protein levels were measured by western blot analysis (Section 2.8).

HCQ pretreatment reduced protein levels pIRAK in control PBMCs stimulated with IFNα (**Figure 22.A**) and JSLE (**Figure 22.B**)-induced NET. These results indicate that IFNα and JSLE-induced NETs signal in a TLR7 and TLR9 – dependent manner.

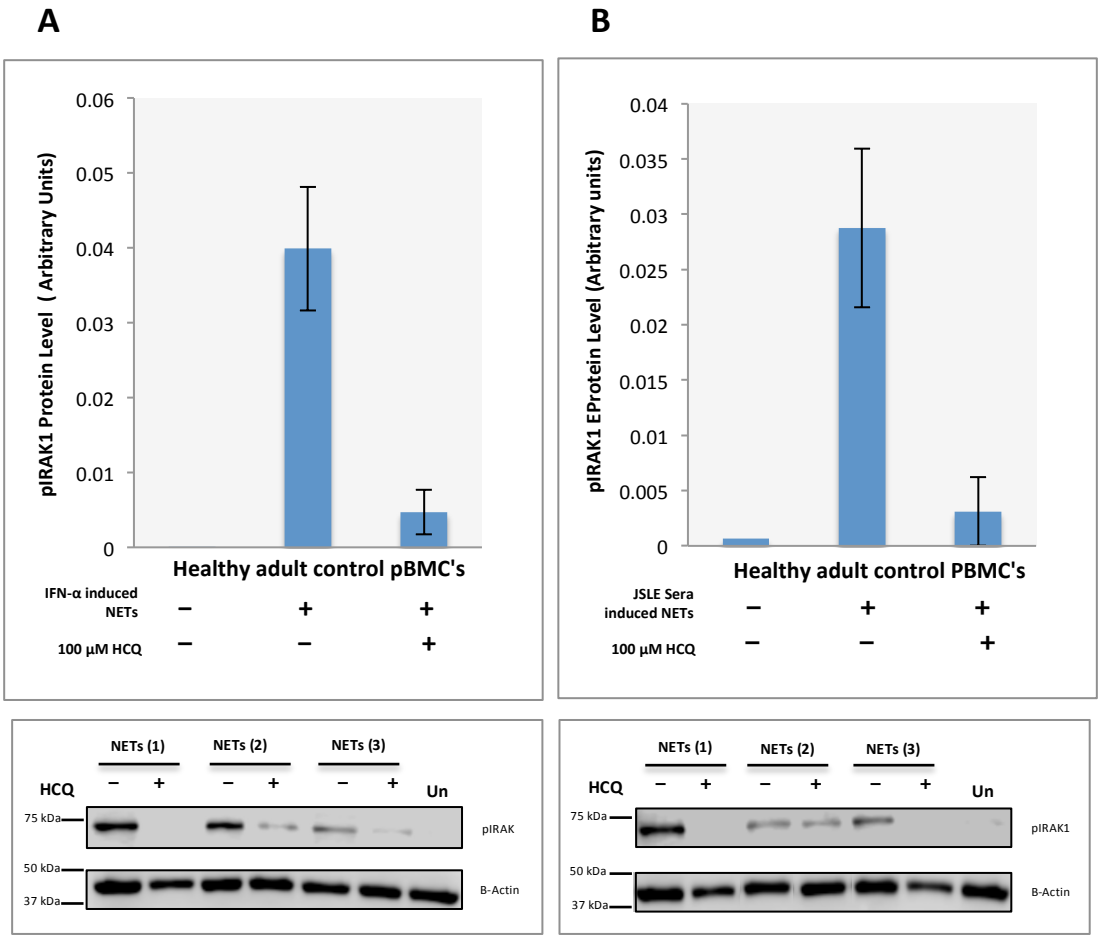


Figure 22: Effect of HCQ pretreatment of adult control PBMCs on pIRAK1 protein levels following stimulation with IFNα and JSLE serum-induced NETs.

PBMCs purified from healthy adult controls were either left unstimulated or incubated with HCQ. The HCQ-treated and untreated cells were either left unstimulated or incubated with IFNα or JSLE serum induced NETs. Cell protein was extracted and pIRAK1 protein levels was measured by western blot analysis and normalized for β-Actin protein levels. (**A**) HCQ pretreatment reduced pIRAK1 protein levels (× 8.5 fold decrease) in cells stimulated with IFNα induced NETs (n=3). **(B**) HCQ pretreatment reduced pIRAK1 protein levels (× 9.2 fold decrease) in cells stimulated with JSLE sera induced NETs (n=3). Un = Unstimulated cells.

3.7 Measuring protein levels of pSTAT1 in adult control PBMCs stimulated with supernatant from NET-stimulated PBMCs

To explore the downstream effects of NET-induced activation of endosomal TLRs and if there was IFNα production, responses compatible with IFNα production was investigated. Supernatant from NET-stimulated PBMCs was incubated with control PBMCs and pSTAT1 protein levels were measured using western blotting and normalized against β-actin expression. STAT1 is phosphorylated following activation of the Type 1 IFN receptors (IFNAR) by IFNα (**Figure 8**).

Purified adult control PBMCs were either left unstimulated or incubated with PMA/IFNα/JSLE sera -induced NETs for 4 and/or 6 hours to induce production of IFNα. Cell free supernatant was removed and stored. Separate control PBMCs were purified and left unstimulated or incubated with a JAK inhibitor to inhibit IFNα signaling. Controls PBMCs pre-treated with or without a JAK inhibitor were either incubated with supernatant from unstimulated PBMCs (control condition) or NET-stimulated PBMCs. Cell protein was extracted and pSTAT1 protein levels was determined by western blot analysis and normalized against β-actin expression levels (Section 2.9).

pSTAT1 protein levels was increased in cells incubated with supernatant from PMBC's stimulated with PMA (**Figure 23.A**), IFNα (**Figure 23.B)** and JSLE sera (**Figure 23.C)** -induced NET compared to the experimental control condition (PBMCs incubated with supernatant from unstimulated PBMCs) (Figure 25) . Pre-treatment of PBMCs with a JAK inhibitor lead to a reduction in pSTAT1 protein levels detected following stimulation with supernatant from NET-stimulated PBMCs (**Figure 23.A, 23.B, 23.C**).

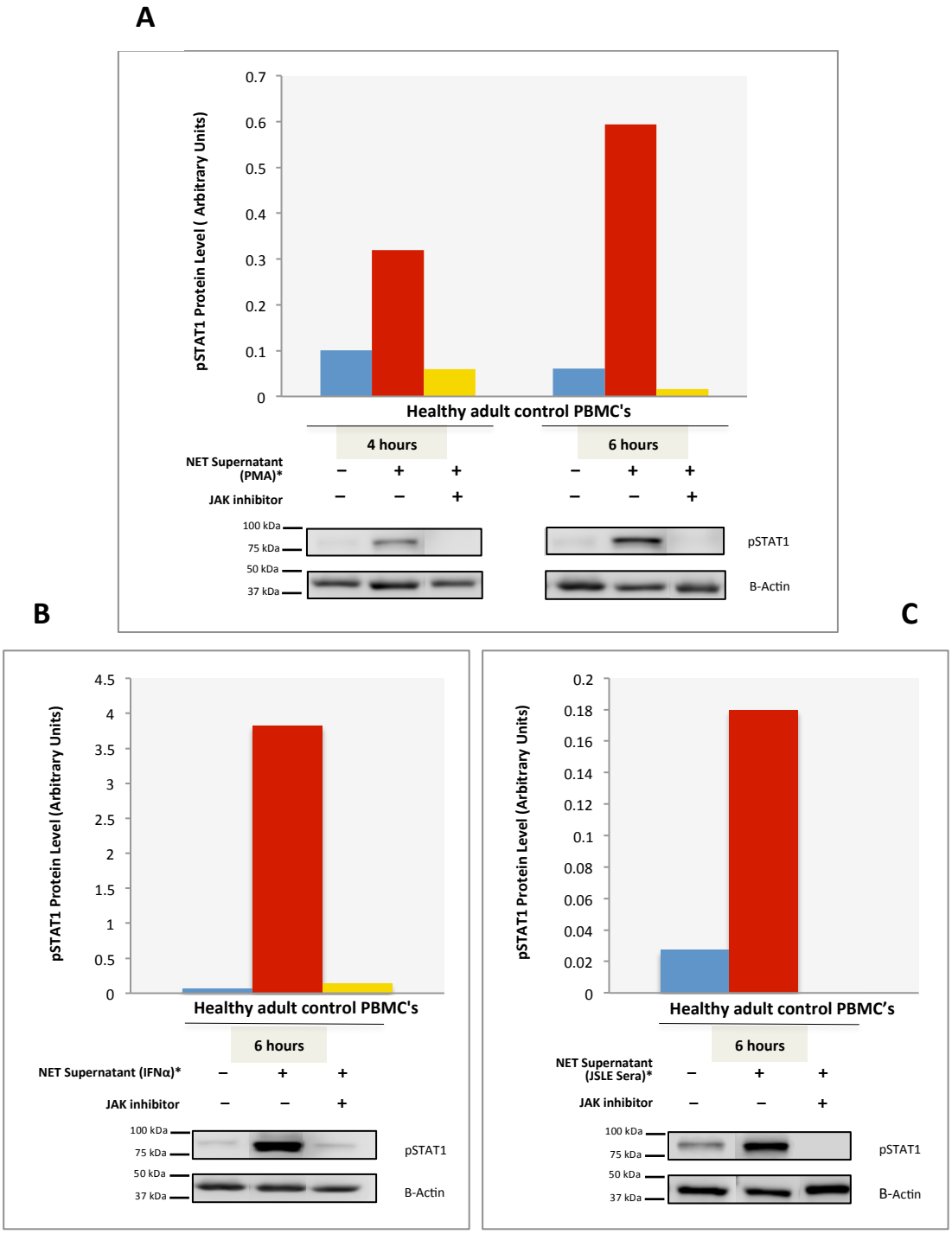


Figure 23: Protein levels of pSTAT1 in adult control PBMCs incubated with supernatant from control PBMCs stimulated with PMA or IFN alpha or JSLE sera-induced NETs

Healthy adult control PBMCs pre-treated ± a JAK inhibitor were incubated with supernatant from control PBMCs stimulated for 4 and/or 6 hours with PMA or IFNα or JSLE Serum-induced NETs. A control condition comprised of PBMCs incubated with supernatant from unstimulated control PBMCs (blue bar). In adult control PBMCs incubated with supernatant from PBMCs stimulated with (**A**) PMA induced NETs there was increased proteins levels of pSTAT1 at both time points compared to the unstimulated condition. In PBMCs that were pretreated with a JAK inhibitor, protein levels of pSTAT1 was drastically reduced following stimulation with the supernatant. Similarly, in control PBMCs incubated with supernatant form control PBMCs stimulated with (**B**) IFNα and (**C**)-JSLE sera induced NETs there was an increase in pSTAT1 protein levels compared to the unstimulated conditions. Proteins levels of pSTAT1 were drastically reduced when supernatant was added to control PBMCs pre-treated with a JAK inhibitor. n=1.

3.8 Exposure of JSLE neutrophils to supernatant from NET-stimulated PBMCs

To further explore the downstream effects of NET-induced TLR activation and if IFNα production was occurring, responses compatible with IFNα production was investigated with regards to NET production as IFNα has been shown to prime neutrophils to release NETs [94]. Accordingly, the interferon environment of the supernatant from NET-stimulated PBMCs was investigated by incubating supernatant from NET-stimulated PBMCs with JSLE neutrophils and the release of NETs from the induction of NETs was visualized using confocal microscopy following staining for DAPI.

PBMCs isolated from healthy adult controls were either left unstimulated or incubated for 6 hours with JSLE sera-induced NETs, to induce IFNα production. Following incubation, cell-free supernatant was removed and stored. Neutrophils were then isolated from JSLE patients and either left unstimulated or treated with JAK inhibitor to inhibit IFNα signaling. JSLE neutrophils ± pretreatment with a JAK inhibitor was either left unstimulated or incubated for 2 hours with cell-free supernatant from PBMCs stimulated with JSLE sera-induced NETs. As positive controls, JSLE neutrophils were stimulated with either 10ng/ml IFNα or 10% JSLE sera. Possible NET formation was subsequently visualized using confocal microscopy by staining for nuclear DNA using DAPI stain (Section 2.10).

No NETs were observed in the unstimulated JSLE neutrophils (**Figure 24.A**). NETs were observed when neutrophils were incubated with JSLE sera (**Figure 24.B**), IFNα (**Figure 24.C**) and supernatant from PBMCs stimulated with JSLE sera-induced NETs (**Figure 24.D**). However, when the supernatant from PBMCs stimulated with JSLE sera induced NETs was incubated with JSLE neutrophils which were pre-treated with a JAK inhibitor, no NETs were observed (**Figure 24.E**).

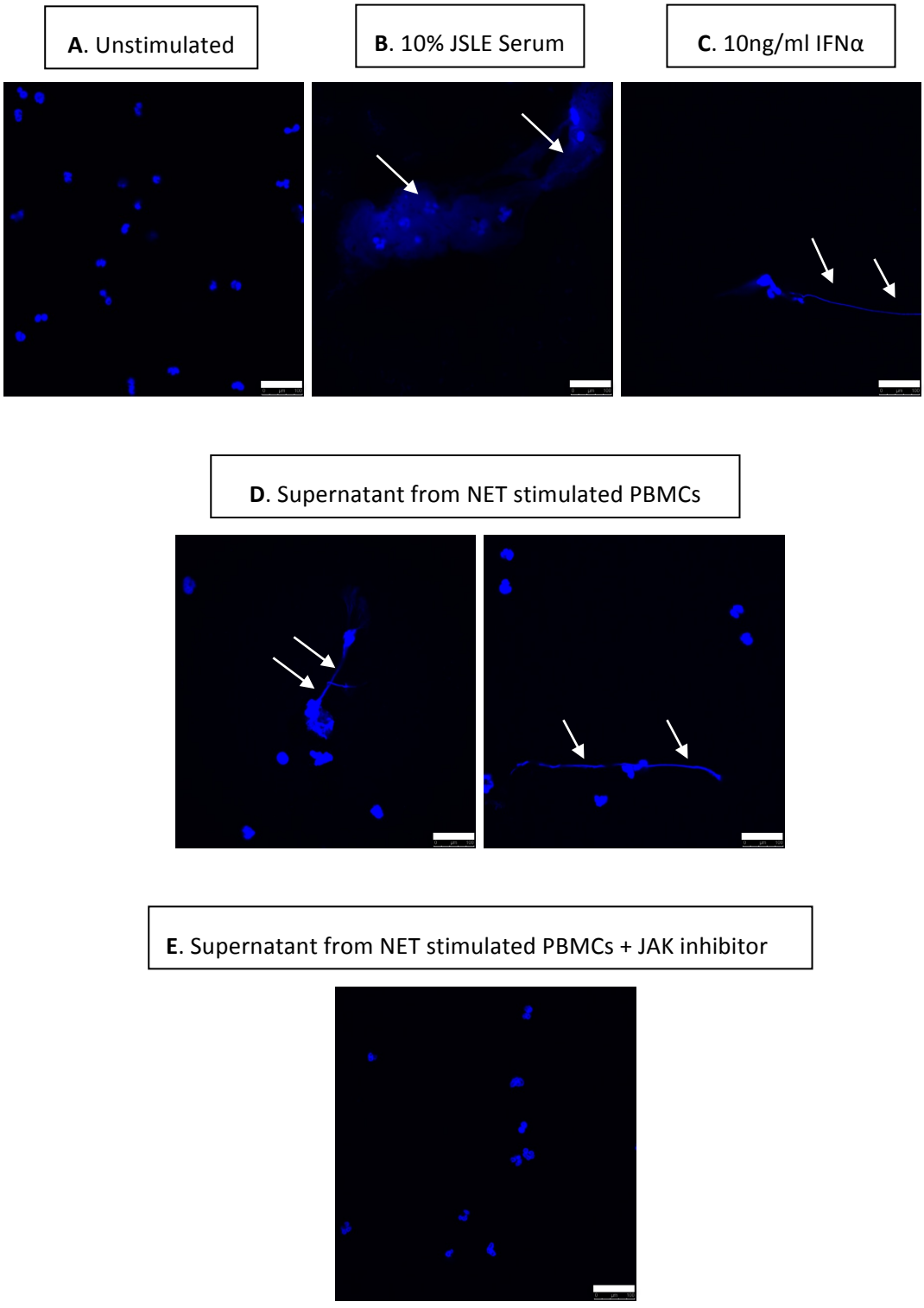


Figure 24: Exposure of JSLE Sera, IFNα or supernant from NET stimulated PBMCs to JSLE neutrophils pre-treated with or without a JAK inhibitor.

Purified JSLE neutrophils pre-treated ± a JAK inhibitor were either left unstimulated or incubated with either 10% JSLE sera, 10ng/ml IFNα or supernatant from control PBMCs stimulated with JSLE sera-induced NETs. NET formation was visualized using confocal microscopy by staining for nuclear material using DAPI (blue) **(A)** No NETs were observed in unstimulated JSLE neutrophils. Incubation of JSLE neutrophils with (**B**) JSLE sera and (**C**) IFNα resulted in NET release (**D**) Exposure of JSLE neutrophils to supernatant from PBMCs stimulated with JSLE sera-induced NETs resulted in NET release. (**E**) However, no NETs were observed when JSLE neutrophils pre-treated with a JAK inhibitor were exposed to supernatant from PBMCs stimulated with JSLE sera-induced NETs. Bottom right hand corner bar represents. Scale Bar = 100µm; White arrows indicate NET release. n=1.

3.9 Analysis of Induction and Reduction of Signalling pathways

For all experiments involving induction (using NETs) or reduction (using HCQ) of signalling pathways, each time there was an increase or decrease in protein levels of signalling proteins compared to the unstimulated condition. Accordingly, combination of results of all induction experiments (n=5) and all reduction experiments (n=5) there was a statistically significant increase or decrease in protein levels compared to the unstimulated condition. Compared to the unstimulated PBMCs, stimulation with NETs lead to a significant increase in pIRAK1 protein levels ($p=0.043$; $n=5$). In PBMCs pre-treated with HCQ, there was a significant reduction in pIRAK1 protein levels following NET stimulation ($p=0.028$; $n=6$). Although following stimulation of signalling pathway using supernatant of NET-stimulated PBMCs there was an increase in the protein levels of pSTAT1 compared to the control condition (cells stimulated with supernatant from unstimulated PBMCs), the increase was not statistically significant ($p=0.068$; $n=4$). Similarly, although in cells pre-treated with a JAK inhibitor there was a reduction in pSTAT1 protein levels following exposure to supernatant of NET-stimulated PBMCs the data shows that there is no significant difference ($p=0.068$; $n=4$).

3.10 Summary of Results

JSLE NETs activated the immune system in a TLR3, 7 and 9- dependent manner (**Figure 19**). HCQ was successful blocking in NET-induced activation of TLR7 and TLR9 (**Figure 22.B**). The supernatant of NET-stimulated PBMCs activated JAK-STAT signalling pathways of control PBMCs resulting in increased proteins levels of pSTAT1 (**Figure 23**) and also stimulated JSLE neutrophils to release NETs (**Figure 24**). A JAK inhibitor was successful in inhibiting both these responses (**Figure 23 and Figure 24.E)**.

3.11 Comparison of results with Clinical and Demographic data.

All results obtained were compared against patient clinical and demographic data. However numbers investigated were too small to give any correlation of statistical significance. Quite interestingly, even with small numbers there was an indication of a negative correlation between pIRAK1 protein levels in healthy control PBMCs stimulated with NETs derived from JSLE neutrophil (n=4) and current drug therapy, HCQ (R^2 = 0.9472). Unfortunately sample sizes investigated were too small to produce any reliable correlations.

4 Discussion

4.1 Purpose and Objective of Study

Activated neutrophils may undergo cell death by NETosis to generate NETs [94]. These structures were initially described in the setting of host defense, as a novel antimicrobial mechanism generated to trap, immobile and kill microorganisms [94]. However, despite their evident protective role an increasing pool of evidence implicates NETs at the center of many pathological conditions, including SLE, serving as a double-edged sword of immunity [64]. It is believed that aberrant NET formation coupled with impaired NET degradation leads to persistence of these structures and their continuous exposure to immune system [94]. Accordingly, dysregulated NETosis is increasingly being speculated to provide a novel source of auto antigens, occurring at the expense of autoimmune responses in susceptible individuals. Unfortunately, there is currently no robust evidence base incorporating this speculation into the disease pathogenesis of SLE.

Once released, the DNA scaffold of NETs is degraded by extracellular DNase I to facilitate subsequent clearance from the circulation by macrophages [158]. This rapid clearance prevents the prolonged exposure of these potentially immunogenic structures to the immune system which can mediate inadvertent immune responses [158]. Therefore it is plausible that if these clearance mechanisms were to fail, it could lead to persistence of these structures in a non-degradable form, leading to presentation of self-antigens by functioning as endogenous TLR ligands. Adult SLE patients demonstrating impaired ability to degrade NETs support this theory [159, 160]. Even in the presence of non-degradable NETs it raises the question of immunogenic antigens in these structures as self DNA is a poor TLR ligand [142]. However, this apparent discrepancy can be explained by the ability

of NETosis to affect immunogenicity of autoantigens, which is described in detail further in the discussion (Section 4.3.1) [168].

However, a large proportion of existing research exploring the role of NETs in Lupus is related adult-onset SLE despite JSLE having a more severe presentation and course. Therefore, research into the exact role of NETosing neutrophils in JSLE will be beneficial, not only to provide new insight into the immune dysregulation driving Lupus pathogenesis, but also potentially open up new avenues for the development of therapeutic strategies in the hope of improving the overall disease outcome of JSLE patients. The hypothesis therefore stated that NETs from JSLE patients are providing a novel source of auto-antigens, which are being detected through endosomal TLRs leading to the activation of the immune system and induction of Type 1 IFN production.

The ability of NETs to activate the immune system in an endosomal TLR-dependent manner by providing a source of autoantigens was investigated by initially measuring protein levels of phosphorylated IRAK1 and IRF3 (TLR7/9 and TLR3-assocaited signaling proteins) in adult control PBMCs stimulated with PMA, IFNα or JSLE sera-induced NETs derived from JSLE or pediatric control neutrophils. The levels of phosphorylation was initially compared between NET-stimulated and unstimulated cells, followed by comparison between cells stimulated by NETs derived from JSLE and pediatric control neutrophils to determine if the origin of NETs affect the extent of immune activation. Once the ability of NETs to activate PBMCs was established, as a proof-of-concept study, to verify that the NET stimulation was TLR dependent, the effect of TLR inhibition on NET induced stimulation was assessed. Lastly, the downstream effects of NET-mediated activation of control PBMCs was explored by adding the supernatant of NET-stimulated PBMCs to control PBMCs or JSLE neutrophils to investigate for responses consistent with the presence of IFNα.

4.2 Overview and interpretation of study findings

The hypothesis stated that NETs could provide a novel source of auto antigens that get detected through endosomal TLRs leading to immune cell activation and Type 1 IFN production. Therefore, the ability of NETs to stimulate control PBMCs was initially determined.

Investigation of IRAK and IRF3 phosphorylation in control PMBC's stimulated with IFNα (**Figure 18**) or JSLE sera (**Figure 19**)- induced NETs demonstrated increased protein levels of phosphorylated IRAK1 and IRF3 compared to unstimulated cells. However, no significant difference was seen in the degree of phosphorylation between cells stimulated with NETs derived from JSLE or pediatric control neutrophils, irrespective of the NET stimuli (**Figure 18 and 19**). This indicates that PMA, IFNα and JSLE sera induced NETs can activate TLRs by providing a source of auto-antigens, regardless of the origin of the neutrophils generating the NETs. These findings suggest that immune cells are detecting autoantigens that are being provided by NETs in a TLR 3, 7 and 9 -dependent manner.

HCQ pretreatment of control PBMCs was carried out to inhibit TLR signaling, which reduced phosphorylation of IRAK1 in a dose-dependent manner in cells stimulated with TLR7/9 (**Figure 20.A and 20.B**) Ligands and PMA-induced NETs (**Figure 20.C**). Similarly, HCQ reduced phosphorylation of IRAK in cells stimulated with IFNα and JSLE sera induced NETs (**Figure 22.A and 22.B**). These findings implicate NETs in triggering activation of immune cells in a TLR7 and TLR9 dependent manner.

The downstream effects of NET-induced TLR activation was observed when the protein levels of phosphorylated STAT1 was increased in control PBMCs exposed to supernatant from NET stimulated PBMCs (**Figure 23)**. As phosphorylation of STAT can be initiated by binding of type1 IFNs to IFNAR, these findings indicate that NET mediated TLR activation

likely induces type 1 IFN production, which can in turn, activate JAK-STAT signaling pathway in PBMCs. In further validation of these results, upon addition of the same supernatant to control PBMCs pretreated with a JAK inhibitor to block IFN-mediated signaling, lead to a reduction in STAT1 phosphorylation (**Figure 23**). Furthermore, exposure of the same supernatant from NET-stimulated PBMCs to JSLE neutrophils led to NET release (**Figure 24.D**).

These findings strongly support the hypothesis that NETs stimulate TLR activation by providing a novel source of auto antigens.

4.3 Incorporation of findings into current literature

Linking NETs to the induction of autoimmune processes is an expanding field of research requiring a great deal of further investigation if these structures are to be as considered therapeutic targets in SLE. Our study has demonstrated a mechanistic pathway for NET signaling and subsequent immune activation which can be incorporated into existing literature regarding this area of study to help further understand the biology of these structures.

4.3.1 Signalling pathways activated in cell subsets by auto antigens exposure

This study demonstrates increased protein levels of phosphorylated IRAK1 and IRF3, in cells stimulated with IFNα (**Figure 18**) or JSLE sera (**Figure 19**)- induced NETs. As phosphorylation of these signaling proteins is a direct result of endosomal TLR7/9 and TLR3 activation, it suggests that NETs are providing auto antigens that are being detected in a TLR3/7/9 dependent manner, and provides a mechanism through which NET-mediated autoimmune response could be driven. This auto antigenic potential of NETs is supported by the observation that development or exacerbation of autoimmune responses frequently follows an infection by pathogens involving NETosis [95]. Consistent with the findings of

this study, Lande et al and Garcia-Romo et al have demonstrated the ability of NET to potently activate pDCs by releasing self-DNA-antimicrobial complexes [78, 151]. The immunogenicity of these complexes was attributed to the NET-derived anti-microbial peptides, especially LL37, which facilitates that uptake of self-DNA into intracellular compartments of pDCs where nucleic acid sensing TLRs are located [78, 151]. Existing data regarding NET induced pDC activation primarily involves the activation of TLR 9 [78, 95]. This present study provides novel insight into the possible involvement of TLR3 in detecting nuclear auto antigens provided by NETs. Consistent with existing data regarding auto antigenic potential of NETs [139], these findings support the hypothesis that NETosing neutrophils trigger endosomal TLR activation through their presentation of immunogenic autoantigens. The increase in phosphorylated IRAK protein levels observed was greater compared to the increase in phosphorylated IRF3 protein levels. This could be on account of the fact that NETs might contain more endogenous ligands for TLRs 7 and 9 leading to greater activation of the TLR 7/9- MyD88-IRAK1 pathway compared to TLR 3. Another possibility is the differential expression of these receptors within PBMC cell subsets [213]. TLR3 is limited to myeloid dendritic cells, whereas TLR7 and TLR9 are co-expressed on pDCs and B-cells [213]. So it is reasonable to speculate that the greater increase in phosphorylated IRAK1 compared to IRF3 could be attributed to the greater frequency of TLR7/ 9 expressing cell subsets within PBMC populations compared to TLR3. Although NETs were shown to increase protein levels of phosphorylated IRF3, we cannot however confirm that it was a TLR3 dependent response as the effect of blocking TLR3 activation with HCQ on NET mediated immune activation could not be optimized and therefore warrants further investigation. Furthermore, a more TLR3 pathway- targeted approach for inhibition could be investigated. TLR3 signals through adaptor protein TRIF[25] , and use of a TRIF-inhibitor to inhibit NET mediated signalling through these receptors could provide convincing evidence for the direct involvement of TLR3 pathway, compared to 100 μM

HCQ, which inhibits all endosomal TLRs. Similarly, MyD88 inhibitor would be more specific for TLR7/9 pathway inhibition [25], and could be a potential avenue to be investigated. However, looking at the effects HCQ is important as this is used quite successfully therapeutically in Lupus, which is described in further detail later in the discussion (Section 4.3.4) and therefore insights into its involvement in TLR inhibition would be useful in understanding its underlying mechanism in Lupus and development of other TLR targeted therapies.

Contrary to what we expected, the source of the neutrophils generating the NETs was not a contributory factor in immune activation observed (**Figure 18 and 19**), which meant that although JSLE neutrophils have been shown to release more NETs [78, 151], they did not lead to production of more immunogenic NETs compared to control neutrophils. An explanation for this could be that it is not the source of the NETs, but the source of the immune cells that get activated by the NETs, which determines the degree of immune activation. In support of this theory, TLRs 3, 7 and 9 have been shown to be upregulated in JSLE PBMCs, potentially making these cell subsets more sensitive to activation [214]. Accordingly, investigating if JSLE derived PBMCs are more sensitive to NET auto antigens compared to control PBMCs could be a potential avenue to be explored in the future. Another possibility is the involvement of low-density granulocytes (LDG) in producing more immunogenic NETs. LDGs, which typically contaminate PBMC fractions during cell separation, represent an alternate neutrophil subset and potent producers of NETs, morphologically and functionally distinct from normal density neutrophils (investigated in this study)[215]. Accordingly, it is possible that NETs generated through LDG is inherently more immunogenic in nature compared to NETs from normal density neutrophils in SLE, and the origin of NETs as a determining factor immune activation could likely still apply.

Neutrophils from SLE patients are reported to release more NETs [78, 151]. In support, we observed that JSLE sera induced more NET DNA that pediatric control sera (**Figure 16.B)**. Furthermore, in murine model of SLE, neutrophils from Lupus prone mice were found to undergo enhanced NETosis in a spontaneous manner compared to control mice [216]. Furthermore, consistent with human SLE for antibody generation and organ damage, the mice were reported to produce anti-NET autoantibodies, and NET-like material containing MPO, DNA and NE were detected in nephritic kidneys as well as non-affected skin [216]. However, data from murine Lupus model can be quite conflicting, with Campbell et al observing disease exacerbation in the presence of mice lacking the ability to generate NETs, implying that disease pathogenesis is not solely attributable to NETs [217].

Once released, NETs are rapidly degraded by action of DNase1 and subsequently cleared from circulation by macrophages [158]. However, in Lupus it is largely believed that defects in these clearance mechanisms coupled with aberrant NET formation leads to persistence of these structures in a non-degradable form leading to prolonged exposure to the immune system [94]. Adult SLE patients demonstrating impaired ability to degrade NETs, owing to the presence of DNase1 inhibitors or anti-NET antibodies that protect NETs from DNase activity have supported this theory [159, 160]. Depletion of these NET-protecting autoantibodies from Lupus sera was associated with increased degradation of NETs [159]. Impaired NET degradation was found to correlate with Anti-NET antibodies, Anti-dsDNA titers and development of Lupus nephritis [159]. A similar study observed the presence of anti-DNase antibodies, which interfered with DNase1 activity [160]. This inability to degrade NETs was reported to fluctuate with disease activity and mainly observed during flares than when in remission [162]. NET derived antimicrobial peptides LLL37 and high mobility group box protein 1 (HMGB1), found in high levels in SLE, have been reported to protect DNA from nuclease degradation [78, 151]. Studies have also reported a genetic

basis to impaired DNase1 activity in Lupus patients, involving mutation of *DNASE1 genes* [218, 219]. The relevance of functional DNase activity is reflected by the fact that DNAse1 deficient mice developed symptoms characteristic of SLE [220]. Therefore, persistence of these effectively non-degradable structures could largely account for the counter-productive role of NETs, as a source of auto antigens potentially mediating autoimmune response. In SLE autoantibodies directed against NET-components include dsDNA, histones, MPO and LL-37 are present [64, 157]. The presence of anti-NET antibodies in Lupus patients is suggestive of a NET mediated autoimmune responses in SLE.

Although NET-pDC interactions are evident, self-DNA is a weak stimulator of TLRs [142], which suggests that despite the presence of NETs in a non-gradable form, the NET DNA should not activate pDCs. Endosomal TLRs discriminate between microbial and self-DNA by the degree of methylation, and only bind to unmethylated but not methylated DNA [142, 143]. Self-DNA is mainly methylated which accounts for the lack of immuno stimulatory potential of self-nuclear material [142]. Accordingly impaired methylation can lead to loss of tolerance to self-DNA and likely resulting in recognition by TLR9 in Lupus [142]. NETosis is speculated to affect the immunogenicity of autoantigens [221]. In support, Hurtado et al reported that self-DNA is hypomethylated during NETosis, rendering them potent activators of TLR9 [168]. PTM of proteins is thought to contribute to the autoimmune process by leading to formation of proteins with neoantigens ('neo-epitopes') which have the capacity to incite autoimmune responses as they appear foreign to T and B-cells and are no longer recognized as self [139, 148, 222]. Also, the processing and presentation of these antigens by APCs may be modified by PTMs [222]. PTMs thereby could potentially account for self-antigens being made into targets of autoimmune responses, in predisposed individuals [148]. A key PTM of particular relevance to Lupus is PAD4-mediated citrullination of histones, which, incidentally is a pre-requisite event for the

formation of NETs [86]. Citrullinated histones have been identified in NETs [86, 223]. Although largely uninvestigated in SLE, NETs may represent an important source of citrullinated histones that can break tolerance and potentially provide a stimulus for autoimmunity [157]. In SLE, autoantibodies against citrullinated H3 and H4 have been reported, which is indicative of their ability to act as autoantibody stimuli [157]. Citrullination of other NET-proteins has been found. LL37, reported to be citrullinated by PAD2/4 was found to be more chemotactic to PBMCs, and with higher pro-inflammatory potential compared to unmodified LL37 [157]. Other PTMs, including acetylation and methylation of histones are being speculated for their immunogenic activity in the context of NETs [139]. An alternate mechanism for the production of neoantigens is the proteolytic cleavage of proteins by proteases whereby peptide bonds between two residues in a protein is cleaved [157]. During NETosis, one such protease, NE, translocates to the nucleus to induce partial degradation of histones, which is essential for chromatin decondensation [82]. The proteolytically cleaved histones produced during this process could potentially be recognized by T and B-cells, leading to production of autoantibodies targeting these modified antigens [157].

Collectively, PTMs and proteolytic cleavage of proteins occurring during NETosis may be responsible for loss of tolerance towards NET-associated proteins and the subsequent generation of autoantibodies targeting these modified proteins [157]. This could be particularly deleterious in SLE, where clearance of these structures is impaired [139], leading to persistent exposure of these altered proteins with increased antigenic and immunogenic potential on the NETs to the immune system.

PBMCs in JSLE patients have high expression of TLR, 3, 7 and 9 [214]. With SLE neutrophils readily releasing NETs and the high expression of endosomal TLRs in Lupus patients, it is reasonable to postulate that NET-TLR interactions could be greatly amplified leading to

chronically activated pDCs, which may in part be responsible for the autoimmune responses characterizing Lupus pathogenesis [224]. As demonstrated in this study (**Figure 18 and 19)** endosomal TLRs are responsible for the recognition of auto-antigens in NETs and accordingly, they may represent an important link between NETs and autoimmunity.

HCQ is a therapeutic intervention in JSLE which functions by effectively inhibiting activation of endosomal TLRs [198]. Accordingly, we explored the effect of HCQ on NET- mediated activation of these receptors. As expected, in a dose-dependent manner, pretreatment of PBMCs with HCQ reduced protein levels of phosphorylated IRAK in cells stimulated with TLR7/9 Ligands (**Figure 20.A and 20.B**) and PMA induced NETs (**Figure 20.C**). Similarly, HCQ effectively inhibited phosphorylation of IRAK1 in cells stimulated with IFNα and JSLE sera induced NETs (**Figure 22.A and 22.B**). These findings confirm that NETs trigger immune cell activation via endosomal TLR7/9 dependent manner, and also corroborates existing evidence regarding the efficacy of HCQ in blocking these pathways. These findings may also shed light into the therapeutic efficacy of HCQ in Lupus, by effectively blocking all NET-meditated pathological response that stem from the detection of autoantigens presented by NETs. Notably, we found that HCQ treatment did not have an effect on cell viability of PBMCs **(Figure 21),** even at high concentrations (100µM), verifying that the inhibition observed was due to direct effects of HCQ on TLR signaling. Owing to time constraints, investigating these experiments with regards to TLR3 could not be pursed in this thesis.

Extracellular trap formation however is not limited to neutrophils and witnessed by other cell subset including mast cells, eosinophils as well as macrophages [59, 62, 69]. Mast cell extracellular traps (MCET) are typically induced by the same stimuli as NETs and were reported to comprise nuclear histones and DNA, LL37 and tryptase [59]. Although not investigated so far, it is reasonable to postulate that extracellular traps from these alternate cell subsets, similarly to NETs, could potentially serve as a source of autoantigens

and immuno stimulatory proteins in SLE, particular with MCET, which are reported to release DNA and the antimicrobial peptide, LL37 [62].

The pathogenic involvement of NETs in Lupus is not limited to their ability to externalize autoantigens. NETosis has been linked with skin, kidney as well as premature vascular damage in Lupus [225]. NETs have been observed in affected kidney and skin biopsies of Lupus patients [225].

4.3.2 How the body responds to auto antigen exposure

Type 1 IFN dysregulation plays a central role in the initiation and perpetuation of autoimmunity in Lupus [226]. pDCs are the primary IFNα producing cells in Lupus [227]. Endosomal TLR signaling is a pathway through which Type 1 IFN production is induced [227]. Accordingly, it can be expected that any mechanism leading to activation of these receptors will undoubtedly lead to an IFNα response. Accordingly, we investigated if NETs acting through endosomal TLRs in PBMCs could generate an IFN-response that in turn could activate IFNAR- mediated signaling in pDCs. As expected, protein levels of phosphorylated STAT1 in control PBMCs was significantly increased (i.e. activation of JAK-STAT signaling pathway) on exposure to supernatant from NET stimulated PBMCs. As this response is consistent with IFNα mediated signaling it is suggestive of the likely presence of IFNα in supernatant of NET stimulated PBMCs. Therefore, chronic IFNα production owing to persistent activation of pDCs could in large be explained by dysregulated NETosis. Consistent with these findings, the ability of these NETs to drive pDCs to produce IFNα has been demonstrated in both adult and pediatric SLE [78, 151]. Notably, both studies also reported the ability of IFNα to prime neutrophils to release more NETs [78, 151]. In support, we demonstrated the ability of the supernatant from NET-stimulated PBMCs to induce activation of the JAK-STAT signaling pathway of control PBMCs and stimulate JSLE neutrophil to release NETs. Both theses responses were absent when the supernatant was

added to control PBMCs and JSLE neutrophils pre-treated with a JAK inhibitor, suggesting that the responses were likely induced by the interferogenic environment of the NET-induced PBMC supernant. (**Figure 24.E**).

Collectively, these finding strengthen the evidence base for the pathogenic role of NETs in Lupus. The presence of a self-amplifying cycle is evident: Lupus neutrophils readily release more NETs; these NETs can potently induce activation of the immune system to produce IFNα which can in turn prime neutrophils to generate further NETs. This self-perpetuating loop of NETosis and IFNα production is likely to work in combination with other auto-inflammatory effects of IFNα leading to the initiation and propagation of disease in Lupus.

Chronic IFNα production is largely attributed to chronic pDC activation in SLE [78]. Regardless of the processes leading to unabated IFNα production, this cytokine in its excess could largely account for the immune dysregulation and resultant loss of self-tolerance observed in Lupus [226]. Elevated IFNα is a likely mediator of Lupus pathology, as development of SLE following IFNα treatment is a widely reported phenomenon [183]. In majority of the cases, symptoms subsided on discontinuation of IFNα treatment, suggestive of a direct causal link between IFN therapy and onset of Lupus [228, 229]. Such autoimmune occurrences have been reported in patients receiving IFNα treatment for other conditions including malignant carcinoid tumor, chronic myelogenous leukemia, juvenile laryngeal papillomatosis and Hepatitis C [228-232]. IFNα therapy has also been reported to exacerbate pre-existing subclinical autoimmune disease [233]. Initiation of pro-autoimmune effects by IFNα is not limited to Lupus. In genetically susceptibility individuals, development of autoimmune thyroid disease has been linked to IFNα therapy [234]. Moreover, in Lupus prone mice lacking IFNα/β Receptor, Lupus-like disease was significantly decreased [235]. This body of evidence implicates a causative role of IFNα as a mediator of autoimmunity in Lupus.

Significantly raised Type 1 IFN titers correlating with disease activity is a common feature of adult SLE [152, 153]. Raised IFNα levels was reported to correlate with higher prevalence of renal involvement, higher number of ACR criteria and SLEDAI score, raised dsDNA titers and erythrocytes sedimentation rate as well as occurrence of autoantibodies against dsDNA, Ro, Sm, and U1 RNP [236]. Dysregulated gene expression of IFN pathway components has been reported in Lupus patients [154]. A large proportion of IFNα regulated genes were shown to be significantly unregulated in peripheral blood mononuclear cells (IFNα signature) of adult SLE and JSLE patients with active disease, thereby reinforcing the critical role of this cytokine in Lupus pathogenesis [154, 155]. In adult SLE, this IFNα signature was found to be a marker for severe Lupus, with levels correlating with disease severity with regards to kidney and/or central nervous system involvement [154]. High serum IFNα activity was shown to be frequently found in both the Lupus patient as well as in their healthy first degree relatives [237]. This implies a genetic basis, and high serum IFNα activity to potentially be a heritable risk factor is Lupus [237]. Genes involved in IFNα transcription and or signaling pathways have been implicated in Lupus pathogenesis, including *IRF5, TYK2, STAT4, IRAK and JAK2* [238-240].

As previously mentioned, an abnormal neutrophil subset (referred to LDGs) has been identified in the peripheral blood of Lupus patients [155, 241]. Microarray analysis revealed increased expresison of neutrophil-specific genes in PBMC fractions from pediatric Lupus patients, commonly referred to as the 'granulopoiesis signature' [155]. This signature has been attributed to the presence of immature neutrophils (granulocytes) in the PBMC fractions of these patients [155]. These low density neutrophils, are distinct from normal-density neutrophil, in that they are immature and also demonstrate distinctive functional features [215]. LDGs show a pro inflammatory phenotype with impaired phagocytic abilities and have been proposed to play a critical role in Lupus pathogenesis through their

increased capacity to release NETs and raised production of Type 1 IFNs and other pro inflammatory cytokines (TNFα) [156, 215, 225]. Accordingly, in Lupus, LDGs may be accountable for the increased externalization of NET auto antigens (dsDNA and LL37) and pro inflammatory cytokines (IL-17) through their increased ability to generate more NETs [225]. Notably, several bactericidal proteins and serine proteases originating from azurophilic granules implicated in NETosis were found to be upregulated in Lupus LDGs [225]. Although the functional implications of this up regulation is not fully understood, increased expression of MPO and elastase, which regulate NETosis [82], could enhance LDG NET release. LDGs have been reported to promote endothelial cytotoxicity [156]. Investigation of the immunogenic potential of NETs released through LDGs compared to normal density neutrophils could be an interesting prospect to add the existing pathogenic model of LDG in SLE.

Biological effects of type 1 IFNs are mediated primarily through the JAK-STAT signaling pathway, which relies upon the signaling molecule Janus Kinase 1 (JAK1) for signal transduction [242]. JAK inhibitors are an emerging class of drugs being developed to block the cytokines signaling through the JAK –STAT pathway [242]. As a proof-of-concept study, the effect of JAK inhibition on the ability of the supernatant from NET-stimulated PBMCs to induce phosphorylation of JAK-STAT signaling pathway molecules (STAT1) was explored. As expected, JAK inhibition effectively blocked phosphorylation of STAT1 in control PBMCs exposed to supernatant of NET stimulated PBMCs (**Figure 23**). This reduction in phosphorylation suggests the possible interferogenic environment of the supernatant of NET-stimulated PBMCs.

These findings add to the existing literature regarding role of NET-pDC interaction in Lupus pathogenesis, by promoting the production of interferon molecules. Furthermore through NETosis, neutrophils may play a large role in producing large quantities of IFNα.

4.3.3 Other sources of auto-antigens

Dysregulated apoptosis was considered the primary source of modified auto antigens until the discovery of NETs [139]. Similar to NETs, aberrant apoptosis coupled with defective clearance of apoptotic debris is reported in SLE patients [243]. This was thought to result in persistence of apoptotic cells, which subsequently undergo secondary necrosis to generate apoptotic blebs, containing chromatin, which had undergone apoptosis -mediated modifications, eventually resulting in autoantigen exposure [145, 243]. In SLE, the exact association and contribution of dysregulated NETosis and apoptosis is unknown and likely to vary between patients. However, is has been postulated that disturbances of both these cell death process are likely to interact with each other and act in concert to induce self-perpetuating cycles leading to initiation and propagation of autoimmunity in SLE [139].

4.3.4 Therapeutic implication of findings

Endosomal TLRs 7 and 9, expressed by both pDCs and =B-cells, have been implicated in disease pathogenesis of SLE [226]. In murine models, Lupus prone mice deficient in TLR7 or TLR9 failed to generate antibodies towards DNA and RNA-containing antigens, respectively [188]. Consistent with the role of endosomal TLRs in Lupus, TLRs 3, 7 and 9 were up regulated in PBMCs fractions of JSLE patients [214]. *IRAK1*, *IRF5* and *TNFAIP3* are Lupus-associated genes which are involved in TLR signaling, which implies a genetic basis for TLRs in prompting disease pathology in Lupus [244]. As the most potent producers of Type1 IFN in Lupus, pDCs can be activated by immune complexes containing self-DNA and Self-RNA in a TLR9 and TLR7-depdent manner, to produce very high levels of IFNα in SLE [227]. Accordingly, blocking these two receptors could be a particularly promising therapeutic target in preventing initiation of IFNα regulated autoimmune response in Lupus. This has led to the development of a range of drugs aimed at preventing activation, signaling and downstream effects of these receptors [244].

HCQ is an anti-malarial drug that is widely being used for the treatment of SLE [196]. In Lupus, HCQ is reported to improve overall survival, disease-free survival, damage accrual and also reduce the risk and severity of clinical flares, delay the onset of SLE, protects against renal damage and early use found to increase these therapeutic benefits [197, 245]. HCQ is the one of the common treatments for cutaneous Lupus, with more than half of the patients responding to treatment with HCQ alone [246]. It is thought to function by either inhibiting endosomal acidification by raising intracellular PH, a condition which is pre-requisite for the activation of endosomal TLRs or by directly binding to nucleic acids and masking their TLR-binding epitopes to exert TLR antagonism [198-201]. Sacre et al reported that HCQ treatment lead to impaired generation of IFNα and TNF-α by adult SLE pDCs in response to TLR-7/9 stimulation, highlighting a mechanistic pathway for their beneficial role in Lupus [247]. Notably, lower concentrations of HCQ in blood of Lupus patients was found to correlate with disease activity and served as a predictor of disease exacerbations [248]. The concentration of HCQ used in this study to effectively block NET-induced TLR activation (51.5 ng/ml) was in the range of the plasma HCQ concentrations found in RA patients (69 – 518 ng/ml) [202] and therefore could potentially be an indication that the therapeutic efficacy of this drug could, at least in part, be attributed to their ability to inhibit NET-signaling. The benefits of this drug extend beyond their Lupus-related effects and are reported to be associated with a reduction in serum cholesterol and blood glucose levels and confer protection against diabetes and thrombotic occurrences [245, 249].

Barrat et al developed oligonucleotide-based dual inhibitor of TLR7 and TLR9, which was observed to inhibit IFNα production by pDCs in response to both DNA and RNA containing immune complexes, which are known activators of these receptors in Lupus [143, 244, 250]. The efficacy of this dual TLR7/9 antagonist was replicated in murine Lupus model, where in this presence of this inhibitory oligonucleotide (ODN), autoantibody production

and overall mortality was reduced in Lupus-prone mice [143, 244]. In order to achieve an optimal therapeutic agent, blockade of both receptors is a pre-requisite to interfere with all DNA and RNA associated stimuli as inhibition of only TLR9 would still leave TLR 7 functional permitting signaling through that receptor [143].

Effective treatment of Lupus often necessitates aggressive approaches with the use of high dose glucocorticoids, which is often associated with debilitating side effects including but not limited to hypertension, obesity and osteoporosis [251, 252]. These drugs exert anti-inflammatory effects through inhibition of NF-κB [251]. It has been theorized that their anti-inflammatory effects are being offset by unknown pro inflammatory responses occurring in SLE [252]. Guiducci et al reported that signaling through TLR7 and TLR9 in pDCs accounts for reduced activity of glucocorticoids, and could be counteracted by dual inhibitors of TLR7 and TLR9, such as immunoregulatory DNA sequences (IRS) 954[250] that restore glucocorticoid sensitivity to pDCs in blocking IFN production [251]. Currently achieved through a combination of HCQ and immunosuppressants, these dual TLR7/9 antagonists could serve an alternate steroid-sparing drug, allowing minimization of dosage without impairing their anti-inflammatory therapeutic effects [252]. This could be particularly beneficial in pediatric patients to avoid glucocorticoid related side effects on their development [252]. These findings add further evidence to the role of TLRs in Lupus pathogenesis, and support the rationale for developing TLR7/9-targeted treatments.

Therapeutic approaches targeting the end-product effectors of TLR signaling, IFNα in particular, has gained attention over recent years. A variety of cytokines are considered key mediators of many autoimmune conditions and use JAK-STAT signaling pathway to exert their effects [242]. The critical function of JAK in this pathway has made them ideal targets for therapeutic interventions to prevent signaling of such cytokines and their pro autoimmune effects [242]. Several JAK inhibitors (Jakinibs) are currently in clinical use,

including Tofacitinib, an approved treatment for RA [242]. Although not yet fully investigated, several studies have suggested the potential efficacy of Jakinibs in Lupus [242, 253, 254]. In Lupus prone mice, treatment with selective JAK2 inhibitor was associated with reduction in proteinuria and serum levels of anti-dsDNA as well as improvement in renal function [253]. Several JAK-STAT pathway related genes, including *JAK1 and JAK2* are up regulated in active SLE [254]. It is conceivable that JAK inhibitors could therefore serve a logical therapeutic option in SLE, given that the disease pathology is considered to be driven by IFNα responses. In support, we show that blocking the signaling of Type 1 IFNs (generated through NET signaling) with a JAK inhibitor (specific for JAK1 at the concentrations used in this study) not only reduced their ability to phosphorylate STAT1 in control PBMCs (**Figure 23**) but also prevented their ability to induce neutrophils to release NETs (**Figure 24.E**). This study provides compelling evidence for the potential beneficial role of JAK inhibitors in SLE with regards to NET-induced IFN-responses.

A lot remains to be understood about these structures, including the therapeutic implication of NET-targeted therapies in diseases like SLE. Although investigation into their therapeutic potential is still in its infancy, several studies have reported the potential benefits associated with NET-targeting treatments [157]. ROS scavengers have been shown to be effective at decreasing NET release, which could be a potential avenue to be explored especially in chronic inflammatory conditions where NET suppression could be beneficial [157]. In cystic fibrosis, not only are NETs ineffective against microbial elimination but also the accumulation of DNA and proteases released by these structures are thought to further compound the chronic airway inflammation by worsening tissue damage and thickening the mucus layer [157]. Aerosolized recombinant human DNase (rhDNase), is a current treatment option for patients with mild CF, which functions by degrading DNA in order to decrease the viscosity of mucous [157]. Anti-histone and anti-protease antibodies are

among a few other NET-targeted treatments used in inflammatory diseases [157]. However, one of the main obstacles with developing NET targeted therapies is the possibility of partial immunodeficiency in its absence, particularly with regards to fungal and protozoal defenses [221].

The limited NET-targeted treatment options are largely reflective of the lack of understanding regarding their underlying mechanisms. Thus, research into their biology will be greatly beneficial. The data in this present study adds to the existing evidence base of NET in the pathogenesis of Lupus, and strengthens the rationale for the development of novel therapies that specifically target NET mediated signaling pathways in pediatric SLE.

4.4 Study Limitation

Our study achieved its original aims, although there were several limitations, which require acknowledgement. In a given healthy peripheral blood sample, pDCs constitute approximately less that 1% of PBMC cell subset populations [175]. This rarity makes it difficult for this cell subset to be studied in large numbers. This experimental limitation meant that PBMC populations containing other cell subsets had to be used for the experiments conducted this study, although investigating the effects of NET stimulation on pDC population alone would have been ideal. Owing to time restrictions, NET-induced IFNα production could not be quantified. Although supernatant from NET stimulated PBMCs was able to induce responses consistent with IFNα mediated signaling when exposed to other PBMCs and neutrophils, we must be cautious in assuming that these response were solely due to IFNα and that it does remain a possibility that NET-induced pDC activation could have led to other cytokine production which could also have contributed to these responses. Lupus patients are largely neutropenic [255]. This restriction together with sample limitations meant generating NETs in large quantities was frequently a difficult task. NETs were found to stimulate PBMCs in a dose-dependent manner, which meant that a

greater stimulation could have been obtained if more samples could have been used. Although we were able to demonstrate NET-induced increased protein levels of phosphorylated IRF3, owing to time restrictions, the proof-of-concept study investigating the ability of HCQ to effectively block NET-mediated TLR3 activation could not be optimized and remains an important experiment, which warrants further investigation.

4.5 Study Strength

These findings add to the growing body of literature and strengthen the evidence base regarding the pathogenic role of NETs in Lupus by autoantigen exposure. It provides a mechanism through which autoantigenic NETs could be getting detected and contributing to the initiation and propagation of autoimmunity, by setting into motion a self-amplifying cycle with regards to IFNα production. It provides novel information regarding the therapeutic success of HCQ in Lupus, potentially by blocking NET-signaling through endosomal TLR pathway, preventing activation of auto inflammatory responses that are likely occurring as a result of NET induced activation of the immune system. With majority of studies exploring NET –pDC interactions implicating TLR7/9 pathways, we demonstrated insight into the additional involvement of TLR3 in detecting nuclear autoantigens provided by NETs. Accordingly, with TLR targeted therapies primarily focusing on TLR7 and TLR9 antagonism, targeting TLR3 may represent a promising avenue for the future. With JAK inhibitors emerging as promising therapeutic options in many interferon-centred autoimmune conditions, we have also shown that JAK inhibitor was successful in blocking responses of IFNα generated through NET-induced signalling, thereby providing compelling evidence for JAK inhibitors in SLE both with regards to NETs and IFNα. The findings of this study add to the limited data concerning the pathological role of NETs in JSLE. Furthermore, shows NET formation in healthy control children and therefore gives some insight into the induction of NETs in a developing immune system where knowledge is

limited. Finally, it supports the rationale behind potential benefits of developing targeted treatments aimed at NET suppression.

4.6 Future directions

Taking into account the limitations of this study it raises many new potential avenues to be explored for future research. First and foremost, the validity of all results obtained in this study could be further enhanced to give more statistical significance by increasing sample number. From our experiments the source of the neutrophils generating the NETs was not a determining factor in activation of immune cells. This raises the possibility that it is not the source of the NETs but the origins of the immune cells, which get activated by these NETs, which determines degree of stimulation. Therefore it may be useful to investigate the degree of activation of PBMCs from JSLE and pediatric control patients by NETs from the same neutrophil source. This would be an interesting research direction to see if JSLE PBMCs are more responsive to immunogenic NETs compared to control PBMCs, considering the up regulation of TLRs 3, 7 and 9 reported in PBMCs from JSLE patients [214]. The source of neutrophils in producing more immune-stimulatory NETs could likely still apply with regards to LDG. This distinct neutrophil subset, typically separating into PBMC populations is shown to release more NETs [215]. Distinct from neutrophils investigated in this study, LDG could be the neutrophil subtype that does produce more immunogenic NETs. So, investigating if LDG derived NETs are more immunostimulatory compared to both normal density JSLE and control neutrophils derived NETs is an interesting research direction to be explored. Also, as HCQ inhibited NET signaling through TLRs, if this inhibition prevents production of Type 1 IFN could be a potential experiment in the future.

Although PBMCs were stimulated with the same concentration of JSLE sera-induced NET DNA, there was a great deal of variation between degree of phosphorylation of both IRAK1

and IRF3. This could be due to the involvement of confounding factors, which could have independently affected the degree of immune activation observed. One such example would be the JSLE sera that were used to stimulate NET release. JSLE sera from different patients with varying disease activity and drug therapies were used; therefore the immune-stimulatory constituents of the sera could have largely varied between Sera samples, indirectly affecting the stimulation observed. Therefore removal of such confounding factors by standardizing the JSLE sera for disease activity and drug therapies could be a potential avenue to be explored.

Although we were able to show increased phosphorylation of IRF3 in PBMCs exposed to NETs we could not however conclude that it was a TLR3 dependent stimulation as the ability of HCQ to block TLR3 activation could not be optimized. As HCQ was not able to inhibit TLR3 activation when incubated for the same length of time that successfully inhibited TLR7/9 singling, a potential direction would be to investigate HCQ incubation for different time points and at higher concentrations as a combination of higher concentration and longer incubation period could be required for HCQ to inhibit TLR 3 signaling. Optimization would allow confirmation that the NETs also act through a TLR3-dependent pathway.

Another potential direction would be to quantify the amount of IFNα released following NET induced activation and investigating the possibility of a dose response relationship between NET DNA content and IFN concentrations. Lastly, although the focus of this study was extracellular traps generated from neutrophils, other cell subset (masT-cell and macrophages) also known to produce extracellular traps [62, 69]. Therefore investigating the possibility of these additional sources of extracellular traps in producing autoantigens would be greatly beneficial to further understanding Lupus pathology.

4.7 Conclusion

Although protective in nature, NETosis, when dysregulated, can have severe consequences with large autoimmune potential owing to the high immunogenicity of these structures. With many gaps in the understanding of the biology of NETs, these structures are increasingly being investigated for their role in inducing activation of immune responses in SLE through autoantigen exposure.

To conclude, this study has demonstrated IFNα and JSLE serum-derived NETs may be an important source of auto antigens in JSLE that are being detected through endosomal TLRs 3, 7 and 9 leading to activation of these receptors. However, the origin of the NETs was not a determining factor in immune activation. The inhibition of TLR 7 and 9 activation using HCQ significantly reduced the ability of NETs to stimulate these receptors indicating that NETs activate the immune system through these endosomal receptors. Exposure of supernatant of NET-stimulated PBMCs lead to significant increase in phosphorylation of STAT1 in controls PBMCs, which was inhibited in the presence of a JAK inhibitor. Lastly, JSLE neutrophils released NETs following incubation with supernatant from control PBMCs stimulated with JSLE sera induced NETs.

This study has provided a mechanistic pathway for the immune-stimulatory effects mediated by NETs, through autoantigen exposure. Furthermore, it highlights a possible mechanism for the therapeutic efficacy of HCQ observed in Lupus patients. These findings not only add to the limited data concerning the pathological role of NETs in JSLE, but also strengthen the rationale for the development of novel therapies that specifically target NET signaling pathways in Lupus, thereby contributing towards improved therapeutic outcomes.

Supplementary Material

Optimization of blocking agents

A variety of blocking agents ranging from non-fat dried milk to highly purified proteins are commercially available. The inadequate use of blocking agent will produce a blot with excessively high background and the excessive use of blocking agent may conceal antibody-antigen interaction or interfere with detection reagents. Thus, it is of utmost importance to obtain the ideal blocking agent which possesses ability to bind to all sites of possible non-specific interaction, without changing or masking the epitope for antibody binding, thus giving a blot with strong specific signal with minimal background. Initially, 5% BSA diluted in 1×TBS-T was used as the blocking agent but too many non-specific bands were generated. Ideal blocking agents was found to be 5% dry milk powder diluted in 1×TBS-T.

Optimization of Primary and Secondary antibodies

Optimization of the primary (1˚ Ab) and secondary antibodies (2˚ Ab) was carried out to determine the optimal dilution of antibody required to obtain best results. Primary and secondary antibodies were tested at various dilutions that were within the manufacturers recommended reference range. Commercially obtained primary and secondary antibodies were used. anti-IRAK1 (phospho T209) antibody, a rabbit polyclonal that detects IRAK1 only when phosphorylated at threonine 209 was initially used (**Supplementary Table 1**). The choice of secondary antibody was based on species in which the primary antibody was raised. Accordingly, as primary antibody used is a rabbit antibody, a HRP-conjugated anti-Rabbit Antibody raised in was used (**Supplementary Table 2**).

Primary Antibody	Antigen	Species	Range of Dilutions tested for 1°	Company, Catalogue Number
Anti-IRAK1 (phospho T209) Antibody	Detects IRAK1 only when phosphorylated at threonine 209	Rabbit	1/250-1/1000	Abcam, ab61799
Anti-IRAK1 (phospho T387) Antibody	Detects IRAK1 only when phosphorylated at threonine 387	Rabbit	1/500	Abcam, ab139739

Supplementary Table 1: Primary Antibodies used for the detection pIRAK1 protein levels.

Two different primary antibodies were tested for the detection of phosphorylated IRAK1: Anti-IRAK1 (phospho T209) Antibody and Anti-IRAK1 (phospho T387) Antibody.

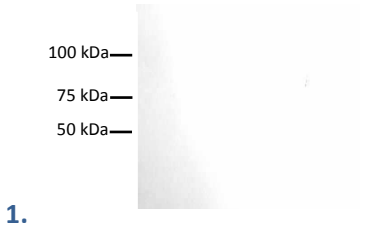
Primary and secondary antibodies were tested at various dilutions (**Supplementary Table 1**) to obtain optimal signal (**Supplementary Figure 1**). Antibody was diluted in blocking buffer and tested at dilutions ranging from 1/250- 1/1000. An initial dilution of 1/1000 of anti-IRAK (phospho T209) antibody and 1/1000 dilution of anti-rabbit antibody was tested. These dilutions did not generate any results and produced an empty blot. Thereafter, experiment was repeated with primary antibody dilution of 1/500, in order to increase its concentration. Secondary antibody concentration was unchanged. Despite producing a signal, the blot had very high background. Thus, in order to reduce background noise western blot was repeated with secondary antibody concentration reduced to 1/3000, and primary antibody concentration remaining unchanged. This generated a blot with ideal bands with clear background. A further experiment was carried out with primary antibody dilution of 1/250 and secondary antibody dilution of 1/3000; however, this is generated excessive amount non-specific bands. Thus, primary antibody dilution of 1/500 was

deemed to be ideal to generate optimal signal without non-specific binding and minimal background noise. An optimal blot was achieved by using a 1/500 primary antibody dilution and a 1/3000 dilution of secondary antibody.

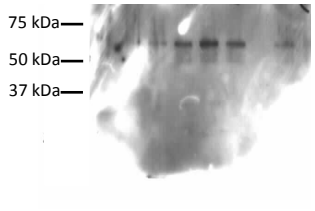
Primary Antibody	Secondary Antibody	Range of Dilutions tested for 1˚ Ab	Range of Dilutions tested for 2˚ Ab	Predicted Molecular weight	Observed Molecular weight
Anti-IRAK (phospho T209) Antibody	ANTI-Rabbit Antibody	1/250-1/1000	1/1000-1/3000	77kDa	77kDa
Anti-IRAK (phospho T387) Antibody	Anti-Rabbit Antibody	1-500	1/3000	76kDa	74kDa

Supplementary Table 2: Primary antibodies tested for the optimization of pIRAK1 detection.

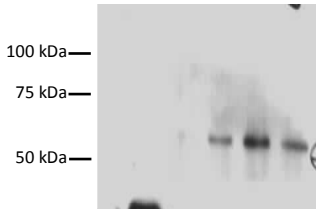
The different primary antibodies tested for the detection of pIRAK1: Anti-IRAK (phospho T209) Antibody and Anti-IRAK (phospho T387) Antibody.



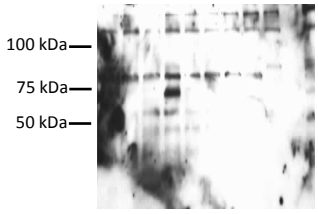
1.



2.



3.



4.

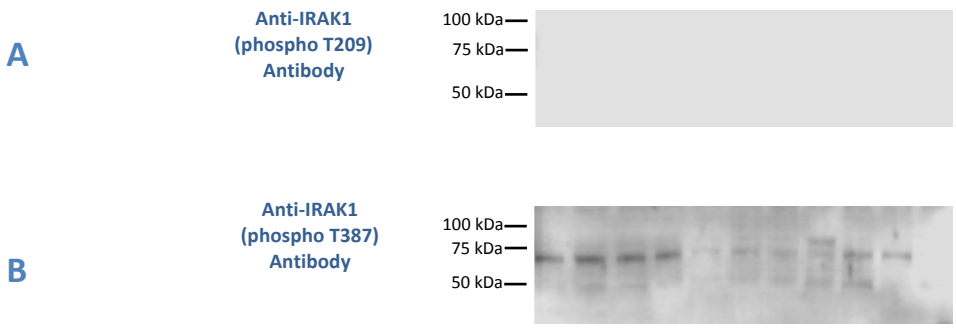
	1.	2.	3.	4.
Anti-pIRAK1 Ab (Dilution)	1/1000	1/500	1/500	1/250
Anti-Rabbit Ab (Dilution)	1/1000	1/1000	1/3000	1/3000
Exposure time	30 minutes	30 minutes	30 minutes	30 minutes

Supplementary Figure 1: Optimization of the primary and secondary antibodies used in the detection of pIRAK1.

Stages of optimization of the primary antibody **(**Anti-IRAK (phospho T209) antibody) and secondary antibody (Anti-Rabbit antibody) to detect phosphorylated IRAK. The exposure time for all experiments was 30 minutes. (1) Primary antibody dilution of 1/1000 and secondary antibody dilution of 1/1000 (2) Primary antibody dilution of 1/500 and secondary antibody dilution of 1/1000 (3) primary antibody dilution of 1/500 and secondary antibody dilution of 1/3000 (4) primary antibody dilution of 1/250 and secondary antibody dilution of 1/3000.

Optimization of Assay

Due to inconsistencies in the results with anti-IRAK (phospho T209) antibody, an alternate antibody, namely anti-IRAK (phospho T387) antibody (**Table 1 and 2**), was tested. Anti-IRAK (phospho T387) antibody binds to an alternate phosphorylation site, that is, a different epitope of the same protein. Where anti-IRAK (phospho T209) antibody only detects IRAK1 only when phosphorylated at threonine 209, anti-IRAK (phospho T387) antibody detects IRAK1 when phosphorylated at threonine 387. A blot initially probed using the first antibody, was re-probed using the new antibody. This antibody detected protein levels of pIRAK1 at its expected level of 74kDa (**Figure 2**).



Supplementary Figure 2: Optimization of the primary antibody used in the detection of pIRAK1.

Two different primary antibodies were tested for the detection of pIRAK: Anti-IRAK (phospho T209) Antibody and Anti-IRAK (phospho T387) Antibody. (**A**) No bands detected when probed with Anti-IRAK1 (Phospho T209) antibody (**B**) bands detected when same western membrane was re-probed with Anti-IRAK (phospho T387) antibody.

References

1. Mak, T.W., *The immune response: Basic and Clinical Principles*. 2005: Elsevier Academic Press.
2. Parham, P., *The Immune System*. 2009: Garland Science.
3. Elgert, K.D., *Immunology: Understanding The Immune System*. 2009: Wiley-Blackwell.
4. Chaplin, D.D., *Overview of the immune response.* J Allergy Clin Immunol, 2010. **125**(2 Suppl 2): p. S3-23.
5. Murphy, K.M., *Janeway's Immunobiology*. Garland Science, Taylor and Francis Group.
6. Goldman, L., *Goldman's Cecil Medicine*. 2012: Elsevier Heath Sciences.
7. Mogensen, T.H., *Pathogen recognition and inflammatory signaling in innate immune defenses.* Clin Microbiol Rev, 2009. **22**(2): p. 240-73, Table of Contents.
8. Medzhitov, R., *Toll-like receptors and innate immunity.* Nat Rev Immunol, 2001. **1**(2): p. 135-45.
9. Kumar, H., T. Kawai, and S. Akira, *Pathogen recognition by the innate immune system.* Int Rev Immunol, 2011. **30**(1): p. 16-34.
10. Medzhitov, R., P. Preston-Hurlburt, and C.A. Janeway, *A human homologue of the Drosophila Toll protein signals activation of adaptive immunity.* Nature, 1997. **388**(6640): p. 394-397.
11. Gay, N.J. and F.J. Keith, *Drosophila Toll and IL-1 receptor.* Nature, 1991. **351**(6325): p. 355-6.
12. Rock, F.L., et al., *A family of human receptors structurally related to Drosophila Toll.* Proc Natl Acad Sci U S A, 1998. **95**(2): p. 588-93.
13. Belvin, M.P. and K.V. Anderson, *A conserved signaling pathway: the Drosophila toll-dorsal pathway.* Annu Rev Cell Dev Biol, 1996. **12**: p. 393-416.
14. Lemaitre, B., et al., *The Dorsoventral Regulatory Gene Cassette spätzle/Toll/cactus Controls the Potent Antifungal Response in Drosophila Adults.* Cell, 1996. **86**(6): p. 973-983.
15. Vasselon, T. and P.A. Detmers, *Toll receptors: a central element in innate immune responses.* Infect Immun, 2002. **70**(3): p. 1033-41.
16. Chuang, T. and R.J. Ulevitch, *Identification of hTLR10: a novel human Toll-like receptor preferentially expressed in immune cells.* Biochim Biophys Acta, 2001. **1518**(1-2): p. 157-61.
17. Takeuchi, O., et al., *TLR6: A novel member of an expanding toll-like receptor family.* Gene, 1999. **231**(1-2): p. 59-65.
18. Du, X., et al., *Three novel mammalian toll-like receptors: gene structure, expression, and evolution.* Eur Cytokine Netw, 2000. **11**(3): p. 362-71.
19. Kawai, T. and S. Akira, *The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors.* Nat Immunol, 2010. **11**(5): p. 373-384.

20. Akira, S. and K. Takeda, *Toll-like receptor signalling.* Nat Rev Immunol, 2004. **4**(7): p. 499-511.

21. McGettrick, A.F. and L.A. O'Neill, *Localisation and trafficking of Toll-like receptors: an important mode of regulation.* Curr Opin Immunol, 2010. **22**(1): p. 20-7.

22. Takeda, K. and S. Akira, *Toll-like receptors in innate immunity.* Int Immunol, 2005. **17**(1): p. 1-14.

23. Williams, A.E., *Immunology : Mucosal and Body Surface Defenses*. 2012: Wiley-Blackwell.

24. Kawai, T. and S. Akira, *Toll-like receptors and their crosstalk with other innate receptors in infection and immunity.* Immunity, 2011. **34**(5): p. 637-50.

25. Blasius, A.L. and B. Beutler, *Intracellular toll-like receptors.* Immunity, 2010. **32**(3): p. 305-15.

26. Hacker, H., et al., *CpG-DNA-specific activation of antigen-presenting cells requires stress kinase activity and is preceded by non-specific endocytosis and endosomal maturation.* Embo j, 1998. **17**(21): p. 6230-40.

27. Kaisho, T. and S. Akira, *Toll-like receptor function and signaling.* J Allergy Clin Immunol, 2006. **117**(5): p. 979-87; quiz 988.

28. Kawai, T. and S. Akira, *Innate immune recognition of viral infection.* Nat Immunol, 2006. **7**(2): p. 131-137.

29. Konat, G.W., *Signaling by Toll-Like Receptors*. 2008: Taylor & Francis Inc.

30. Barton, G.M. and J.C. Kagan, *A cell biological view of Toll-like receptor function: regulation through compartmentalization.* Nat Rev Immunol, 2009. **9**(8): p. 535-42.

31. Shrivastav, M. and T.B. Niewold, *Nucleic Acid sensors and type I interferon production in systemic lupus erythematosus.* Front Immunol, 2013. **4**: p. 319.

32. O'Neill, L.A.J., D. Golenbock, and A.G. Bowie, *The history of Toll-like receptors [mdash] redefining innate immunity.* Nat Rev Immunol, 2013. **13**(6): p. 453-460.

33. Kawai, T., et al., *Unresponsiveness of MyD88-Deficient Mice to Endotoxin.* Immunity, 1999. **11**(1): p. 115-122.

34. Suzuki, N., S. Suzuki, and W.C. Yeh, *IRAK-4 as the central TIR signaling mediator in innate immunity.* Trends Immunol, 2002. **23**(10): p. 503-6.

35. Kollewe, C., et al., *Sequential autophosphorylation steps in the interleukin-1 receptor-associated kinase-1 regulate its availability as an adapter in interleukin-1 signaling.* J Biol Chem, 2004. **279**(7): p. 5227-36.

36. Rich, T., *Toll and Toll-Like Receptors:: An Immunologic Perspective*. 2005: Kluwer Academic/Plenum Publishers.

37. Kawai, T., et al., *Interferon-alpha induction through Toll-like receptors involves a direct interaction of IRF7 with MyD88 and TRAF6.* Nat Immunol, 2004. **5**(10): p. 1061-8.

38. Uematsu, S., et al., *Interleukin-1 receptor-associated kinase-1 plays an essential role for Toll-like receptor (TLR)7- and TLR9-mediated interferon-{alpha} induction.* J Exp Med, 2005. **201**(6): p. 915-23.

39. Uematsu, S. and S. Akira, *Toll-like receptors and Type I interferons.* J Biol Chem, 2007. **282**(21): p. 15319-23.

40. Kawai, T. and S. Akira, *TLR signaling.* Cell Death Differ, 2006. **13**(5): p. 816-25.

41. Noppert, S.J., K.A. Fitzgerald, and P.J. Hertzog, *The role of type I interferons in TLR responses.* Immunol Cell Biol, 2007. **85**(6): p. 446-457.

42. Mesa, M.A. and G. Vasquez, *NETosis.* Autoimmune Diseases, 2013. **2013**: p. 7.

43. Németh, T. and A. Mócsai, *The role of neutrophils in autoimmune diseases.* Immunology Letters, 2012. **143**(1): p. 9-19.

44. Brinkmann, V. and A. Zychlinsky, *Beneficial suicide: why neutrophils die to make NETs.* Nat Rev Microbiol, 2007. **5**(8): p. 577-82.

45. Brown, G.D., *Immunology of Fungal Infections*. 2007: Springer.

46. Schatten, G., *Current Topics in Developmental Biology*. Vol. 74. 2006: Elsevier.

47. Mayer-Scholl, A., P. Averhoff, and A. Zychlinsky, *How do neutrophils and pathogens interact?* Curr Opin Microbiol, 2004. **7**(1): p. 62-6.

48. Edwards, S.W., *Biochemistry and Physiology of the Neutrophil*. 1994: Cambridge University Press.

49. Summers, C., et al., *Neutrophil kinetics in health and disease.* Trends Immunol, 2010. **31**(8): p. 318-24.

50. Papayannopoulos, V. and A. Zychlinsky, *NETs: a new strategy for using old weapons.* Trends in Immunology, 2009. **30**(11): p. 513-521.

51. Knight, G., *Haematology*. 2010: Oxford University Press.

52. Pham, C.T.N., *Neutrophil serine proteases: specific regulators of inflammation.* Nat Rev Immunol, 2006. **6**(7): p. 541-550.

53. Mantovani, A., et al., *Neutrophils in the activation and regulation of innate and adaptive immunity.* Nat Rev Immunol, 2011. **11**(8): p. 519-531.

54. Kobayashi, S.D., et al., *Neutrophils in the innate immune response.* Arch Immunol Ther Exp (Warsz), 2005. **53**(6): p. 505-17.

55. Kolaczkowska, E. and P. Kubes, *Neutrophil recruitment and function in health and inflammation.* Nat Rev Immunol, 2013. **13**(3): p. 159-175.

56. Brinkmann, V., *Neutrophil extracellular traps kill bacteria.* Science, 2004. **303**: p. 1532-1535.

57. Fuchs, T.A., et al., *Novel cell death program leads to neutrophil extracellular traps.* J Cell Biol, 2007. **176**(2): p. 231-41.

58. Steinberg, B.E. and S. Grinstein, *Unconventional roles of the NADPH oxidase: signaling, ion homeostasis, and cell death.* Sci STKE, 2007. **2007**(379): p. pe11.

59. Remijsen, Q., et al., *Dying for a cause: NETosis, mechanisms behind an antimicrobial cell death modality.* Cell Death Differ, 2011. **18**(4): p. 581-8.

60. Yousefi, S., et al., *Catapult-like release of mitochondrial DNA by eosinophils contributes to antibacterial defense.* Nat Med, 2008. **14**(9): p. 949-53.

61. von Kockritz-Blickwede, M., et al., *Phagocytosis-independent antimicrobial activity of mast cells by means of extracellular trap formation.* Blood, 2008. **111**(6): p. 3070-80.

62. Wartha, F. and B. Henriques-Normark, *ETosis: a novel cell death pathway.* Sci Signal, 2008. **1**(21): p. pe25.

63. Wartha, F., et al., *Neutrophil extracellular traps: casting the NET over pathogenesis.* Curr Opin Microbiol, 2007. **10**(1): p. 52-6.

64. Kaplan, M.J. and M. Radic, *Neutrophil extracellular traps: double-edged swords of innate immunity.* J Immunol, 2012. **189**(6): p. 2689-95.

65. Urban, C.F., et al., *Neutrophil extracellular traps contain calprotectin, a cytosolic protein complex involved in host defense against Candida albicans.* PLoS Pathog, 2009. **5**(10): p. e1000639.

66. Brinkmann, V. and A. Zychlinsky, *Neutrophil extracellular traps: is immunity the second function of chromatin?* J Cell Biol, 2012. **198**(5): p. 773-83.

67. Cooper, P.R., L.J. Palmer, and I.L. Chapple, *Neutrophil extracellular traps as a new paradigm in innate immunity: friend or foe?* Periodontol 2000, 2013. **63**(1): p. 165-97.

68. Miyata, T. and X. Fan, *A second hit for TMA.* Blood, 2012. **120**(6): p. 1152-4.

69. Guimarães-Costa, A.B., et al., *ETosis: A Microbicidal Mechanism beyond Cell Death.* Journal of Parasitology Research, 2012. **2012**: p. 11.

70. Pilsczek, F.H., et al., *A novel mechanism of rapid nuclear neutrophil extracellular trap formation in response to Staphylococcus aureus.* J Immunol, 2010. **185**(12): p. 7413-25.

71. Ramos-Kichik, V., et al., *Neutrophil extracellular traps are induced by Mycobacterium tuberculosis.* Tuberculosis (Edinb), 2009. **89**(1): p. 29-37.

72. Tacchini-Cottier, F., *Neutrophils in Infectious Diseases*. 2011: Bentham Science Publishers.

73. Guimaraes-Costa, A.B., et al., *Leishmania amazonensis promastigotes induce and are killed by neutrophil extracellular traps.* Proc Natl Acad Sci U S A, 2009. **106**(16): p. 6748-53.

74. Urban, C.F., et al., *Neutrophil extracellular traps capture and kill Candida albicans yeast and hyphal forms.* Cell Microbiol, 2006. **8**(4): p. 668-76.

75. Patel, S., et al., *Nitric oxide donors release extracellular traps from human neutrophils by augmenting free radical generation.* Nitric Oxide, 2010. **22**(3): p. 226-34.

76. Gupta, A.K., et al., *Activated endothelial cells induce neutrophil extracellular traps and are susceptible to NETosis-mediated cell death.* FEBS Lett, 2010. **584**(14): p. 3193-7.

77. Clark, S.R., et al., *Platelet TLR4 activates neutrophil extracellular traps to ensnare bacteria in septic blood.* Nat Med, 2007. **13**(4): p. 463-9.

78. Lande, R., et al., *Neutrophils activate plasmacytoid dendritic cells by releasing self-DNA-peptide complexes in systemic lupus erythematosus.* Sci Transl Med, 2011. **3**(73): p. 73ra19.

79. Amulic, B. and G. Hayes, *Neutrophil extracellular traps.* Current Biology. **21**(9): p. R297-R298.

80. Zawrotniak, M. and M. Rapala-Kozik, *Neutrophil extracellular traps (NETs) - formation and implications.* Acta Biochim Pol, 2013. **60**(3): p. 277-84.

81. Li, P., et al., *PAD4 is essential for antibacterial innate immunity mediated by neutrophil extracellular traps.* J Exp Med, 2010. **207**(9): p. 1853-62.

82. Papayannopoulos, V., et al., *Neutrophil elastase and myeloperoxidase regulate the formation of neutrophil extracellular traps.* J Cell Biol, 2010. **191**(3): p. 677-91.

83. Parker, H. and C.C. Winterbourn, *Reactive oxidants and myeloperoxidase and their involvement in neutrophil extracellular traps.* Front Immunol, 2012. **3**: p. 424.

84. Kouzarides, T., *Chromatin Modifications and Their Function.* Cell, 2007. **128**(4): p. 693-705.

85. Leshner, M., et al., *PAD4 mediated histone hypercitrullination induces heterochromatin decondensation and chromatin unfolding to form neutrophil extracellular trap-like structures.* Front Immunol, 2012. **3**: p. 307.

86. Wang, Y., et al., *Histone hypercitrullination mediates chromatin decondensation and neutrophil extracellular trap formation.* J Cell Biol, 2009. **184**(2): p. 205-13.

87. Logters, T., et al., *The clinical value of neutrophil extracellular traps.* Med Microbiol Immunol, 2009. **198**(4): p. 211-9.

88. Menegazzi, R., E. Decleva, and P. Dri, *Killing by neutrophil extracellular traps: fact or folklore?* Blood, 2012. **119**(5): p. 1214-6.

89. Khan, F.H., *The Elements of Immunology*. 2009: Pearson Education.

90. DeFranco, A., *Immunity: The Immune Response in Infectious and Inflammatory Disease*. 2007.

91. Abbas, A.K., *Cellular and Molecular Immunology*. Elsevier Saunders.

92. Goodnow, C.C., et al., *Cellular and genetic mechanisms of self tolerance and autoimmunity.* Nature, 2005. **435**(7042): p. 590-7.

93. Darrah, E. and F. Andrade, *NETs: the missing link between cell death and systemic autoimmune diseases?* Front Immunol, 2012. **3**: p. 428.

94. Yu, Y. and K. Su, *Neutrophil Extracellular Traps and Systemic Lupus Erythematosus.* J Clin Cell Immunol, 2013. **4**.

95. hafez, A.E., *Could Neutrophil Extracellular Traps Elucidate the Mysteries of Pathogenesis?* J Clin Exp Pathol.

96. Watson, L., et al., *Disease activity, severity, and damage in the UK Juvenile-Onset Systemic Lupus Erythematosus Cohort.* Arthritis Rheum, 2012. **64**(7): p. 2356-65.

97. Tsokos, G., *Systemic Lupus Erythematosus: A Companion to Rheumatology*. 2007: Mosby Elsevier.

98. Font, J., et al., *Systemic lupus erythematosus (SLE) in childhood: analysis of clinical and immunological findings in 34 patients and comparison with SLE characteristics in adults.* Ann Rheum Dis, 1998. **57**(8): p. 456-9.

99. Brunner, H.I., et al., *Difference in disease features between childhood-onset and adult-onset systemic lupus erythematosus.* Arthritis Rheum, 2008. **58**(2): p. 556-62.

100. Levy, D.M. and S. Kamphuis, *Systemic lupus erythematosus in children and adolescents.* Pediatr Clin North Am, 2012. **59**(2): p. 345-64.

101. Watson, L., F. Gohar, and M.W. Beresford, *Diagnosis and management of juvenile-onset SLE.* Paediatrics and Child Health. **21**(12): p. 539-545.

102. Habibi, S., M.A. Saleem, and A.V. Ramanan, *Juvenile systemic lupus erythematosus: review of clinical features and management.* Indian Pediatr, 2011. **48**(11): p. 879-87.

103. Stichweh, D., E. Arce, and V. Pascual, *Update on pediatric systemic lupus erythematosus.* Curr Opin Rheumatol, 2004. **16**(5): p. 577-87.

104. Amaral, B., et al., *A comparison of the outcome of adolescent and adult-onset systemic lupus erythematosus.* Rheumatology (Oxford), 2014. **53**(6): p. 1130-5.

105. Ravelli, A., N. Ruperto, and A. Martini, *Outcome in juvenile onset systemic lupus erythematosus.* Curr Opin Rheumatol, 2005. **17**(5): p. 568-73.

106. Tucker, L.B., et al., *Adolescent onset of lupus results in more aggressive disease and worse outcomes: results of a nested matched case-control study within LUMINA, a multiethnic US cohort (LUMINA LVII).* Lupus, 2008. **17**(4): p. 314-22.

107. Tucker, L.B., et al., *Adult- and childhood-onset systemic lupus erythematosus: a comparison of onset, clinical features, serology, and outcome.* Br J Rheumatol, 1995. **34**(9): p. 866-72.

108. Tsai, C.L., et al., *Age-related differences in clinical outcomes for acute asthma in the United States, 2006-2008.* J Allergy Clin Immunol, 2012. **129**(5): p. 1252-1258.e1.

109. Macdermott, E.J., A. Adams, and T.J. Lehman, *Systemic lupus erythematosus in children: current and emerging therapies.* Lupus, 2007. **16**(8): p. 677-83.

110. Tucker, L.B., *Making the diagnosis of systemic lupus erythematosus in children and adolescents.* Lupus, 2007. **16**(8): p. 546-9.

111. Liu, C.-C., et al., *Mining the complement system for lupus biomarkers.* Clinical and Applied Immunology Reviews, 2005. **5**(3): p. 185-206.

112. Morgan, T.A., et al., *Children and adolescents with SLE: not just little adults.* Lupus, 2013. **22**(12): p. 1309-19.

113. Beresford, M.W. and J.E. Davidson, *Adolescent development and SLE.* Best Pract Res Clin Rheumatol, 2006. **20**(2): p. 353-68.

114. Carreno, L., et al., *Immunological and clinical differences between juvenile and adult onset of systemic lupus erythematosus.* Lupus, 1999. **8**(4): p. 287-92.

115. Hiraki, L.T., et al., *Clinical and laboratory characteristics and long-term outcome of pediatric systemic lupus erythematosus: a longitudinal study.* J Pediatr, 2008. **152**(4): p. 550-6.

116. Belostotsky, V.M. and M.J. Dillon, *Systemic lupus erythematosus in children.* Current Paediatrics, 1998. **8**(4): p. 252-257.

117. Barsalou, J., T.J. Bradley, and E.D. Silverman, *Cardiovascular risk in pediatric-onset rheumatological diseases.* Arthritis Res Ther, 2013. **15**(3): p. 212.

118. Ferraz, M.B., et al., *Evaluation of the 1982 ARA lupus criteria data set in pediatric patients. Committees of Pediatric Rheumatology of the Brazilian Society of*

Pediatrics and the Brazilian Society of Rheumatology. Clin Exp Rheumatol, 1994. **12**(1): p. 83-7.

119. Brunner, H.I., et al., *Sensitivity of the Systemic Lupus Erythematosus Disease Activity Index, British Isles Lupus Assessment Group Index, and Systemic Lupus Activity Measure in the evaluation of clinical change in childhood-onset systemic lupus erythematosus.* Arthritis Rheum, 1999. **42**(7): p. 1354-60.

120. Perilloux, B.C., et al., *Antinuclear antibody (ANA) and ANA profile tests in children with autoimmune disorders: a retrospective study.* Clin Rheumatol, 2000. **19**(3): p. 200-3.

121. Kumar, Y., A. Bhatia, and R.W. Minz, *Antinuclear antibodies and their detection methods in diagnosis of connective tissue diseases: a journey revisited.* Diagnostic Pathology, 2009. **4**: p. 1-1.

122. Mina, R. and H.I. Brunner, *Pediatric lupus--are there differences in presentation, genetics, response to therapy, and damage accrual compared with adult lupus?* Rheum Dis Clin North Am, 2010. **36**(1): p. 53-80, vii-viii.

123. Hoffman, I.E., et al., *Juvenile-onset systemic lupus erythematosus: different clinical and serological pattern than adult-onset systemic lupus erythematosus.* Ann Rheum Dis, 2009. **68**(3): p. 412-5.

124. Rood, M.J., et al., *Childhood-onset Systemic Lupus Erythematosus: clinical presentation and prognosis in 31 patients.* Scand J Rheumatol, 1999. **28**(4): p. 222-6.

125. Arbuckle, M.R., et al., *Development of autoantibodies before the clinical onset of systemic lupus erythematosus.* N Engl J Med, 2003. **349**(16): p. 1526-33.

126. Lipsky, P.E., *Systemic lupus erythematosus: an autoimmune disease of B cell hyperactivity.* Nat Immunol, 2001. **2**(9): p. 764-766.

127. Banchereau, J. and V. Pascual, *Type I interferon in systemic lupus erythematosus and other autoimmune diseases.* Immunity, 2006. **25**(3): p. 383-92.

128. Yurasov, S., et al., *Defective B cell tolerance checkpoints in systemic lupus erythematosus.* J Exp Med, 2005. **201**(5): p. 703-11.

129. Pisetsky, D.S. and N. Jiang, *The generation of extracellular DNA in SLE: the role of death and sex.* Scand J Immunol, 2006. **64**(3): p. 200-4.

130. Chan, O.T., et al., *A novel mouse with B cells but lacking serum antibody reveals an antibody-independent role for B cells in murine lupus.* J Exp Med, 1999. **189**(10): p. 1639-48.

131. Crispin, J.C., et al., *T cells as therapeutic targets in SLE.* Nat Rev Rheumatol, 2010. **6**(6): p. 317-25.

132. Crispin, J.C., A. Martinez, and J. Alcocer-Varela, *Quantification of regulatory T cells in patients with systemic lupus erythematosus.* J Autoimmun, 2003. **21**(3): p. 273-6.

133. Valencia, X., et al., *Deficient CD4+CD25high T regulatory cell function in patients with active systemic lupus erythematosus.* J Immunol, 2007. **178**(4): p. 2579-88.

134. Linker-Israeli, M., et al., *Elevated levels of endogenous IL-6 in systemic lupus erythematosus. A putative role in pathogenesis.* J Immunol, 1991. **147**(1): p. 117-23.

135. Wan, S., C. Xia, and L. Morel, *IL-6 produced by dendritic cells from lupus-prone mice inhibits CD4+CD25+ T cell regulatory functions.* J Immunol, 2007. **178**(1): p. 271-9.

136. Tillack, K., et al., *T lymphocyte priming by neutrophil extracellular traps links innate and adaptive immune responses.* J Immunol, 2012. **188**(7): p. 3150-9.

137. Rottman, J.B. and C.R. Willis, *Mouse models of systemic lupus erythematosus reveal a complex pathogenesis.* Vet Pathol, 2010. **47**(4): p. 664-76.

138. Su, K.Y. and D.S. Pisetsky, *The role of extracellular DNA in autoimmunity in SLE.* Scand J Immunol, 2009. **70**(3): p. 175-83.

139. Pieterse, E. and J. Van Der Vlag, *Breaking immunological tolerance in Systemic Lupus Erythematosus.* Frontiers in Immunology, 2014. **5**.

140. Ganguly, D., et al., *Self-RNA-antimicrobial peptide complexes activate human dendritic cells through TLR7 and TLR8.* J Exp Med, 2009. **206**(9): p. 1983-94.

141. Barton, G.M., J.C. Kagan, and R. Medzhitov, *Intracellular localization of Toll-like receptor 9 prevents recognition of self DNA but facilitates access to viral DNA.* Nat Immunol, 2006. **7**(1): p. 49-56.

142. Anders, H.J., *A Toll for lupus.* Lupus, 2005. **14**(6): p. 417-22.

143. Barrat, F.J. and R.L. Coffman, *Development of TLR inhibitors for the treatment of autoimmune diseases.* Immunol Rev, 2008. **223**: p. 271-83.

144. Rahman, A.H. and R.A. Eisenberg, *The role of toll-like receptors in systemic lupus erythematosus.* Springer Semin Immunopathol, 2006. **28**(2): p. 131-43.

145. Cocca, B.A., A.M. Cline, and M.Z. Radic, *Blebs and apoptotic bodies are B cell autoantigens.* J Immunol, 2002. **169**(1): p. 159-66.

146. Shao, W.H. and P.L. Cohen, *Disturbances of apoptotic cell clearance in systemic lupus erythematosus.* Arthritis Res Ther, 2011. **13**(1): p. 202.

147. Kaplan, M.J., *Neutrophils in the pathogenesis and manifestations of SLE.* Nat Rev Rheumatol, 2011. **7**(12): p. 691-9.

148. Dwivedi, N. and M. Radic, *Citrullination of autoantigens implicates NETosis in the induction of autoimmunity.* Ann Rheum Dis, 2014. **73**(3): p. 483-91.

149. Khandpur, R., et al., *NETs are a source of citrullinated autoantigens and stimulate inflammatory responses in rheumatoid arthritis.* Sci Transl Med, 2013. **5**(178): p. 178ra40.

150. Bosch, X., *Systemic Lupus Erythematosus and the Neutrophil.* New England Journal of Medicine, 2011. **365**(8): p. 758-760.

151. Garcia-Romo, G.S., et al., *Netting neutrophils are major inducers of type I IFN production in pediatric systemic lupus erythematosus.* Sci Transl Med, 2011. **3**(73): p. 73ra20.

152. Ytterberg, S.R. and T.J. Schnitzer, *Serum interferon levels in patients with systemic lupus erythematosus.* Arthritis Rheum, 1982. **25**(4): p. 401-6.

153. Hooks, J.J., et al., *Immune interferon in the circulation of patients with autoimmune disease.* N Engl J Med, 1979. **301**(1): p. 5-8.

154. Baechler, E.C., et al., *Interferon-inducible gene expression signature in peripheral blood cells of patients with severe lupus.* Proc Natl Acad Sci U S A, 2003. **100**(5): p. 2610-5.

155. Bennett, L., et al., *Interferon and granulopoiesis signatures in systemic lupus erythematosus blood.* J Exp Med, 2003. **197**(6): p. 711-23.

156. Denny, M.F., et al., *A distinct subset of proinflammatory neutrophils isolated from patients with systemic lupus erythematosus induces vascular damage and synthesizes type I IFNs.* J Immunol, 2010. **184**(6): p. 3284-97.

157. Kaplan, M.J., *NETosis: At the Intersection of Cell Biology, Microbiology, and Immunology*. Frontiers E-books.

158. Farrera, C. and B. Fadeel, *Macrophage clearance of neutrophil extracellular traps is a silent process.* J Immunol, 2013. **191**(5): p. 2647-56.

159. Hakkim, A., et al., *Impairment of neutrophil extracellular trap degradation is associated with lupus nephritis.* Proc Natl Acad Sci U S A, 2010. **107**(21): p. 9813-8.

160. Yeh, T.M., et al., *Deoxyribonuclease-inhibitory antibodies in systemic lupus erythematosus.* J Biomed Sci, 2003. **10**(5): p. 544-51.

161. Meng, W., et al., *Deoxyribonuclease Is a Potential Counter Regulator of Aberrant Neutrophil Extracellular Traps Formation after Major Trauma.* Mediators of Inflammation, 2012. **2012**: p. 8.

162. Leffler, J., et al., *Neutrophil extracellular traps that are not degraded in systemic lupus erythematosus activate complement exacerbating the disease.* J Immunol, 2012. **188**(7): p. 3522-31.

163. Means, T.K., et al., *Human lupus autoantibody-DNA complexes activate DCs through cooperation of CD32 and TLR9.* J Clin Invest, 2005. **115**(2): p. 407-17.

164. Baccala, R., et al., *TLR-dependent and TLR-independent pathways of type I interferon induction in systemic autoimmunity.* Nat Med, 2007. **13**(5): p. 543-51.

165. Chamilos, G., et al., *Cytosolic sensing of extracellular self-DNA transported into monocytes by the antimicrobial peptide LL37.* Blood, 2012. **120**(18): p. 3699-707.

166. Lande, R., et al., *Plasmacytoid dendritic cells sense self-DNA coupled with antimicrobial peptide.* Nature, 2007. **449**(7162): p. 564-569.

167. Stacey, K.J., et al., *The molecular basis for the lack of immunostimulatory activity of vertebrate DNA.* J Immunol, 2003. **170**(7): p. 3614-20.

168. Hurtado, E., *DNA from neutrophil extracellular traps is hypomethylated.* La Presse Medicale, 2013. **42**(4): p. 759.

169. Krug, A., et al., *Identification of CpG oligonucleotide sequences with high induction of IFN-α/β in plasmacytoid dendritic cells.* European Journal of Immunology, 2001. **31**(7): p. 2154-2163.

170. Ronnblom, L. and G.V. Alm, *A pivotal role for the natural interferon alpha-producing cells (plasmacytoid dendritic cells) in the pathogenesis of lupus.* J Exp Med, 2001. **194**(12): p. F59-63.

171. Ronnblom, L., M.L. Eloranta, and G.V. Alm, *Role of natural interferon-alpha producing cells (plasmacytoid dendritic cells) in autoimmunity.* Autoimmunity, 2003. **36**(8): p. 463-72.

172. Niewold, T.B., *Interferon alpha-induced lupus: proof of principle.* J Clin Rheumatol, 2008. **14**(3): p. 131-2.

173. Rönnblom, L., M.-L. Eloranta, and G.V. Alm, *The type I interferon system in systemic lupus erythematosus.* Arthritis & Rheumatism, 2006. **54**(2): p. 408-420.

174. Obermoser, G. and V. Pascual, *The interferon-alpha signature of systemic lupus erythematosus.* Lupus, 2010. **19**(9): p. 1012-9.

175. Liu, Y.J., *IPC: professional type 1 interferon-producing cells and plasmacytoid dendritic cell precursors.* Annu Rev Immunol, 2005. **23**: p. 275-306.

176. Ronnblom, L. and V. Pascual, *The innate immune system in SLE: type I interferons and dendritic cells.* Lupus, 2008. **17**(5): p. 394-9.

177. Fitzgerald-Bocarsly, P. and D. Feng, *The role of type I interferon production by dendritic cells in host defense.* Biochimie, 2007. **89**(6-7): p. 843-55.

178. Blanco, P., et al., *Induction of dendritic cell differentiation by IFN-alpha in systemic lupus erythematosus.* Science, 2001. **294**(5546): p. 1540-3.

179. Farkas, L., et al., *Plasmacytoid dendritic cells (natural interferon- alpha/beta-producing cells) accumulate in cutaneous lupus erythematosus lesions.* Am J Pathol, 2001. **159**(1): p. 237-43.

180. Tucci, M., et al., *Glomerular accumulation of plasmacytoid dendritic cells in active lupus nephritis: role of interleukin-18.* Arthritis Rheum, 2008. **58**(1): p. 251-62.

181. Aghemo, A., M.G. Rumi, and M. Colombo, *Pegylated interferons [alpha]2a and [alpha]2b in the treatment of chronic hepatitis C.* Nat Rev Gastroenterol Hepatol, 2010. **7**(9): p. 485-494.

182. Clark, J.D., M.E. Flanagan, and J.-B. Telliez, *Discovery and Development of Janus Kinase (JAK) Inhibitors for Inflammatory Diseases.* Journal of Medicinal Chemistry, 2014. **57**(12): p. 5023-5038.

183. Niewold, T.B., *Interferon Alpha in Systemic Lupus Erythematosus.* Journal of Biomedicine and Biotechnology, 2010. **2010:** p.8.

184. Ronnblom, L., *Systemic Lupus Erythematosus and the Type 1 Interferon System.* Arthritis Res Ther, 2003. **5:** p.68-75.

185. Manicassamy, S. and B. Pulendran, *Dendritic cell control of tolerogenic responses.* Immunol Rev, 2011. **241**(1): p. 206-27.

186. Jego, G., et al., *Plasmacytoid dendritic cells induce plasma cell differentiation through type I interferon and interleukin 6.* Immunity, 2003. **19**(2): p. 225-34.

187. Braun, D., I. Caramalho, and J. Demengeot, *IFN-alpha/beta enhances BCR-dependent B cell responses.* Int Immunol, 2002. **14**(4): p. 411-9.

188. Ronnblom, L. and K.B. Elkon, *Cytokines as therapeutic targets in SLE.* Nat Rev Rheumatol, 2010. **6**(6): p. 339-47.

189. Ganguly, D., et al., *The role of dendritic cells in autoimmunity.* Nat Rev Immunol, 2013. **13**(8): p. 566-577.

190. Ardoin, S.P. and L.E. Schanberg, *The management of pediatric systemic lupus erythematosus.* Nat Clin Pract Rheumatol, 2005. **1**(2): p. 82-92.

191. Cimaz, R., *Pediatrics in Systemic Autoimmune Diseases*. 2008: Elsevier.

192. Malleson, P. and J. Tekano, *Diagnosis and management of systemic lupus erythematosus in children.* Paediatrics and Child Health, 2008. **18**(2): p. 61-69.

193. Marks, S.D. and K. Tullus, *Modern therapeutic strategies for paediatric systemic lupus erythematosus and lupus nephritis.* Acta Pædiatrica, 2010. **99**(7): p. 967-974.

194. Hochberg, M.C., *Rheumatology*. 2010: Elsevier Health Sciences.

195. Unsal, E., *Current Opinions in Pediatric Rheumatology*. 2006: Nova Publishers.

196. Abarientos, C., et al., *Hydroxychloroquine in systemic lupus erythematosus and rheumatoid arthritis and its safety in pregnancy.* Expert Opin Drug Saf, 2011. **10**(5): p. 705-14.

197. *A randomized study of the effect of withdrawing hydroxychloroquine sulfate in systemic lupus erythematosus. The Canadian Hydroxychloroquine Study Group.* N Engl J Med, 1991. **324**(3): p. 150-4.

198. Kyburz, D., F. Brentano, and S. Gay, *Mode of action of hydroxychloroquine in RA-evidence of an inhibitory effect on toll-like receptor signaling.* Nat Clin Pract Rheumatol, 2006. **2**(9): p. 458-9.

199. Costedoat-Chalumeau, N., et al., *Hydroxychloroquine: a multifaceted treatment in lupus.* Presse Med, 2014. **43**(6 Pt 2): p. e167-80.

200. Lafyatis, R., M. York, and A. Marshak-Rothstein, *Antimalarial agents: Closing the gate on toll-like receptors?* Arthritis & Rheumatism, 2006. **54**(10): p. 3068-3070.

201. Kuznik, A., et al., *Mechanism of endosomal TLR inhibition by antimalarial drugs and imidazoquinolines.* J Immunol, 2011. **186**(8): p. 4794-804.

202. Miller, D.R., et al., *Plasma hydroxychloroquine concentrations and efficacy in rheumatoid arthritis.* Arthritis Rheum, 1987. **30**(5): p. 567-71.

203. Sinha, R. and S. Raut, *Pediatric lupus nephritis: Management update.* World J Nephrol, 2014. **3**(2): p. 16-23.

204. Doria, A., et al., *Long-term prognosis and causes of death in systemic lupus erythematosus.* Am J Med, 2006. **119**(8): p. 700-6.

205. Chatham, W.W. and R.P. Kimberly, *Treatment of lupus with corticosteroids.* Lupus, 2001. **10**(3): p. 140-7.

206. Mahmood, T. and P.C. Yang, *Western blot: technique, theory, and trouble shooting.* N Am J Med Sci, 2012. **4**(9): p. 429-34.

207. Avison, M., *Measuring Gene Expression*. 2007: Taylor and Francis Group.

208. Cheng, L., *Molecular Genetic Pathology*. 2008: Human Press.

209. King, D.J., *Applications And Engineering Of Monoclonal Antibodies.* 1998.

210. Manchenko, G.P., *Handbook of Detection of Enzymes on Electrophoretic Gels.*

211. van Engeland, M., et al., *A novel assay to measure loss of plasma membrane asymmetry during apoptosis of adherent cells in culture.* Cytometry, 1996. **24**(2): p. 131-9.

212. Zhang, G., et al., *Early detection of apoptosis using a fluorescent conjugate of annexin V.* Biotechniques, 1997. **23**(3): p. 525-31.

213. Trivedi, S. and E.L. Greidinger, *Endosomal Toll-like receptors in autoimmunity: mechanisms for clinical diversity.* Therapy, 2009. **6**(3): p. 433-442.

214. Midgley, A., C. Thorbinson, and M.W. Beresford, *Expression of Toll-like receptors and their detection of nuclear self-antigen leading to immune activation in JSLE.* Rheumatology (Oxford), 2012. **51**(5): p. 824-32.

215. Carmona-Rivera, C. and M.J. Kaplan, *Low-density granulocytes: a distinct class of neutrophils in systemic autoimmunity.* Semin Immunopathol, 2013. **35**(4): p. 455-63.

216. Knight, *Lupus-Prone Mice Demonstrate Enhanced Neutrophil Extracellular Trap Formation: Implications for Autoantibody Formation and Organ Damage.*

217. Campbell, A.M., M. Kashgarian, and M.J. Shlomchik, *NADPH oxidase inhibits the pathogenesis of systemic lupus erythematosus.* Sci Transl Med, 2012. **4**(157): p. 157ra141.

218. Yasutomo, K., et al., *Mutation of DNASE1 in people with systemic lupus erythematosus.* Nat Genet, 2001. **28**(4): p. 313-4.

219. Bodano, A., et al., *Study of DNASE I gene polymorphisms in systemic lupus erythematosus susceptibility.* Ann Rheum Dis, 2007. **66**(4): p. 560-1.

220. Napirei, M., et al., *Features of systemic lupus erythematosus in Dnase1-deficient mice.* Nat Genet, 2000. **25**(2): p. 177-81.

221. Radic, M. and T.N. Marion, *Neutrophil extracellular chromatin traps connect innate immune response to autoimmunity.* Semin Immunopathol, 2013. **35**(4): p. 465-80.

222. Liu, C.L., et al., *Specific post-translational histone modifications of neutrophil extracellular traps as immunogens and potential targets of lupus autoantibodies.* Arthritis Res Ther, 2012. **14**(1): p. R25.

223. Chirivi RGS, v.R.J., Jenniskens GJ, Pruijn GJ, Raats JMH, *Citrullination: A Target for Disease Intervention in Multiple Sclerosis and other Inflammatory Diseases?* J Clin Cell Immunol, 2013. **4**.

224. Warde, N., *Autoimmunity: the role of neutrophils in SLE: untangling the NET.* Nat Rev Rheumatol, 2011. **7**(5): p. 252.

225. Villanueva, E., et al., *Netting neutrophils induce endothelial damage, infiltrate tissues, and expose immunostimulatory molecules in systemic lupus erythematosus.* J Immunol, 2011. **187**(1): p. 538-52.

226. Pascual, V., L. Farkas, and J. Banchereau, *Systemic lupus erythematosus: all roads lead to type I interferons.* Curr Opin Immunol, 2006. **18**(6): p. 676-82.

227. Trinchieri, G., *Type I interferon: friend or foe?* J Exp Med, 2010. **207**(10): p. 2053-63.

228. Ronnblom, L.E., G.V. Alm, and K.E. Oberg, *Possible induction of systemic lupus erythematosus by interferon-alpha treatment in a patient with a malignant carcinoid tumour.* J Intern Med, 1990. **227**(3): p. 207-10.

229. Schilling, P.J., et al., *Development of systemic lupus erythematosus after interferon therapy for chronic myelogenous leukemia.* Cancer, 1991. **68**(7): p. 1536-7.

230. Tolaymat, A., et al., *Systemic lupus erythematosus in a child receiving long-term interferon therapy.* J Pediatr, 1992. **120**(3): p. 429-32.

231. Wahid Fadilah, S.A., et al., *The development of systemic lupus erythematosus following interferon-α therapy for hepatitis C infection.* APLAR Journal of Rheumatology, 2006. **9**(3): p. 286-289.

232. Fukuyama, S., et al., *Systemic Lupus Erythematosus After [alpha]-Interferon Therapy for Chronic Hepatitis C: A Case Report and Review of the Literature.* Am J Gastroenterol, 2000. **95**(1): p. 310-312.

233. Conlon, K.C., et al., *Exacerbation of symptoms of autoimmune disease in patients receiving alpha-interferon therapy.* Cancer, 1990. **65**(10): p. 2237-42.

234. Prummel, M.F. and P. Laurberg, *Interferon-alpha and autoimmune thyroid disease.* Thyroid, 2003. **13**(6): p. 547-51.

235. Santiago-Raber, M.L., et al., *Type-I interferon receptor deficiency reduces lupus-like disease in NZB mice.* J Exp Med, 2003. **197**(6): p. 777-88.

236. Kirou, K.A., et al., *Activation of the interferon-alpha pathway identifies a subgroup of systemic lupus erythematosus patients with distinct serologic features and active disease.* Arthritis Rheum, 2005. **52**(5): p. 1491-503.

237. Niewold, T.B., et al., *High serum IFN-alpha activity is a heritable risk factor for systemic lupus erythematosus.* Genes Immun, 2007. **8**(6): p. 492-502.

238. Sigurdsson, S., et al., *Polymorphisms in the tyrosine kinase 2 and interferon regulatory factor 5 genes are associated with systemic lupus erythematosus.* Am J Hum Genet, 2005. **76**(3): p. 528-37.

239. Cham, C.M., K. Ko, and T.B. Niewold, *Interferon Regulatory Factor 5 in the Pathogenesis of Systemic Lupus Erythematosus.* Clinical and Developmental Immunology, 2012. **2012**: p. 11.

240. Ronnblom, L., *Potential role of IFNalpha in adult lupus.* Arthritis Res Ther, 2010. **12 Suppl 1**: p. S3.

241. Hacbarth, E. and A. Kajdacsy-Balla, *Low density neutrophils in patients with systemic lupus erythematosus, rheumatoid arthritis, and acute rheumatic fever.* Arthritis Rheum, 1986. **29**(11): p. 1334-42.

242. O'Shea, J.J., et al., *Janus kinase inhibitors in autoimmune diseases.* Ann Rheum Dis, 2013. **72 Suppl 2**: p. ii111-5.

243. Bouts, Y.M., et al., *Apoptosis and NET formation in the pathogenesis of SLE.* Autoimmunity, 2012. **45**(8): p. 597-601.

244. Horton, C.G., Z.-j. Pan, and A.D. Farris, *Targeting Toll-Like Receptors for Treatment of SLE.* Mediators of Inflammation, 2010. **2010**.

245. Wallace, D.J., et al., *New insights into mechanisms of therapeutic effects of antimalarial agents in SLE.* Nat Rev Rheumatol, 2012. **8**(9): p. 522-33.

246. Ruzicka, T., et al., *Treatment of cutaneous lupus erythematosus with acitretin and hydroxychloroquine.* Br J Dermatol, 1992. **127**(5): p. 513-8.

247. Sacre, K., L.A. Criswell, and J.M. McCune, *Hydroxychloroquine is associated with impaired interferon-alpha and tumor necrosis factor-alpha production by plasmacytoid dendritic cells in systemic lupus erythematosus.* Arthritis Res Ther, 2012. **14**(3): p. R155.

248. Costedoat-Chalumeau, N., et al., *Low blood concentration of hydroxychloroquine is a marker for and predictor of disease exacerbations in patients with systemic lupus erythematosus.* Arthritis Rheum, 2006. **54**(10): p. 3284-90.

249. Costedoat-Chalumeau, N., et al., *Why all systemic lupus erythematosus patients should be given hydroxychloroquine treatment?* Joint Bone Spine, 2010. **77**(1): p. 4-5.

250. Barrat, F.J., et al., *Nucleic acids of mammalian origin can act as endogenous ligands for Toll-like receptors and may promote systemic lupus erythematosus.* J Exp Med, 2005. **202**(8): p. 1131-9.

251. Guiducci, C., et al., *TLR recognition of self nucleic acids hampers glucocorticoid activity in lupus.* Nature, 2010. **465**(7300): p. 937-941.

252. Fulmer, T., *Sparing steroids in lupus.* SciBX, 2010. **3**(26).

253. Wang, S., et al., *Jak/STAT signaling is involved in the inflammatory infiltration of the kidneys in MRL/lpr mice.* Lupus, 2010. **19**(10): p. 1171-80.

254. Kawasaki, M., et al., *Possible role of the JAK/STAT pathways in the regulation of T cell-interferon related genes in systemic lupus erythematosus.* Lupus, 2011. **20**(12): p. 1231-9.

255. Midgley, A., et al., *The role of neutrophil apoptosis in juvenile-onset systemic lupus erythematosus.* Arthritis & Rheumatism, 2009. **60**(8): p. 2390-2401.