# Investigating the IL-17 Family and Th17-related Cytokines in Juvenile-onset Systemic Lupus Erythematosus

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

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# Abbreviations

**ACR -** American College of Rheumatology

°C - degrees Celcius

**Anti-dsDNA -** Anti-double stranded DNA

**APC -** Antigen presenting cell

**B-actin -** Beta actin

**B2M -** Beta-2-microglobulin

**BILAG -** British Isles Lupus Assessment Group

**BSA -** Bovine serum albumin

**CD -** Cluster of differentiation

**cDNA -** Complementary strand of DNA

**Ct -** Cycle threshold

**DTT -** Dithiothrecil

**EAE -** Autoimmune encephalomyelitis

**ELISA -** Enzyme-linked immunosorbent assay

**FCS -** Foetal calf serum

**Foxp3 -** Forkhead box protein 3

**FSC -** Forward scatter

**GATA3 -** GATA binding protein 3

**HMGB1 -** High mobility group box protein 1

**IFN -** Interferon

**Ig -** Immunoglobulin

**IL -** Interleukin

**IL-17R -** Interleukin-17 receptor

**JIA -** Juvenile Idiopathic Arthritis

**JSLE -** Juvenile-onset Systemic Lupus Erythematosus

**kDa -** KiloDalton

**MHC -** Major histocompatibility complex

**mL -**Mililitre

**PAMP -** Pathogen-associated molecular pattern

**PBMC -** Peripheral blood mononuclear cells

**PBS -** Phosphate buffered saline

**pDC -** Plasmacytoid dendritic cell

**PEG -** Polyethylene glycol

**PRR -** Pattern recognition receptor

**qPCR -** Quantitative polymerase chain reaction

**RORγt** - Retinoic acid-receptor-related orphan receptor-γt

**SA-PE -** Streptavidin-phycoerythrin

**SDS -** Sodium dodecyl sulphate

**SEM -** Standard error of mean

**SFMC -** Synovial fluid monouclear cells

**SLE -** Systemic Lupus Erythematosus

**SSC -** Side scatter

**T-bet -** T box transcription factor

**TBS -** Tris Buffer Saline

**Tc cell -** CD8+ Cytotoxic T cell

**TCR -** T cell receptor

**TGF -** Tissue growth factor

**Th cell -** CD4+ T helper cell

**TLR -** Toll-like receptor

**TNF -** Tumour necrosis factor

**Treg -** T regulatory cell

µl - Microlitre

# Abstract


## Investigating the IL-17 Family and Th17-related Cytokines in Juvenile-onset Systemic Lupus Erythematosus

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**Background** Juvenile-onset systemic lupus erythematosus (JSLE) is a multi-system autoimmune disease characterised by severe disease and poorer prognosis compared to adult-onset disease. Currently, its aetiopathogenesis is unknown but is postulated to be a result of a loss of peripheral tolerance. T helper 17 (Th17) cells produce pro-inflammatory cytokines Interleukin-17A (IL-17A) and IL-21 and are upregulated by IL-23. These cytokines have been shown to factor in the inflammatory manifestations of adult SLE. Six members of the IL-17 family have been identified so far, as well as IL-17 receptor subtypes. Although research on IL-17 family members is scarce, existing studies have implicated IL-17B, IL-17C and IL-17 receptor E (IL-17RE) in autoimmune pathology.

**Objective** To optimise the measurement of the IL-17 family (IL-17A, IL-17B, IL-17C and IL-17RE) and Th17-related cytokines (IL-21 and IL-23) in order to investigate their roles in the pathogenesis of JSLE.

**Methods** Peripheral blood components were isolated from JSLE, healthy paediatric control and juvenile idiopathic arthritis (JIA) patients. Spike-and-recovery validation experiments were conducted using JSLE and control serum and plasma in an IL-17A single plex and IL-17A, IL-21 and IL-23 ELISAs. mRNA expression of IL-17A was analysed by qPCR in $CD4^+$ T cells from JSLE and control patients. Western blot analysis of IL-17A protein was optimised and measured in the PBMCs, $CD4^+$ T cells and neutrophils of JSLE, control and JIA patients. JSLE and control PBMC mRNA expression of IL-17B, IL-17C and IL-17RE were quantified using qPCR, then mRNA expression of IL-17C and IL-17RE in CD3/CD28 stimulated JSLE and control PBMCs were analysed.

**Results** 102 patients (JSLE: n=31; healthy controls: n=66; JIA: n=4; lupus nephritis: n=1) were involved in this study, with multiple episode samples from JSLE and JIA patients. Poor recovery and matrix interference were detected in all IL-17A, IL-21 and IL-23 immunoassays. Protein IL-17A expression of neutrophils was significantly decreased in JSLE patients ($p=0.03$). IL-17A neutrophil expression was validated using purified neutrophils from healthy adult donors. IL-17B was not expressed in any samples. No significant differences were found between JSLE and control $CD4^+$ cells or PBMCs at an mRNA and protein level for IL-17A, IL-17C or IL-17RE expression. IL-17RE expression was significantly decreased in stimulated JSLE PBMCs ($p=0.007$), with fold decreases in IL-17C and IL-17RE of JSLE relative to controls. Western blotting of IL-17C and IL-17RE protein expression was optimised in JSLE and control $CD4^+$ T cells.

**Conclusions** Findings show IL-17A is not significantly increased in the peripheral blood of JSLE patients and that IL-17A is expressed by neutrophils. IL-17B is not expressed in human PBMCs while IL-17C and IL-17RE are, but are not significantly increased in JSLE PBMCs. Future immunoassays must be validated for recovery potential and existing data should be interpreted with caution. This study involved the validation and rigorous optimisation of novel methods investigating the IL-17 family and Th17-related cytokines, contributes significantly to limited research in the context of paediatric autoimmune disease, Th17 and the IL-17 family, and opens up new avenues for future directions in these fields.

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# Introduction

## 1.1 The Immune System

All cells in the immune system originate from the bone marrow and can be differentiated from two types of progenitor stem cells– myeloid (neutrophils, basophils, eosinophils, macrophages and dendritic cells) and lymphoid (B cells, T cells and Natural Killer cells) (1, 2). Precursor T cells migrate to the thymus from the bone marrow where differentiation into CD4+ and CD8+ T cells occurs while B cells mature within the bone marrow (1). The key functions of an effective immune system are to identify and mount an attack against a large variety of pathogens whilst avoiding attacking the organism's own tissues in the process (2, 3). The immune system can be broken down into three different levels –the external defences, the innate immune system and the adaptive immune system. The external defences consist of physical barriers to pathogens such as the epidermis, the mucous membranes lining the internal organs, and different types of fluids secreted at various surfaces which are made unconducive for access of these pathogens (2). The innate and adaptive immune systems will be explored in more depth below.

### 1.1.1 Innate Immunity

The role of the innate immune system is to provide non-specific resistance to invading pathogens and it is present from birth. Involved in the innate line of defence are internal anti-microbial proteins, phagocytes, Natural Killer (NK) cells and inflammation.

#### *1.1.1.1 Anti-microbial proteins*

The complement system comprises one type of anti-microbial protein; it consists of a group of normally dormant proteins in blood plasma and on plasma membranes. When activated, complement proteins set off a cascade of events to upregulate immune reactions such as cytolysis, phagocytosis and chemotactic recruitment of inflammatory cells (1). Complement component 3 (C3) b is formed from the splitting of C3 and links to the microbial agent to act as an opsonin, an opsonin enhances the binding of a phagocytic cell to its target cell in order to

increase its susceptibility to phagocytosis (4). Phagocytosis involves the engulfment, ingestion and removal of cells or foreign material by a group of specialised cells – phagocytes, which will be covered in more detail in section 1.1.1.2 below (5). Activated phagocytes bind to C3b-opsonised microbes and along with capillary dilatation and exudation of plasma proteins and oedema, an acute inflammatory response is formed (4). Eosinophils have C3b surface receptors to launch an extracellular attack of oxygen metabolites on parasites (2). Complement component 5 (C5) is activated to form C5a, a chemoattractant for neutrophils (3, 4). C3a and C5a are inflammatory agents which induce the release of histamine, leukotriene $B_4$ and tumour necrosis factor (TNF) from mast cells and contribute to neutrophil chemotaxis and capillary permeability (4).

Interferons (IFNs) are another type of anti-microbial protein which are released by lymphocytes, macrophages and fibroblasts infected with virus (6). IFNs are split into Type I IFNs (α and β groups) and IFN-γ, the latter of which activates macrophages to attack intracellular pathogens (6). Type I IFNs induce the production of antiviral proteins in order to render uninfected neighbouring cells antiviral and inhibit viral replication, in addition they increase presentation of viral proteins to cytotoxic T lymphocytes by upregulating major histocompatibility complex (MHC) class I molecules (6).

There are natural control mechanisms in place which endeavour to counteract invading pathogens and maintain immune homeostasis. Acute phase proteins such as C-reactive protein, serum amyloid A protein and coagulation proteins are mobilised in response to pro-inflammatory cytokines secreted in the blood to modulate inflammation and help with repair of affected tissue (4).

#### *1.1.1.2 Phagocytes*

During an infection, migration of phagocytes to the site of inflammation occurs. Phagocytes comprise of mononuclear (monocytes, macrophages, dendritic cells) and polynuclear phagocytes (neutrophils, basophils, eosinophils) which bind onto bacteria, foreign antigens or target cells and ingest them through the process of phagocytosis (3). Neutrophils are non-dividing and survive in the circulation for

approximately six hours before apoptosing, with granules containing myeloperoxidase, nonoxidative antimicrobial effectors and lysozymes which facilitate in the anti-microbial destruction of engulfed pathogens (3, 7). Apoptosis is programmed cell death which maintains normal physiology in the body; here the cells actively assist in activating a cascade of reactions which result in a pre-determined cell death pathway (8). Basophils and eosinophils are found in much smaller numbers compared to their neutrophil counterpart and are involved in mediation of parasitic or allergic response (3). Monocytes leave the blood stream to enlarge and develop into active macrophages.

Phagocytic cells have pattern recognition receptors (PRR) on their surfaces which are activated by pathogen-associated molecular patterns (PAMPs) and bind specifically to microbial surfaces which express these PAMPs, these receptors recognise a broad spectrum of ligands as long as they share a common molecular pattern (4, 6). Epithelial cells and innate effector cell subsets such as antigen presenting cells (APCs) also express PRRs on their surfaces (6). Under the class of PRRs, there are toll-like receptors (TLRs) which can act as sensors for extracellular infections by microbial pathogens (9).

Phagocytosis is initiated with chemotaxis (movement according to certain chemicals in the cellular micro-environment) which consists of phagocytes stimulated to accumulate at the site of inflammation by moving up a chemotactic gradient of chemoattractant cytokines (chemokines) which may originate from invading microbes, damaged tissue cells, activated complement proteins or lymphocytes (3). At the site of inflammation, inflammatory cytokines such as TNF-α and Interleukin (IL)-1 are released as well as bradykinin and histamine which increase the blood volume flowing to the site by means of vasodilation (3). It is followed by a PAMP recognition stage where the phagocyte adheres to the bacterial cell wall by means of opsonisation, or with complement or antibody signalling (10). The plasma membrane of the phagocyte extends pseudopod projections, temporary outgrowth extensions, that guide the bacterium into a phagosome sac in order to engulf it (2). The phagosome merges with lysosomes containing the enzyme lysozyme, and a series of oxidative and non-oxidative reactions

subsequently break down the microbe (1). PAMP binding also releases host proteins which recruit and prime macrophages and dendritic cells to interact with lymphocytes to initiate an adaptive immune response which will be explained further below (see section 1.1.2) (2).

#### *1.1.1.3 Dendritic cells*

The dendritic cells behave as APCs when activated by recognition of PAMPs by their cell surface PRRs (1). This is usually triggered by release of heat shock proteins in necrosis or IFN-α from virally infected cells (1). When activated, dendritic cells up-regulate B7 co-stimulatory molecules (CD80 and CD86) which signal for lymphocyte activation (4). Dendritic PRRs include TLRs, mannose receptors and lipopolysaccharide receptors which recognise yeast cell wall mannans and bacteria (10). There are three subtypes of dendritic cells which are present in the body at steady state that is before infection. These include migratory dendritic cells and resident lymphoid dendritic cells, which act as APCs for T cell stimulation. Plasmacytoid dendritic cells (pDCs) are a type of steady pre-cursor dendritic cell which produce type I IFNs and can mature to APCs (11, 12).

#### *1.1.1.4 Toll-Like Receptors*

Mentioned before as a subset of PRR in 1.1.1.2, TLRs are transmembrane proteins which contain an extracellular leucine-rich repeat domain as well as a Toll-IL-1R domain (13). Ten TLRs are expressed in humans (TLR1-10) and each is thought to have the ability to recognise a specific set of ligands (3). These TLRs are primarily expressed on macrophages and dendritic cells but are also found on T cells, B cells, neutrophils, eosinophils, keratinocytes and epithelial cells (14). The function of TLRs are to signal foreign antigens which induces an inflammatory cellular response resulting in the enhanced expression of inflammatory cytokines, type I IFNs and chemokines (15).

#### *1.1.1.5 Mast cells*

Mast cell receptors have a high affinity to IgE antibodies and play a role in triggering an inflammatory response in atopic diseases such as hay fever, asthma and eczema (4). The allergen binds to the IgE receptor and activates the cell to

secrete histamine, leukotrienes and prostaglandins which are inflammatory mediators (4).

#### *1.1.1.6 Natural Killer cells*

Macrophages recognise apoptotic cells via the CD14 receptor and through the binding of C1q to surface nucleosome blebs in order to remove these cells by phagocytosis without triggering an inflammatory response (16). This is different to necrosis, where cells are subjected to extreme deviation from normal physiological environment such as infection or heat, these cells swell rapidly, organelles break down and ultimately the plasma membrane ruptures (16). Activated Natural Killer (NK) cells work through perforin/granzyme granules and Fas-mediated pathway to eliminate virally infected cells by apoptosis (4). Apoptosis is mediated by the activation of the caspase endonuclease cascades which results in digestion of DNA (4). NK cells also contain TNF-α in their granules which can induce apoptotic cell death or engage Fas via the Fas-ligand to signal apoptosis (4). The NK cells have two ways of recognising target cells- they have Fc receptors which bind to IgG on target cells and killer-inhibiting receptors which recognise MHC class I molecules on normal cell surfaces which are absent on abnormal cells, these killer-inhibiting receptors would not be engaged on encountering abnormal cells and are thus a positive signal for cytolysis (4).

### 1.1.2 Adaptive Immunity

The adaptive immune system consists of specialised cells, lymphocytes recruited by the innate immune system, which mount a highly specific immune response to pathogen invasion (17). This protection is provided long-term as subsets of lymphocytes are able to remember, recognise and eliminate the same specific pathogens encountered in the future (9). Adaptive immune response occurs in the secondary lymphoid tissues – lymph nodes, spleen and mucosa-associated lymphoid tissue. Initiation of the adaptive immune system occurs when an immature dendritic cell engulfs a pathogen at the site of infection and is activated to form a phagocytic APC (18). The APCs migrate to the local draining lymph node where these phagocytic cells break down pathogens to form antigenic

components, which bind to Class II MHC membrane glycoproteins and insert into the APC cell membrane for activation of T lymphocytes (18).

#### *1.1.2.1 B cells*

B lymphocytes are first sensitized to antigens by APCs which endocytose the antigen and then express it on the cell surface via Class II MHC proteins (3). CD4+ T helper cells recognise these peptide-MHC complexes, become activated and present co-stimulatory molecules such as CD154 (CD40 ligand) (10, 19, 20). CD154 binds to CD40 on the B cell, up-regulating IL-2, IL-4, IL-5 receptors from helper T cells as well as IL-8, IL-12 and TNF-α production from dendritic cells in order to signal B cell activation, antigen presentation and antibody switching (10, 19, 20). If the target cell is somatic, that is a cell that does not participate in reproduction, cytotoxic T effector cells will be mobilized to remove it. However if the target is an APC, CD154 can activate it to express B7 molecules which go on to up-regulate antibody secretion from B cells and macrophage activity (6).

B cells use the antibody molecule as their receptor to recognise epitopes on the surface of the target antigen. Interaction between the epitope and receptor stimulates the B cell differentiation of plasma cells and memory B cells (10, 19). The plasma cells produce and secrete antibodies whose receptors are of the same specificity for the target epitopes on the sensitized B cell (10, 19). Memory B cells are kept in reserve to mount an antibody-mediated response in future presentations of the target antigen, upon subsequent exposure they divide into antibody-secreting plasma cells (19). The antibody produced binds to its corresponding antigen to form an antigen-antibody complex in order to cause the removal of the antigen by neutralisation, agglutination and precipitation, complement activation, phagocytic attraction, opsonisation, and inhibiting bacterial and viral adhesion and further inflammation (10).

#### *1.1.2.2 T cells*

T lymphocytes fall into three major functional groups – T helper cells (Th), cytotoxic T cells (Tc) and suppressor T cells (Ts) (21). Cytotoxic T cells are involved in cell-mediated immunity and directly attack antigens in the peripheral tissue with perforin, granzyme and TNF lytic granules (10). Suppressor T cells uphold peripheral tolerance by moderating T and B cell response whilst helper T cells, which express cluster of differentiation (CD) markers CD4 and CD25, activate B cells to produce antibodies (4, 15). CD markers are membrane protein markers which consist of sub-clusters of monoclonal antibodies, they help determine T cell response to antigens held by Class I or Class II MHCs (19). T cells recognise a complex structure of an antigen peptide bound to an MHC molecule, a cell-surface glycoprotein which binds to either protein fragments synthesized within the cell (Class I) or protein that has been digested proteolytically by the cell (Class II) (15). All T lymphocytes have a CD3 receptor complex marker on their membrane; Tc cells and Ts cells also express CD8 and bind to Class I MHC molecules while T helper cells express CD4 markers and respond to Class II MHC proteins (4).

For complete T cell activation and proliferation, two signals are necessary. The first is interaction between the antigen-specific T cell receptor (TCR) and a peptide-MHC molecule on the surface of the APC. The second is the antigen-nonspecific interaction between co-stimulatory molecules CD80 and CD86 on the APC and CD28 on the T cell. Activation of T cells without co-stimulation would lead to T cell deletion or anergy (6, 15). Anergy is when a cell is rendered hyporesponsive following interaction with an antigen, its proliferation and effector functions are disabled and growth is ceased (22). Self-antigens do not have PAMPs and therefore are not able to induce co-stimulatory activity. Hence T cells directed at self-antigens are rarely activated, showing that innate immunity plays a part in regulating self-tolerance in the adaptive immune response (6).

#### 1.1.2.2.1 T regulatory cells

Under the induction of Tissue Growth Factor-β1 (TGF-β1), Naive CD4+ T cells have the potential to differentiate into T regulatory cells (Tregs) which are generated in the thymus. Tregs suppress T effector cell differentiation, proliferation and induce apoptosis of T cells, playing a crucial role in upholding peripheral tolerance (23). Tregs target $CD4^+$, $CD8^+$ cells, and other cell types such as B cells, monocytes and NK cells (24).

Forkhead box protein 3 (Foxp3) is a transcription factor necessary for the induction of naive $CD4^+$ T cells into $CD4^+CD25^+$ T regulatory cells and serves as a marker for Tregs. It has been shown that increased expression of Foxp3 correlates with increased regulatory function. However, although all Tregs express Foxp3, not all Foxp3 $CD4^+$ T cells can be considered as suppressive Tregs, as activated naive CD4+ T cells can also express Foxp3 in humans (25).

Recent studies have shown that $CD4CD25^{high}$Foxp3 Tregs have a strong propensity to differentiate into IL-17 producing cells under the influence of IL-6, IL-1β, IL-21 and IL-23 and these cells reflected a high expression of retinoic acid-receptor-related orphan receptor-γt (RORγt) which will be explained in section 1.3.4 (26, 27). This newfound plasticity of the Foxp3 Treg suggests that while upholding peripheral tolerance, it may also play a role in inflammation and autoimmunity.

#### 1.1.2.2.2 T helper cell subsets

Th cells arise from a common pre-cursor naive CD4+ cell which, dependent on the cytokine environment, will differentiate into one of three subtypes Th1, Th2 or Th17 cells. Th1 cells secrete IL-2, IL-3, lymphotoxin, granulocyte macrophage colony-stimulating factor (GM-CSF), IFN-γ and TNF-α (18). Th1 cells promote Type I responses which involve phagocytic removal of microbes and the activation of macrophages: IFN-γ, lymphotoxin and TNF-α all upregulate nitrous oxide synthetase in macrophages to increase their phagocytic ability. IFN-γ also facilitates antibody switching in B cells, activates the complement cascade and binds to Fc receptors on macrophages to promote phagocytosis (18).

Th2 cells secrete IL-4, IL-5, IL-6 and IL-13; these cytokines activate mast cells and eosinophils which mediate atopy and parasitic infection. In addition Th2 cells inhibit macrophage activation (28-31). IL-4 and IL-5 up-regulate IgE, IgG and IgM antibody synthesis by B lymphocytes (31, 32). The differentiation of naive T cells into Th1 or Th2 are mutually exclusive processes. Th1 is induced by IL-12 which also inhibits development of Th2 while IL-4 induces Th2, and with IL-10 inhibits the generation of Th1 (28, 30). Stimulation of TLRs on macrophages or DCs will result in the production of IL-12 which would consequently cause a skew toward Th1 cell differentiation (14). T helper cell differentiation is very dependent on master transcription factors such as T box transcription factor (T-bet) for Th1 and GATA binding protein 3 (GATA3) for Th2, which are activated by the induction cytokines IFN-γ (Th1), IL-12 and IL-4 (Th2) via "Signal Transducers and Activators of Transcription" (STAT) proteins STAT-1, STAT-4 and STAT-6 respectively to initiate the differentiation process (33, 34).

Th17 cells are the third T helper cell subset and secrete IL-6, IL-17A, IL-17F, IL-21 and IL-22 which are thought to be involved in pro-inflammatory reactions, especially in autoimmune disease; it will be explored further below in section 1.3 (35).

## 1.2 Juvenile-onset Systemic Lupus Erythematosus

### 1.2.1 Epidemiology and clinical features

Systemic lupus erythematosus (SLE) is a multi-system autoimmune inflammatory disease which affects approximately 25 per 100,000 people in the United Kingdom (36). Most patients are women who present between 20-30 years of age however approximately 20% of SLE patients present during childhood (37). Juvenile-onset SLE (JSLE) prevails in approximately 3.3-8.8 per 100,000 children and is of greater incidence in non-Caucasian populations especially Black African and Asian populations (38). Both adult-onset SLE and JSLE have been shown to be more prevalent in females, with a ratio of 8:1 females to males in adults but 3:1 to 6:1 in children depending upon age and cohort; this lower pre-pubertal sex ratio suggests a probable hormonal factor contributing to the disease onset (38, 39).

The American College of Rheumatology (ACR) criteria defined a person as having SLE if they fulfil four or more of the eleven criteria stated in the 1997 revised classification of SLE (40), as shown in Table 1. SLE is characterised by diverse clinical manifestations, often following a relapsing and remitting course of progression. JSLE patients most commonly present with the fever, lymphadenopathy and renal involvement (41).

SLE is a debilitating lifelong condition, which can impact significantly on a person's quality of life. Quality-of-life scores are lower in patients with JSLE compared to healthy children and paediatric patients with other autoimmune diseases (42). Parents and JSLE patients have also reported significant impact in their attendance and performance at school (43).

JSLE carries a worse prognosis and increased disease severity amongst the younger the age patients compared to adult-onset disease (44, 45). High mortality rates have been reported in JSLE patients, in one study even twice as high as in adult SLE patients (46, 47). In addition, JSLE patients seem to have more active disease over the duration of their disease compared to adult SLE patients (48). Studies have shown that JSLE patients are more likely than adult SLE patients to have neurological and kidney disease, with increased renal damage and

haematological dysfunction (46, 48, 49). Even within the paediatric populations, a younger age (<2 years) denoted a significantly higher prevalence of target organ disease and thrombocytopaenia and anaemia (45).

**Table 1: Revised ACR Classification of SLE 1997(40, 50).**

| Revised ACR Classification Criteria |
|---|
| 1. Malar Rash |
| 2. Discoid rash |
| 3. Photosensitivity |
| 4. Oral ulcers |
| 5. Nonerosive Arthritis |
| 6. Pleuritis or Pericarditis |
| 7. Renal Disorder<br>Proteinuria > 0.5g per day<br>Cellular casts |
| 8. Neurologic Disorder<br>Seizures<br>Psychosis |
| 9. Hematologic Disorder<br>Haemolytic anaemia<br>Leucopenia<br>Lymphopaenia<br>Thrombocytopaenia |
| 10. Immunologic Disorder<br>Anti-dsDNA antibodies<br>Anti-Sm antibodies<br>Antiphopholipid antibodies<br>- Anticardiolipin antibodies<br>- Lupus anticoagulant positive |
| 11. Positive Antinuclear Antibody |

### 1.2.2 Management

JSLE is complex with varying disease manifestations at different stages in individual patients; therefore treating it poses a challenge. The mainstay of therapeutic treatment is the employment of immunosuppressive medication in order to combat the autoimmune properties of the disease. Currently, there is a great paucity of published studies in relation to the management specifically of JSLE, the treatment measures mostly based on extrapolated conclusions drawn from an extensive number of adult SLE trials.

Hydroxychloroquine is an anti-malarial which suppresses neutrophil chemotaxis and is frequently used as an adjunct to non-steroidal anti-inflammatory medication or steroid therapy. It reportedly reduced SLE flares in a double-blind randomised

controlled trial in adult SLE patients and may be protective of lipid profile risks associated with SLE (51, 52). For JSLE patients with significant organ involvement, glucocorticoids are the mainstay of treatment and have been reported to decrease circulating dendritic cells and dampen IFN-α expression in adult SLE patients (53). However prolonged use of high-dose glucocorticoids in children has to be balanced out against worrying adverse effects such as premature atherosclerosis and compromised growth and loss of bone mass (54, 55).

This has led to the use of steroid-sparing immunosuppressive agents such as azathioprine, mycophenolate mofetil (MMF) and cyclophosphamide. Studies examining JSLE patients found that MMF use was effective in a majority of patients and reduced disease activity scores (56, 57). In refractory disease, oral or intravenous infusions of cyclophosphamide has proven to be of benefit with adult lupus nephritis patients; the risk of gonadal damage to patients with cyclophosphamide appears to be less of a concern in the younger age group, but must be taken into consideration (52, 53). Rituximab is a monoclonal antibody directed against B cells and used as a therapeutic option in resistant disease. Rituximab was administered to JSLE patients and found to be efficacious in an uncontrolled study (58). Other novel biologic therapies are also being trialled in SLE, and paediatric trials of these agents are warranted.

The early onset and severe disease in JSLE patients would translate to mean that these patients carry a longer disease burden and therefore larger risk to developing adverse effects associated with these medications. Reported by Tucker et al, JSLE patients received increased doses of corticosteroids and more JSLE patients received IV cyclophosphamide throughout the duration of their disease compared to adult SLE patients (46). Other studies have also shown that significantly more JSLE patients compared to adult patients received corticosteroids and immunosuppressive medication such as azathioprine and methotrexate (41, 48). Although a key to treatment of severe disease, this prolonged use of high-dose immunosuppressive medication in JSLE patients compared to adults highlights the need for rigorous trials investigating the use of these drugs and their long term effects in specifically JSLE patients.

### 1.2.3 Aetiology

The exact aetiology and pathogenesis of SLE is unknown; however factors such as exposure to ultraviolet radiation, infection and chemicals have been investigated. It is hypothesised that SLE is a result of loss of self-tolerance which develops from a cascade of events that occur within innate and adaptive immunity (59). Tolerance is the ability of an organism to recognise and remove a foreign antigen without attacking its own cells. Increased apoptosis and impaired clearance of this apoptotic material have been demonstrated in both adult SLE and JSLE patients; it is thought that the build-up of apoptotic material increases presentation of autoantigens by dendritic cells to T and B lymphocytes (60-62). Lupus T cells are activated by exposure to these autoantigens and drive B lymphocytes to stimulate production of autoantibodies (59). These autoantibodies interact with nucleosomes from apoptotic material to form immune complexes which deposit on target organs, causing inflammation (63). The resultant tissue damage releases more apoptotic cells, which in the presence of antinuclear antibodies, may cause more autoantigens to be taken up and presented by B cells for further T cell activation, driving a exacerbating positive feedback loop (59, 64).

### 1.2.4 T Cells in SLE

CD8+ T cells have been found to have impaired cytoxicity however were found in increased numbers in patients with active SLE (65). CD4+ T cells in SLE have been shown to have abnormal cytokine production and T follicular helper cells are found in high number in SLE which may drive autoantibody production in B cell germinal centres (23, 66).

There is decreased activation threshold in SLE T cells as well as faster and early signalling in SLE T cells stimulated through TCR/CD3. This suggests abnormal T cell function as it results in higher levels of intracellular calcium and hence increased expression of the CD40 ligand (23, 66). Expression of CD40 ligand has been reported to be increased in SLE and this is thought to lead to prolonged co-stimulation of T cells, thereby driving B cell antibody production (67).

As described in section 1.1.2.2, autoreactive cells normally undergo cell death or are rendered anergic. However SLE autoreactive T cells display resistance to anergic induction, decreased apoptosis of autoreactive cells and poor clearance of apoptotic material and these may contribute to abnormal antigen processing (66, 68). There is increased spontaneous apoptosis and necrosis of SLE T cells which may further exacerbate the inflammatory process in SLE due to an increased supply of apoptotic material available to be presented as autoantigens to drive the inflammatory process in SLE (23). In addition, studies indicate possible reduced numbers of regulatory T cells and this is coupled with reduced clearance of autoreactive material to inhibit peripheral tolerance (66, 69).

In SLE, pDCs are activated via TLRs to produce IFN-α and IFN-β, which drives Th1 related inflammation (66, 70). This could suggest a Th1/Th2 imbalance as a pathological explanation for SLE. Defective IL-12 production and increased spontaneous IL-10 production from SLE B cells are a possible explanation for the skew from Th1 to Th2 immune responses which encourage B cell hyperactivity and autoantibody production (71). However it was found that deficiency in Th17-related cytokine IL-23, was protective in mouse models of joint inflammation (collagen-induced arthritis), further enforced by findings that Th1-related cytokine IL-12 was redundant for the induction of disease (72). These conclusions were supported by studies demonstrating a dominant role of IL-23 in mouse models of inflammatory bowel disease and multiple sclerosis (experimental allergic encephalitis), previously thought to be a Th1-driven autoimmune diseases (73-75). This considered, the Th1/Th2 concept was unable to provide an adequate explanation for the development of autoimmune disease (76). In the past decade, many studies have emerged investigating the role of Th17 and its pro-inflammatory cytokines; these suggest that Th17 may be a crucial component in the pathogenesis of autoimmune disease and will be discussed in 1.3 and 1.4 below.

## 1.3 T Helper 17 cell (Th17)

Th17 is a subset of CD4+ T helper cells, shown to be distinct from the Th1 and Th2 lineage (77). IL-6 and IL-1β together with TGF-β initiate Th17 differentiation and these Th17 cells produce IL-6, IL-17A, IL-17F, IL-22 and IL-21 cytokines (Figure 1) (35). The IL-23 receptor (IL-23R) is found on Th17 cells but not on naive $CD4^+$ T cells, therefore IL-23 is postulated to be involved in maturing and enhancing committed Th17 cells (33). In addition, IL-23 promotes IL-17 transcription and upregulates RORγT activity which is essential for the maintenance of Th17 cells (78, 79). Th17 cells have been shown to express IL-1R in order to enable its cells to respond to IL-1 for differentiation and cytokine expression (80, 81). IL-17A promotes B-cell differentiation and autoantibody production, demonstrating Th17 as the main T effector subset in inflammation and autoimmunity as shown in Figure 1, IL-17A will be discussed later in section 1.4 along with lesser known IL-17 subsets (82, 83). In a multitude of previous studies, significantly raised numbers of Th17 cells have been found in the skin, kidneys, circulating blood and urine of SLE and JSLE patients, which suggests that Th17 and its secreted cytokines may hold the key to the aetiology of SLE (84-91).

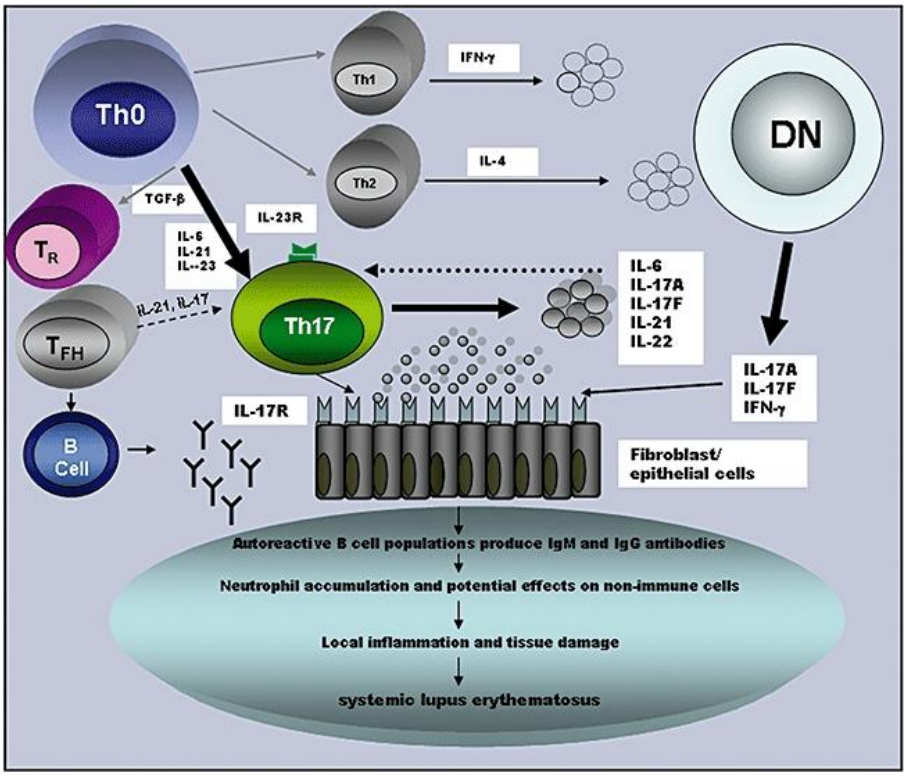



**Figure 1: Proposed model for the role of Th17 and IL-17A cytokines in SLE.**

**Naïve CD4$^+$ T cells differentiate into Th17 cells under the influence of IL-6, IL-1β and TGF-β and are maintained by IL-23. The Th17 cells secrete cytokines IL-6, IL-17A, IL-17F, IL-21 and IL-22. IL-17A is thought to play a role in SLE by promoting B cell differentiation and autoantibody production thereby causing inflammation and tissue damage. This image is taken from Nalbandian et al 2009 (92).**

### 1.3.1 Interleukin-21

IL-21 is produced by CD4+ and NK cells and its receptor IL-21R is expressed on CD4+T cells, CD8+ T cells, Th17 and T follicular helper (TFH) cells (93). IL-21 is induced by IL-6 and RORγt to amplify Th17 differentiation and maintenance, by upregulating its own receptor and IL-23R expression (94, 95). It counteracts Treg cells by down-regulating Foxp3 induction in TGF-β stimulated cells and opposes Treg inhibition of T cell activation and proliferation (96).

SLE T cells secrete higher percentages of IL-21 compared to healthy controls although this did not correlate with disease activity (97). Active and inactive SLE patients have been found to have higher serum IL-21 levels and more IL-21 expressing T cells in comparison to healthy controls. The increased level of IL-21

also correlated with IL-17A CD4+ T cell percentages (97). In addition, IL-21 and IL-21R polymorphisms are associated with increased risk in developing SLE (98). As Th17 cells are a source of IL-21, it is likely that increased IL-21 production encourages the propensity for Th17 cell differentiation to produce more IL-17 which in turn amplifies IL-21 response in an autocrine loop (97). In addition, TGF-β1 in synergy with IL-21 or IL-6 instead may promote Th17 differentiation and suppress Foxp3 transcription factor expression, thereby decreasing Treg cell numbers (99).

Bubier et al demonstrated a severe SLE-like phenotype of hypergammaglobulinaemia, renal disease and premature morbidity in BXSB-Yaa mice which have increased expression of IL-21, while mice that were IL-21 deficient exhibited none of these SLE-like features (100). Blocking the IL-21 pathway has been shown to be successful in animal models of SLE: treatment with IL-21R.Fc reduced renal disease (proteinuria and glomerular basement membrane thickening), skin lesions, lymphadenopathy and circulating auto-antibodies in MRL-Fas$^{lpr}$ mice (101). Remarkably, CD4+ and CD8+ T-cells in the spleen of these mice were also reduced after treatment with IL-21R.Fc, supporting the idea of IL-21 blockade as a novel therapeutic approach (101).

### 1.3.2 Interleukin-23

IL-23 comprises of two subunits IL-12 p40 and IL-23 p19 that link to form its heterodimer cytokine produced by T cells as shown in Figure 2 (102). The receptor of IL-23, IL-23R also shares a common subunit with the IL-12 receptor – IL-12Rβ1(102). IL-23 has been shown to be raised in the serum of patients with autoimmune diseases such as systemic sclerosis or at increased mRNA expression in psoriatic skin lesions (102, 103).

Previously polymorphisms in IL-23 receptor (IL-23R) have been found to be associated with other autoimmune diseases such as inflammatory bowel disease and systemic sclerosis, however several studies have shown no association between IL-23R and SLE in Chinese, Hungarian, Spanish and Korean populations (104-107). A study by Huang et al demonstrated a significantly higher mRNA expression of IL-23 P19 in the peripheral blood mononuclear cells (PBMCs) of adult patients with active

SLE compared to inactive SLE, and this was suppressed by treatment of corticosteroids (108). Recent studies have reported elevated levels of IL-23 expressing T cells in the PBMCs of lupus patients compared to healthy controls which correlated with IL-17 T cell levels (109). In addition, these IL-23 cell percentages were significantly higher than that of inflammatory control psoriatic patients (88, 109). In addition, IL-23 has been shown to be raised in the serum of patients with active SLE with cutaneous manifestations and serositis compared to those without (110). Plasma concentrations of IL-23 have also been shown to be significantly increased in patients with SLE (88, 111). It was demonstrated in a study that IL-23 receptor deficiency in lupus mouse models reduced the production of IL-17A and autoantibodies, preventing the development of signs of lupus nephritis and renal damage (112). These studies suggest that IL-23 upregulation may play a role in the pathogenesis of SLE and therefore tackling the IL-23/IL-17 axis may be a potential therapeutic target in lupus.

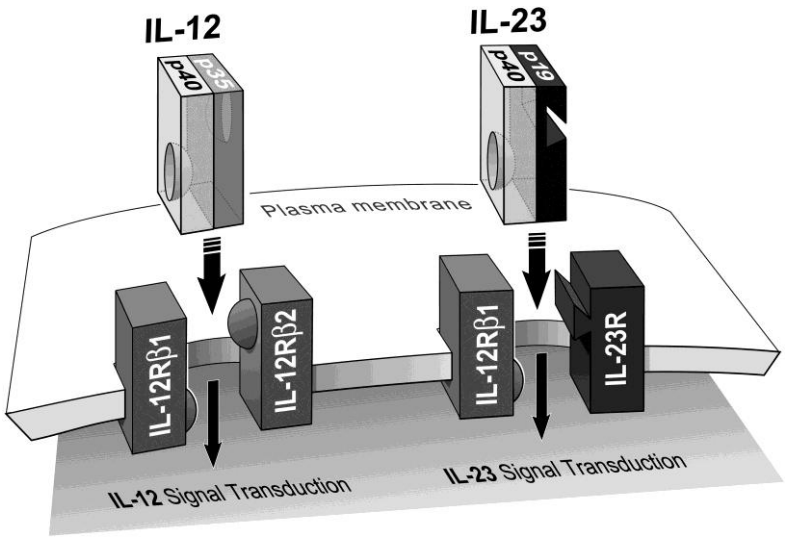



**Figure 2: IL-23 heterodimeric cytokine and its binding to its receptor**

**IL-23 is a heterodimer cytokine which consists of two subunits – IL-12p40 and IL-23p19, while the IL-23 receptor shares a common subunit with the IL-12 receptor – IL-12Rβ1. IL-23 promotes IL-17 transcription and maintains Th17 cells. Image taken from Torti et al 2007 (102).**

### 1.3.3 Interleukin-6

Significantly raised plasma and serum IL-6 levels have been shown in patients with SLE compared to healthy controls and also in active SLE compared to inactive SLE although the level did not correlate with disease activity (91, 110, 113). Furthermore, the combination of a low concentration of TGF-β in addition to IL-21 and IL-6 has been shown to upregulate the IL-23 receptor to promote the differentiation of Th17 cells (114). IL-6 inhibits TGF-β-driven generation of Foxp3+ Treg cells, and instead switches the transcription programme to express naive T cells as IL-17 producing T cells (35). However at high concentrations, TGF-β suppresses IL-23 receptor expression and favours the production of Foxp3+ Treg cells (114). This implies that IL-6 may work as a signal to determine the differentiation of pro-inflammatory (Th17) or suppressive (Treg) cells (92).

In a murine model of lupus it has been demonstrated that IL-6 induces Treg cells to become IL-17 producing effector T cells and by down-regulating IL-6 stimulation the Tregs become resistant to this change (115). It was also shown in a rheumatoid arthritis mouse model that IL-6 blockade inhibited the differentiation of Th17 cells which further substantiates its therapeutic use in SLE patients (116).

Tocilizumab is a monoclonal antibody which suppresses IL-6 signalling by binding to IL-6R receptors; it has been shown to improve disease activity in autoimmune disease such as rheumatoid arthritis and juvenile idiopathic arthritis (JIA) (117). It is possible that blocking IL-6 activity opposes its inhibitory function on T regulatory cell development, hence restoring Treg balance (35). Tocilizumab proved promising in an open-label study with SLE patients where it improved or even resolved arthritis, significantly reduced anti-double-stranded DNA antibody levels and had a positive effect on disease activity scores (118).

### 1.3.4 Retinoic acid-receptor-related orphan receptor-γt (RORγt)

RORγt is a transcription factor induced by TGF-β and IL-6 along the STAT3 pathway that is solely expressed in Th17 cells and is vital for IL-17A and IL-17F production (35, 92). RORγt in Th17 parallels T-bet and GATA3 master regulators in Th1 and Th2 differentiation respectively (70). These transcription factors seem to share a reciprocal relationship where T-bet can suppress GATA3 and RORγt expression while IFN-γ and IL-4 can suppress Th17 differentiation (70). RORγt activation also upregulates the expression of the IL-23 receptor, which would further encourage IL-17 production (119). mRNA expression of RORγt has been reported to be higher in SLE adult patients compared to healthy controls (90). In addition STAT3 and phosphorylated STAT3 have been shown to be upregulated in the T lymphocytes of SLE patients compared to controls on both mRNA and protein levels (120). Th17 cell differentiation is hence very dependent on a presence and concentrations of a significant number of transcription factors and proteins within the cytokine milieu.

## 1.4 The IL-17 Cytokine Family

Th17 cells produce the pro-inflammatory cytokine IL-17A which has been found in increased concentrations in the peripheral blood plasma, serum and $CD4^+$ T cells of adult SLE patients (70, 88, 121). IL-17 comprises of a group of six relatively recently identified cytokines: IL-17A, IL-17B, IL-17C, IL-17D, IL-17E and IL-17F. IL-17A and IL-17F are closely associated with each other sharing approximately 50% of gene sequence identity while IL-17B-to-D share 23-29% homology with IL-17A (122, 123). In addition the IL-17 family also comprises of five receptors: IL-17RA, IL-17RB, IL-17RC, IL-17RD and IL-17RE. IL-17A and IL-17F, which both bind to the same receptors IL-17RA and IL-17RC, drive inflammation and autoimmunity and are produced by CD4+ and CD8+ T cells, Natural Killer cells and neutrophils (92, 94). IL-6 increases IL-17 production by memory T cells, suggesting that it parallels IL-12/Th1 in inducing Th17 differentiation, while IL-23 activates and upregulates IL-17 secreting potential in naive T cells committed to Th17 differentiation (92, 124). Current evidence indicates that IL-17A may factor in the immunopathogenesis of inflammatory clinical manifestations of SLE (70). However research on other members of the IL-17 family is scarce, especially in the context of SLE.

### 1.4.1 IL-17A

IL-17A is involved in the immune response against primarily bacterial and fungal pathogens such as *Klebsiella pneumoniae and Candida albicans* (35, 92). IL-17A increases production of chemokines such as IL-8 and growth-regulated oncogene alpha and growth factors, granulocyte colony-stimulating factor (G-CSF) and GM-CSF, where these chemokines and growth factors recruit and develop neutrophils and monocytes (92, 125). This induces IL-6 and prostaglandin-E production, which encourages effector T cell infiltration and activation and enhances the immune response (92, 125). IL-17A also participates in the adaptive immune response by proliferating plasma cells and maintaining B cell survival (126). This increases autoantibody synthesis and proliferation as evidenced by increased anti-dsDNA and IgG production in lupus PBMCs compared in healthy controls (127). In addition it stimulates macrophages to produce IL-1β and TNF-α inflammatory cytokines which amplify tissue pro-inflammatory response (125). IL-6 is a

differentiation factor of Th17, hence increased induction by IL-17A would commit more naive T cells to the Th17 lineage (35). IL-17A has been shown to play a role in autoimmune conditions such as JIA, rheumatoid arthritis and psoriasis thus demonstrating that IL-17A and Th17 cells may act as pro-inflammatory agents in autoimmune disease (94, 128, 129). Adding to this, two recent double-blind, randomised placebo-controlled trials have reported significant improvements in psoriasis severity scores in patients treated with an anti-IL-17 monoclonal antibody or an anti-IL-17RA antibody, indicating the therapeutic value in tackling this pathway in autoimmune disease (130, 131). Previously, IL-17A has been found at significantly higher levels in the synovium and synovial fluid compared to the peripheral blood of patients with rheumatoid arthritis and JIA, indicating that IL-17A may be more localised to the site of inflammation (128, 132).

Adult SLE patients have been found to have increased numbers of IL-17A producing T cells and raised IL-17A, IL-17F and IL-23 plasma and serum levels in comparison to healthy controls (23, 82, 84, 88, 92, 110). In addition, these raised levels of IL-17A showed correlation with disease activity scores (91, 133). Increased IL-17A levels have also been found in the circulation and tissues of both murine models of lupus and adult lupus patients (70). Stimulation of CD4+ T cells with CD3/CD28 resulted in increased IL-17A in SLE adult patients compared to healthy controls while there were no differences between control and psoriatic patients, indicating that SLE CD4+ T cells expand more readily and skew toward IL-17A production (88, 109). Lupus patients have also been shown to have increased IL-17A expression in their kidneys and urine sediments, suggesting that IL-17A could play a role in target organ damage (121). Its strong presence at target organs may be causing an increased deposition of autoantibody-nucleosome immune complexes causing tissue inflammation (127). In addition, IL-17A has been found in a vast majority of skin tissue samples of patients with cutaneous and systemic lupus which correlated with IFN-α expression (134). Animal studies have shown that blocking IL-17A reduces lupus manifestations, which may prove to be therapeutically useful for SLE patients (92).

### 1.4.2 IL-17B

IL-17B and IL-17C have previously been found to stimulate the secretion of TNF-α and IL-1β from THP-1, a monocytic cell line (135). In a study reported by Yamaguchi et al, mRNA expression of IL-17A, IL-17B, IL-17C and IL-17F have been found to be elevated and associated with IL-6, IL-23 TNF-α and IL-1β production in the arthritic paws of murine arthritis models (136). Adding to this, flow cytometry found that CD4+ T cells from the arthritic paws significantly expressed IL-17A and IL-17F, while IL-17B was only expressed in the chondrocytes of the inflamed joints of these mice (136). This suggests that CD4+ T cells may express members of the IL-17 family mainly at the site of inflammation. The study also reported that blocking IL-17B, as well as IL-17A, suppressed the progression of arthritis in these mice, suggesting therapeutic potential (136).

### 1.4.3 IL-17C

In the study mentioned in section 1.4.2 by Yamaguchi et al, it was found that within mice with inflammatory arthritic paws, IL-17C was expressed not only by macrophages and dendritic cells but also by CD4+ cells (136). IL-17C has been shown to bind to an IL-17RA-IL-17RE heterodimer receptor cytokine to induce the expression of genes encoding chemokines such as IL-8 and pro-inflammatory cytokines such as TNF and IL-1β, bearing inflammatory similarities to IL-17A (135, 137, 138).

A recent study has shown that mRNA expression of IL-17C is increased in the central nervous system of mice with autoimmune encephalomyelitis (EAE) and that knocking out IL-17C in these mice showed reduced disease incidence and severity, as well as decreased expression of Th17 related cytokines IL-17A, IL-17F, IL-22 (139). In addition, increasing concentrations of IL-17C led to a significantly raised expression of IL-17A and IL-17F protein production in Th17 cells (139). However this study also found that IL-17C expression was increased in the localised inflamed central nervous system tissues as compared to the spleen, which led to the conclusion that Th17 cells may migrate to affected tissues where it is potentiated by IL-17C for local tissue inflammation. This is further supported by a study reporting

increased IL-17C expression in synovial fluid mononuclear cells (SFMCs) and PBMCs of patients with rheumatoid arthritis (140).

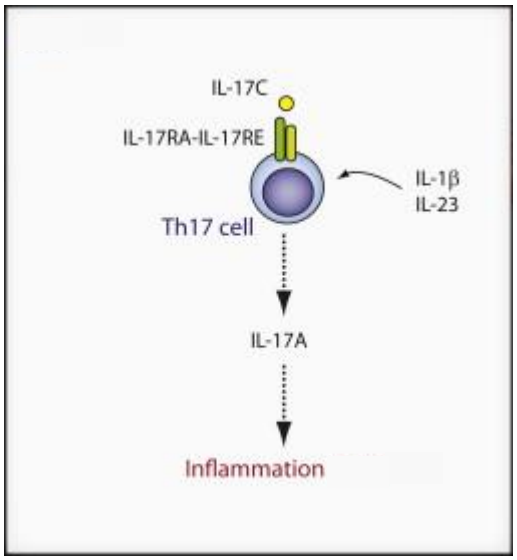



**Figure 3: IL-17C binds to an IL-17RA-IL-17RE heterodimer (139).**

**IL-17C has been shown to bind to an IL-17RA-IL-17RE heterodimer receptor cytokine to induce the expression of chemokines and pro-inflammatory cytokines to potentiate the production of IL-17A. Image adapted from Haines et al 2011 (141).**

### 1.4.4 IL-17RE

It has been found in previous studies that IL-17RE mRNA expression is upregulated in Th17 cells and not expressed in naive T cells, and that IL-6 and TGF-β cytokines regulate this expression while IL-1 and IL-23 promote it (139, 142). In addition Chang et al found that in the presence of IL-1β and IL-23, IL-17C binds to a IL-17RA - IL-17RE heterodimer to potentiate IL-17A cytokine production, and that increased IL-17RE signalling led to raised susceptibility to EAE in mice (see Figure 3) (139). Several other studies have also implicated IL-17RE as a crucial subunit of the heterodimer receptor through which IL-17C signals (137, 138).

Although the current evidence base is limited, the conclusions drawn from the aforementioned studies indicate IL-17 cytokine subsets may have a part to play in autoimmune pathology and would therefore be useful to explore in our subset of patients with juvenile-onset SLE.

## 1.5 Summary

The introduction sections above discuss the crucial potential role T cells play in the aetiopathogenesis of lupus, and how abnormal T cell function may contribute to its pathogenesis. Significant findings have been reported in IL-17A, IL-21 and IL-23 expression in adult-onset SLE and other autoimmune conditions. In addition, IL-17B, IL-17C and IL-17RE have been shown to factor in autoimmune pathology. Therefore, the current available evidence base highlights the importance of studying T cells, especially Th17 cells and its related cytokines in the context of JSLE.

## 1.6 Aim and Hypothesis

### 1.6.1 Aim

To optimise the measurement of the IL-17 family and Th17-related cytokine expression at an mRNA and protein level, in order to investigate their roles in the pathogenesis of JSLE in our subset of patients.

### 1.6.2 Hypothesis

Increased expression of Th17-related cytokines, namely IL-17A, IL-21 and IL-23 cytokines, and IL-17 family cytokines, IL-17B, IL-17C and IL-17RE are present in the peripheral blood of our subset of JSLE patients, thereby indicating a crucial role of Th17 cells and the IL-17 family in the pathogenesis of JSLE.

# Methods

## 2.1 Patient Demographics and Blood Sample Collection

Written and informed patient assent and parental/guardian consent were obtained from all participants who were recruited from wards and outpatient clinics at Alder Hey Children's National Health Service (NHS) Foundation Trust, a copy of the UK JSLE Cohort Study and Respository patient information sheet and parental consent consent form are detailed in Appendices 1 and 2. All JSLE patients fulfilled the revised ACR criteria for SLE diagnosis as stated in 1.2.1 prior to the age of 17 years. All JSLE patients had been recruited to the UK JSLE Cohort Study & Repository (described in Watson et al 2012) for which full ethical approval was granted (ethical approval letter listed in Appendix 3) (143). Healthy controls were paediatric patients, without intercurrent infection or autoimmune history, who were attending for elective day surgery and who had been evaluated beforehand for non-inflammatory musculoskeletal features. Inflammatory paediatric controls were JIA patients who met the International League of Associations for Rheumatology Criteria for JIA (144).

5-10 mL of venous blood was collected during routine blood clinical monitoring according to the Study protocol, anonymised and transferred immediately after phlebotomy to the laboratory for processing which will be further discussed in 2.2.

## 2.2 Blood Sample Processing

### 2.2.1 Polymorphprep™ isolation of PBMCs and neutrophils

Patient venous blood samples were layered at a 1:1 ratio over Polymorphprep™ (Axis Shield, Norway) and centrifuged at 1800 rpm for 30 minutes at 20°C, allowing the rotor to decelerate without brake. This produced two distinct layers of PBMCs and neutrophil granulocyte which were then isolated individually and washed in 10 mL of RPMI-1640 (Lonza, Switzerland) at 2000 rpm for 10 minutes with brake. The media was discarded and resultant pellets were re-suspended in 1 mL of RPMI-1640 media and 9 mL of 1% ammonium chloride lysis buffer for three

minutes in order to lyse red blood cells. These were subsequently topped up with RPMI-1640 media and centrifuged at 2000 rpm for 5 minutes. If the PBMC sample was not required for immediate use it was frozen down by adding 1 mL of freezing media (10% dimethyl sulfoxide in foetal calf serum (FCS) before storing the sample at -80°C overnight, after this time it was placed in liquid nitrogen storage.

### 2.2.2 Plasma and serum

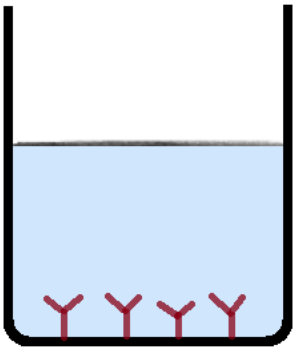
Plasma and serum were collected from patient venous blood samples obtained with or without anticoagulant respectively. The blood samples were centrifuged at 2000 rpm for 10 minutes. Plasma and serum were then pipetted in 100-200 µL aliquots and stored in -80°C conditions.

## 2.3 Enzyme-linked Immunosorbent Assay (ELISA)

An enzyme-linked immunosorbent assay (ELISA) is used to detect the amount of a target analyte in a liquid sample. ELISAs used in this thesis followed a sandwich ELISA methodology. All ELISAs were conducted and reagents made up according to manufacturers' instructions. As shown in Figure 4, a cytokine-specific monoclonal antibody is first coated overnight on a 96-well plate. The plate is washed the following day in a series of buffer washes according to manufacturer's instructions using Bio-Tek ELx50 microplate strip washer. Standards and samples are added to the wells where the targeted cytokine binds to the capture antibody. Weakly positive antibodies are removed in a series of buffer washes. Following this, a biotinylated anti-human detection antibody is added to form an antibody-antigen-antibody complex. Avidin-horseradish peroxidase and a substrate solution is added to each well to metabolise a blue coloured product in proportion to the amount of specific cytokine present in each sample. A stop solution consisting of $H_2SO_4$ then changes the reaction colour to yellow and the absorbance is read at 450 nm with spectrophotometer (Bio-Tek ELx800, BioTek, USA) and compared to the standard curve in order to quantify the cytokine of interest.

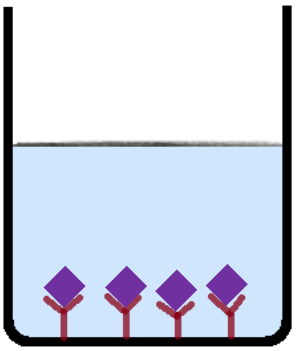
Figure 4: Enzyme-linked Immunosorbent Assay (ELISA)

1



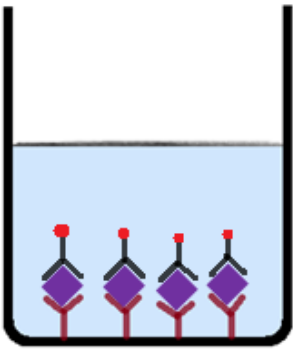
Wells coated with capture antibody

2



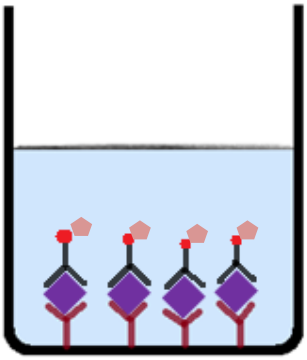
Target cytokine binds to capture antibody

3



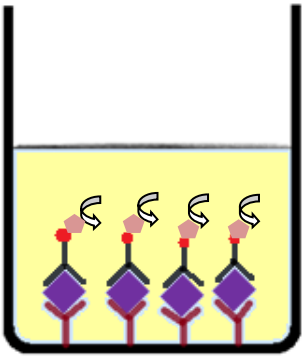
Biotinylated detection antibody forms antibody-antigen-antibody complex

4



Avidin HRP binds to detection antibody

5



Addition of substrate and subsequent addition of stop solution changes the solution from blue to yellow. The plate is then analysed with a photometer

## 2.4 Spike-and-recovery Experiments

A spike-and-recovery assessment is used to determine the difference in analyte recovery between a sample matrix and the standard diluent. Generally 80 to 120% is considered an acceptable range of analyte recovery. A linearity-of-dilution experiment involves diluting the sample, spiking each dilution level with the same amount of analyte and measuring its recovery of the spike against recovery at each dilution level. Percentage recovery of respective cytokines in spiked and unspiked samples were then calculated and compared against the concentration of spiked sample diluents.

### 2.4.1 Spike-and-recovery ELISAs

Spike-and-recovery and linearity-of-dilution experiments were conducted using IL-17A (Human IL-17A ELISA Ready-Set-Go Kit, eBioscience, USA), IL-21 (Human IL-21 ELISA MAX Deluxe, Biolegend, USA) and IL-23 (Human IL-23 DuoSet, R&D Systems, USA) ELISA kits. Three JSLE plasma samples and one control plasma samples were assayed in the IL-21 and IL-23 spike-and-recovery ELISAs, while two JSLE serum and two JSLE plasma samples were used in the IL-17A ELISA. JSLE patient and healthy control samples were randomly selected prior to experiment. The ELISAs were conducted according to manufacturer's instructions and following the principles stated in 2.3.

### 2.4.2 IL-17A Single Plex Spike-and-recovery

A spike-and-recovery assessment with IL-17A was performed with 5 JSLE serum samples with a single plex Bio-Plex Pro™ Assay (Bio-Rad, UK), a bead-based technology of similar principle to that of an ELISA. In an IL-17A single plex assay, capture antibodies directed against IL-17A covalently bind to specific colour coded magnetic beads; these coupled beads are allowed to react with the sample containing IL-17A. The assay is washed several times in order to exclude any unbound cytokines and a biotinylated detection antibody directed against different epitopes on IL-17A is then added to form an antibody-antigen-antibody sandwich complex. Streptavidin-phycoerythrin (SA-PE) conjugate is added as a fluorescent indicator to form the detection complex. The beads are then read individually by a

specialised microtiter plate reader to detect IL-17A and the concentrations are calculated from a prepared standard curve.

Briefly, a provided vial of standards was reconstituted in Bio-Plex standard diluent and serially diluted according to manufacturer's instructions. The serum samples were diluted to 1 in 2, 1 in 4, 1 in 8 and 1 in 16 concentrations in the Bio-Plex standard diluent and spiked with 2000 pg of IL-17A standard. The 96-well filter plate were pre-wet and vacuum filtered, and 50 µL of 1X diluted coupled beads in assay buffer were added to each well.

The wells were washed twice by adding 100 µl of wash buffer each time to each well and the liquid was removed by vacuum filtration on a calibrated vacuum apparatus and the plate was blotted on a clean paper towel in between washes. Then 50 µL of diluted standard or sample was added to each well, and the plate was covered and incubated on a shaker at room temperature at 1,100 rpm for 30 seconds then 300 rpm for 30 minutes. The wells were washed three times following the same wash method previously described and 25 µL of 1X diluted detection antibodies was added to each well. The plate was covered and incubated on a shaker at room temperature at 1,100 rpm for 30 seconds then 300 rpm for 30 minutes.

The plate was washed three times and 50 µL of 1X diluted streptavidin-PE was added to each well. The plate was covered and incubated on the shaker at room temperature at 1,100 rpm for 30 seconds then 300 rpm for 10 minutes. The wells were washed three times, 125 µL of assay buffer was added to each well and the plate was sealed and incubated at room temperature on a shaker for 30 seconds at 1100 rpm.

The plate was then read using the Bio-Plex 200 System (Bio-Rad, USA) reader at low RP1 target default setting on the Bio-Plex Manager 6.0 (Bio-Rad, USA) software to detect IL-17A at bead region 76. Percentage recovery of respective cytokines in spiked and unspiked samples were then calculated and compared against the concentration of spiked sample diluents.

## 2.5 Isolation of $CD4^+$ T Helper Cells

PBMCs were isolated from anti-coagulated peripheral blood as described in 2.2.1 above.

### 2.5.1 Magnetic labelling and separation with CD14 Microbeads

$CD14^+$ monocytes weakly express CD4 antigen therefore these cells must be separated out of the PBMCs before $CD4^+$ cells can be isolated. Microbeads (Miltenyi Biotec GmbH, Germany) were used to magnetically label monocytes and these were then separated using a MACS MS Column (Miltenyi Biotec GmbH, Germany) according to manufacturer instructions. Briefly, cell numbers were determined using a haemocytometer and the cell suspension was spun at 300xg for 10 minutes and resulting pellet was resuspended in 80 µL per $10^7$ cells of sterilised wash buffer solution consisting of 0.5% FCS in Phosphate buffered saline (PBS) with 2mM EDTA. 20 µL per $10^7$ cells of CD14 Microbeads (Miltenyi Biotec GmbH, Germany) was added to the suspension which was then incubated for 15 minutes at 4ºC. Cells were then washed with wash buffer and centrifuged at 300xg for 10 minutes; resulting pellets were resuspended in 500µL wash buffer solution.

A MS column (Miltenyi Biotec, GmbH, Germany) was placed in the magnetic fields of the MACS Separator and washed with 500 µL of wash buffer solution. The $CD14^+$ labelled cell suspension was pipetted onto the MS column and rinsed three times with 500 µL of 0.5% FCS buffer solution. The buffer solution was added each time only when the column reservoir was empty. The flow-through of CD14-negative unlabelled cells was collected and centrifuged at 2000 rpm for 5 minutes and the resulting pellet was suspended in 1 mL of RPMI with 10% FCS.

### 2.5.2 Magnetic separation of monocyte-depleted PBMCs with CD4 Microbeads

Cell numbers of monocyte-depleted PBMCs were determined using a haemocytometer before the cell suspension was centrifuged at 300xg for 10 minutes. The resulting pellet was resuspended in 80 µl per $10^7$ cells of buffer solution consisting of 0.5% FCS in PBS with EDTA. The cells were labelled with 20 µl per $10^7$ cells of CD4 Microbeads (Miltenyi Biotec, GmbH, Germany) and then incubated for 15 minutes at 4ºC. Cells were then washed with wash buffer and

centrifuged at 300xg for 10 minutes and the resulting pellet was resuspended in 500 µL of wash buffer.

CD4-labelled cells were separated using the magnetic MS column (Miltenyi Biotec, GmbH, Germany) as described in 2.5.1 above. 1 ml of wash buffer was added to the positive fraction and centrifuged at 2000 rpm for 5 minutes at 4˚C. The supernatant was removed and resulting pellet resuspended in 1 mL RPMI with 10% FCS and transferred to an eppendorf tube. The eppendorf was centrifuged at 8000 rpm for 10 minutes at 4˚C and $CD4^+$ RNA is extracted from the solution based on the procedure described in 2.6 below. Alternatively, a cell count of $CD4^+$ cells is performed, the eppendorf is centrifuged at 8000rpm for 3 minutes at room temperature and $CD4^+$ protein is prepared using the protein sample preparation process described in 2.11.2 below.

## 2.6 RNA Extraction

RNA was extracted using the RNeasy kit (Qiagen, UK) according to manufacturer's instructions. Red blood cell lysed PBMCs or $CD4^+$ cells were resuspended in 1 mL of RPMI-1640 and centrifuged at 4°C for 10 minutes at 8000 rpm. 350 µL of lysis buffer consisting of 1% beta-mercaptoethanol in Buffer RLT (Qiagen, UK) was used to resuspend with the sample. The lysate was pipetted directly onto a QIAshredder spin column (Qiagen, UK) and centrifuged for 2 minutes at 10,100 rpm. 350 µL of 70% ethanol was mixed in with the lysate to encourage selective binding of RNA. The sample was then transferred to an RNeasy spin column (Qiagen, UK) and centrifuged at 10,100 rpm for 20s, the flow-through was discarded and this process was repeated with 700 µL of Buffer RW1 (Qiagen, UK) followed by 500 µL of Buffer RPE (Qiagen, UK) to wash the spin column membrane. Then 500 µL of Buffer RPE was added to the column which was centrifuged for 2 minutes. The RNeasy spin column was transferred to a new collection tube and centrifuged to eliminate Buffer RPE carryover. The column was then placed onto a clean eppendorf and 40 µL of RNAse-free water (Qiagen, UK) was added directly to the spin column membrane and centrifuged for 1 minute to elute the RNA. The resultant flow-through was stored at -80°C.

## 2.7 RNA Quantification

A NanoDrop ND-100 spectrophotometer and software (Thermo Scientific, USA) was used to determine the quantity and concentration of RNA extracted. The spectrophotometer was initialised with 1 µL of RNAse-free water and then blanked with 1 µL of RNAse-free water. 1 µL of the RNA sample was then loaded onto the lower optical surface of the spectrophotometer and measured. The optical surfaces were cleaned with 1 µL of RNAse-free water in between multiple samples. The concentration of the RNA sample was measured in ng/µL. The absorbance was read at 260 nm and 280 nm and the quality was expressed as a 260/280 ratio which is the sample absorbance value obtained at 260 nm divided by the value obtained at 280 nm. A range of 1.8-2.2 is a generally accepted range for RNA purity, any deviation from this range suggests the presence of nucleic acid contamination. All RNA was stored at -80˚C after NanoDrop measurement.

## 2.8 Complementary DNA (cDNA) Synthesis

Complementary strands of DNA were synthesized from RNA by the process of reverse transcriptase. 100 -200 ng of RNA in a total volume of 14 µL was used for the cDNA synthesis. The volume of RNA solution needed was calculated using NanoDrop concentration results and RNAse-free water was titrated according to RNA volumes for a total volume of 14 µL per sample. 1 µL of random primers (Promega, USA) was then added to each 14 µL which were subsequently heated up to 70°C for exactly 5 minutes on pre-heated heat-blocks. After this time, 5 µL of M-MLV RT 5X Buffer (Promega, USA), 1.25 µL of PCR nucleotide mix (Promega, USA), 0.5 µL of RNasin Ribonuclease Inhibitor (Promega, USA), 1µL of M-MLV Reverse Transcriptase (Promega, USA) and 2.25 µL of RNAse-free water were added to each sample solution. The samples were then heated up to 42°C on pre-heated heat-blocks for exactly 1 hour and 75 µL of RNAse-free water was added to each 25 µL of cDNA to make up a 1 in 4 dilution factor. The cDNA samples were stored in a -20°C freezer or used immediately for PCR to avoid degradation.

## 2.9 Quantitative Real-Time Polymerase Chain Reaction (qPCR)

### 2.9.1 Polymerase Chain Reaction (PCR)

PCR is an experimental technique designed to exponentially amplify specific short DNA sequences within a larger DNA molecule. Real-time quantitative PCR (qPCR) allows measurement of the amplification process during the early phases of the reaction as opposed to traditional PCR which only measures at the end-point, which is not as sensitive or precise.

In both PCR and qPCR, the sample is heated to 95°C at the start in order to activate the Taq polymerase molecule. PCR amplification then follows on and it consists of three stages. At the denaturation stage, the sample is heated up to 95°C, breaking bonds in the helical structure so that the two strands of the DNA molecule separate. The annealing stage involves the sample being cooled down to 50-60°C and a pair of primers that are complementary to the specific DNA sequence on each of the two strands of DNA anneal to their target sites. During the extension phase, the sample is heated to 72°C and a thermo-stable Taq polymerase is employed to extend the cDNA strand by base-pairing free-floating nucleotides in a 5' to 3' direction. The process is repeated for 30-40 rounds, where newly formed cDNA strands act as templates for doubling the numbers of newly synthesised strands each round.

### 2.9.2 SYBR Green qPCR

SYBR Green Real-Time PCR amplifies a specific DNA segment and quantifies the amount of fluorescence given off by each sample and compares it back to its standard curve. SYBR Green binds to double-stranded DNA and is released when the double strand denatures during the PCR reaction, on completion of polymerisation and extension of amplified double strands, SYBR Green binds to these amplified cDNA and fluoresces. SYBR Green increases the intensity of fluorescent emissions as the amount of double stranded amplicons produced increases. A dissociation curve is graphed at the end stage of the run which purpose is to describe the melting point of the sample. As SYBR Green only binds to double stranded DNA, there is a drop in the fluorescence measured as the strands separate

over a specific narrow temperature range as they are heated up. If there is only one product of the reaction, the dissociation curve would demonstrate one major drop in fluorescence represented by one dominant peak on a graph comparing fluorescence to temperature, multiple peaks would indicate multiple products or contamination (see Figure 5 for an example of this).

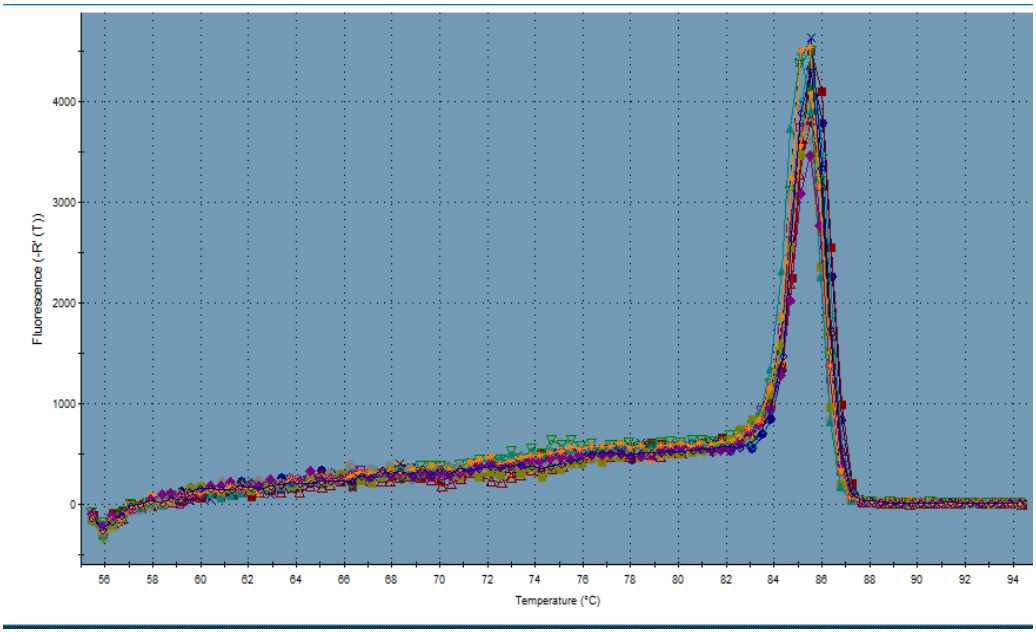



**Figure 5: One dominant peak on a qPCR dissociation curve**

**An example of a dissociation curve, from an assay of JSLE and healthy control CD4+ RNA samples that have been measured for B-actin expression using SYBR Green qPCR. A drop in fluorescent emission of SYBR Green occurs when double-stranded DNA separate. If there is only one major product, this is indicated by one dominant peak on the dissociation curve as pictured. However several peaks indicate multiple products or contamination.**

Standard curve volumes consisting of equal amounts of cDNA samples were calculated and prepared at 1 in 2, 1 in 5, 1 in 10 and 1 in 20 dilution levels. Specific reference gene primers were selected and 0.75 µL of both forward and reverse primers (Eurofins MWG Operon, Germany) were pipetted into PCR plate wells. Primer sequences are listed in Table 2 below.

**Table 2: SYBR Green qPCR primer sequences**

| Primer set | Forward sequence | Reverse Sequence |
|---|---|---|
| **IL-17A** | 5'GAATCTCCACCGCAATGACGACCC-3' | 5'-GTTGATGCAGCCCAAGTGGCG-3' |
| **Beta actin** | 5'-AGATCAAGATCATTGCTCCTCCTG-3' | 5'-CATTTGCGGTGGACGATGGA3' |
| **Beta-2-microglobulin** | 5'TGCCTGCCGTGTGAACCATGT-3' | 5'-TGCGGCATCTTCAAACCTCCATGA-3' |
| **RPL13A** | 5'-TTTCCAAGGGGCTGGCGAAG-3' | 5'TTCCGGCCCAGCAGTACCTGTT-3' |

3.5 µL of RNAse-free water and 10 µL of PerfeCTa SYBR Green FastMix (Quanta Biosciences, USA) are then added to each well. 5 µL of H2O (as a non-template control), standard sample or cDNA sample are added in triplicates to each well and capped. The plate is then pulse centrifuged at 1200 rpm for 20 seconds. The plate is then run through the thermal profile shown in Figure 6 below and analysed using the MxPro – Mx3005P software (Stratagene, USA).

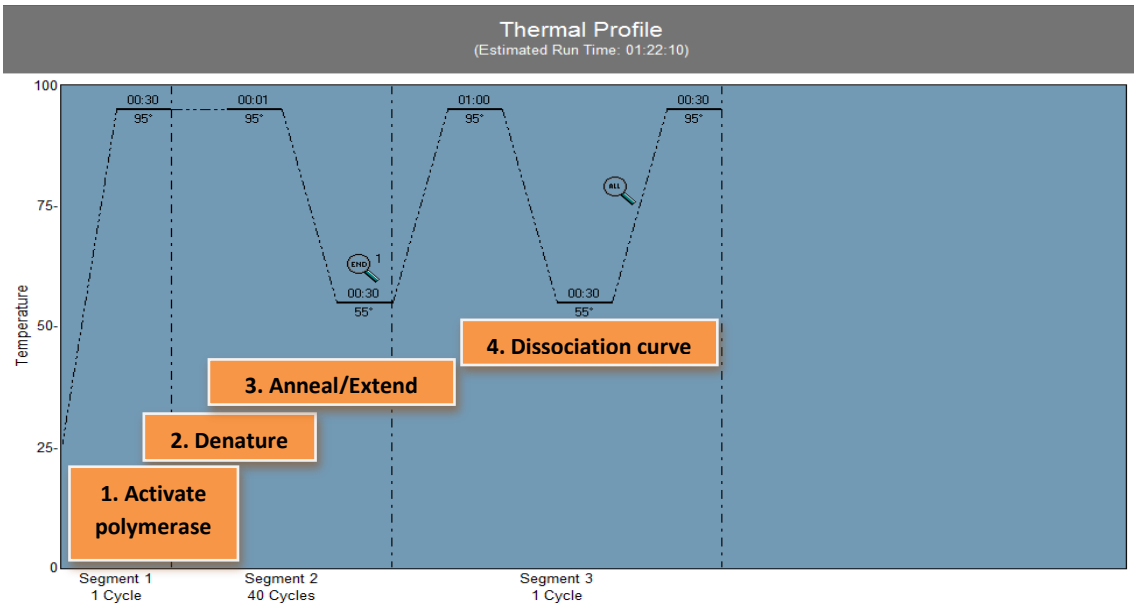



**Figure 6: Thermal profile and stages of SYBR Green qPCR cycle**

**SYBR Green qPCR experiments were conducted using IL-17A, B-actin, RPL13A and Beta-2-microglobulin primers following this thermal profile. The sample mixtures are initially heated to 95°C for 30s to fully activate the DNA Taq polymerase. This is followed by 40 cycles of 95°C denaturing and 55°C annealing and extension stages for PCR amplification. The last segment of the thermal cycle involves the generation of a dissociation curve by denaturing a DNA double helix.**

### 2.9.3 Taqman Probe qPCR

However, while SYBR Green binds to any double stranded DNA molecule, the Taqman Probe qPCR assay is more specific to a pre-determined target. An oligonucleotide, Taqman Probe, which is designed to anneal to a specific sequence of template between the forward and reverse primers, is added to the PCR reagent master mix. During extension of the PCR phase, Taq polymerase adds nucleotide bases to a growing chain of DNA while removing obstacles downstream. The Taqman Probe consists of a reporter at the 5' end and the quencher at the 3' end. The reporter is suppressed by the quencher while the probe is intact. When the Taq polymerase collides with the Taqman Probe, the probe is cleaved and the reporter starts to fluoresce as it is separated from the quencher. The increase in reporter signal is measured by the sequence detection software over time and is directly proportional to the amount of amplicons in a given sample.

**Table 3: Taqman primers and probes used**

| Taqman primer and probe | Assay probe number |
|---|---|
| **IL-17B** | HS00975262_m1 |
| **IL-17C** | HS00171163_m1 |
| **IL-17RE** | HS00979824_m1 |
| **18S** | HS03928990_g1 |

Taqman Probe qPCR was employed to measure mRNA expression of IL-17B, IL-17C and IL-17RE against housekeeping gene 18S in JSLE and control PBMC samples. Housekeeping gene choice was based on a previous study which found in three housekeeping genes that normalised results were the most reliable and stable across both resting and activated T lymphocytes (145). The probe numbers of commercially available Taqman primer and probe mix used are listed in Table 3. 1.25 µL of the specific primer and Taqman probe mix (Applied Biosystems, Roche, USA), 12.5 µL of TaqMan® Universal PCR Master Mix, No AmpErase® (Applied Biosystems, Roche, USA) and 6.25 µL of RNAse-free water are added to each well. 5 µL of H2O (as a non-template control) or cDNA sample are added in triplicates to

each well and capped. The plate is then pulse centrifuged at 1200rpm for 20 seconds. The plate is then run through the thermal profile shown in Figure 7 below and analysed using the MxPro – Mx3005P software (Stratagene, USA).

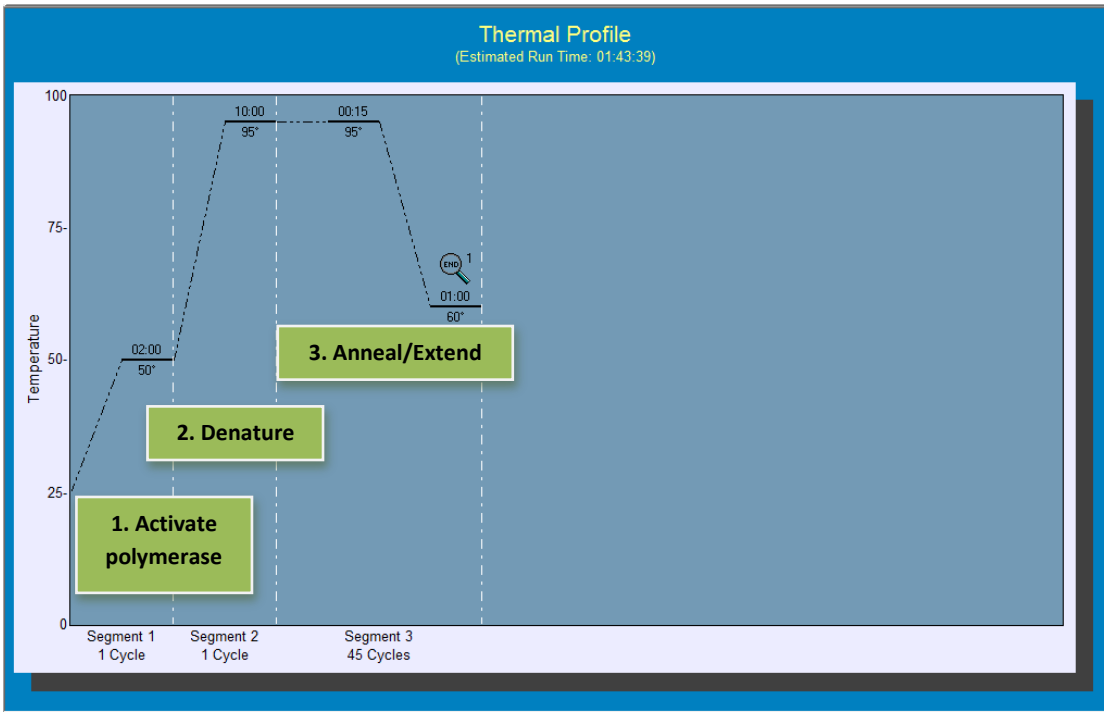



**Figure 7: Thermal profile and stages of Taqman Probe qPCR cycle**

**qPCR experiments using Taqman probes for IL-17B, IL-17C, IL-17RE and 18S in JSLE and control PBMCs were conducted following the thermal profile. The samples mixtures were heated up to 95°C for 10 minutes for polymerase activation, followed by 45 cycles of 95°C denaturing and 60°C annealing and extension stages for PCR amplification.**

### 2.9.4 Interpretation

In qPCR there is a doubling of fluorescence over each cycle, efficiency measures how effectively this amplification cycle occurs. The threshold line is the point at which the fluorescent emissions reach intensity above background level and the cycle number at which this threshold is crossed is called the cycle threshold (Ct); hence the higher the Ct value the less mRNA is detected in the sample. The amplification plot of a qPCR curve tends to consist of an exponential phase followed by a plateau phase. Results were obtained from triplicate samples and expressed as mean expression for the cytokine of interest relative to the expression of the housekeeping gene. In SYBR Green qPCR, a standard curve is generated by

correlating the amount of fluorescence expressed by five standards, which consist of serially diluted samples, against the concentrations of these standards (see Figure 8 as an example). In 100% efficiency, all the values will fall exactly on this correlation curve. For this study, 90-110% efficiency was taken to be acceptable range.

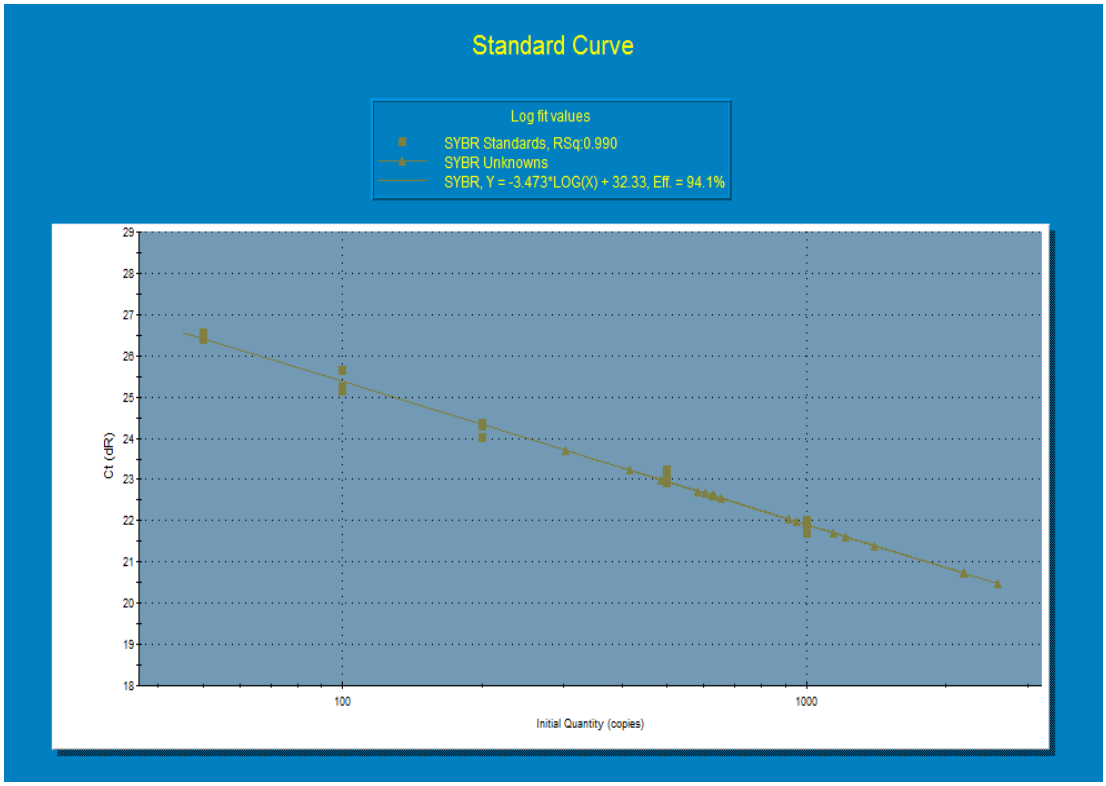



**Figure 8: Standard curve of SYBR Green qPCR experiment**

**The standard curve measures the efficiency of the amplification reaction and involves plotting Ct values of five serial dilutions of the same standard against their relative concentrations. An efficiency of 100% occurs when all values fall onto the same line.**

Interpretation of results for IL-17A SYBR Green qPCR experiments involved taking into consideration the use of a standard curve, hence qPCR normalisation values were calculated using the relative standard curve method by dividing the number of gene copies of each target gene by the number of gene copies of the internal control. While for the IL-17 family TaqMan Probe qPCR experiments, relative expression was calculated as $2^{\Delta Ct}$ where ΔCt equated to the Ct value of the target gene subtracted from the Ct value of the internal control. These values were

then analysed for statistical differences between patient groups. Relative quantification of the fold change of JSLE samples were compared against the control calibrator of value 1 where relative quantification was calculated according to $2^{-\Delta\Delta Ct}$, where ΔΔCt equated to the difference in ΔCt between the sample and the calibrator, ΔCt was the difference in Ct values between the target gene and internal control in each sample (146). A relative quantification value of less than 0.5 or more than 2.0, a minimum two-fold change, was considered to be significant.

## 2.10 T cell activation with CD3/CD28 beads

The aim of T cell stimulation is to induce T cell expansion and propagation without the need of an antigen whilst still retaining its antigen specificity (147). Monoclonal antibodies to CD3 induce $CD4^+$ cell growth by triggering the TCR while those to CD28 acts synergistically with CD3 in initiating and augmenting T cell cloning and efficiency (147, 148). In addition, CD3/CD28 stimulation stabilises and inhibits the degradation of cytokine mRNA, thereby enhancing the expression of the T effector cytokines (149, 150).

PBMCs isolated from JSLE and control patients were stimulated using the T cell Activation/Expansion Kit (Miltenyi Biotec, GmBH, Germany) according to manufacturer's instructions. Briefly, 25µL of Anti-Biotin MACSiBead™ Particles (consisting of biotinylated antibodies against CD2, CD3 and CD28 suspended in a solution of 5% FBS in PBS with 2 mM EDTA) was added, per $5X10^6$ PBMCs, to 100 µL of culture medium, which consisted of 10% FBS in RPMI 1640, and centrifuged at 300xg for 5 minutes. The supernatant was aspirated and the resulting pellet resuspended in 100 µL of culture medium. The isolated PBMCs were pelleted and resuspended in 900 µL per $5X10^6$ cells of culture medium. The resuspended Anti-Biotin MACSiBead Particles were mixed with the 900 µL PBMC suspension. 100 µL of this cell mixture was pipetted into each well of a 96 well plate and incubated at 37°C, at 5-10% carbon dioxide, for 2 days. 100 µL of culture medium was added to each well due to evaporation, and then RNA was extracted from the supernatant according to methods stated in section 2.6.

## 2.11 Western Blot Analysis

The Western blot is a procedure used to measure proteins and involves running a complex protein mixture down a polyacrylamide gel then electrically transferring these separated biological samples across to a PVDF membrane. The membrane is then a replica of the protein composition and pattern, making it possible to detect target proteins on this membrane by using a specific antibody-antigen interaction. The experiment consists of sample preparation, electrophoresis separation of proteins, transfer of proteins and membrane staining.

### 2.11.1 Protein quantification of plasma samples

Protein samples are quantified using a protein assay kit (QuantiPro BCA Assay Kit, Sigma-Aldrich, USA). This assay works on the principle of protein to form a $Cu^{2+}$-protein complex in alkaline conditions and then being able to reduce $Cu^{2+}$ to $Cu^{1+}$ based proportionately on the amount of protein present. Bicinchoninic acid (BCA) is then able to form a purple-blue complex with the reduced $Cu^{1+}$.

First, a standard curve of seven standards is prepared by diluting 40 µL of 1000 ug/mL Bovine Serum Albumin (BSA) stock to 960 µL of PBS to make up a top standard of 40µg/mL. This is then serially diluted to create standard curve values of 20 µg/ml, 10 µg/mL, 5 µg/mL, 2.5 µg/mL, 1.25 µg/mL and 0.625 µg/mL. Samples are diluted in PBS to give 150 µL of sample solution per well at 1 in 1600 and 1 in 3200 dilution. Samples and standards are applied to wells in duplicate.

75 µL of QuantiPro Buffer QA (Sigma-Aldrich, USA) consisting of sodium carbonate, sodium tartate and sodium bicarbonate in 0.2M of NaOH is added to 75 µL of QuantiPro BCA QB (Sigma-Aldrich, USA) consisting of 4% BCA solution mixed with 3 µL of 4% copper (II) sulphate pentahydrate solution to give 150 µL of reagent mix per well. The plate is incubated at 37˚C for two hours or 60˚C for one hour on shaking and then absorbance is read at 540 nm with a spectrophotometer Bio-Tek ELx800 (BioTek, USA).

### 2.11.2 Protein sample preparation

$CD4^+$ cell, PBMC and neutrophil samples were diluted at 100 µL of buffer per $1x10^6$ cells, using a denaturing and reducing buffer consisting of anionic denaturing detergent sodium dodecyl sulphate (SDS), 10% dithiothrecil (DTT) and 1% protease inhibitor. Plasma was diluted to 1 in 10, 1 in 20, 1 in 50 and 1 in 100 dilutions using the same SDS buffer solution. The buffer is needed to enable access of the antibody to its protein of interest within the complex protein structure. The SDS binds to the proteins and breaks up the non-covalent bonds between them to form long SDS-polypeptide chains of negative charge, and the DTT is used to reduce disulphide bridge formation between proteins. These sample mixtures are vortexed, boiled on a heat block at 100°C for three minutes, vortexed once again and placed immediately on ice.

### 2.11.3 Electrophoresis separation

10 µL of the SDS-protein sample solutions are pipetted into wells of 12% Polyacrylamide Mini-PROTEANTGX Precast gels (BioRad, USA) submerged in 1X Tris-glycine running buffer. 10 µL of Precision Plus Protein Standards All Blue (BioRad, USA) is added to the first well for measurement. An electric field (negative to positive) of 150V is applied across the gel using Bio-Rad Model 1000/500 power supply (BioRad, USA), causing the negatively charged polypeptide chains to migrate down toward the positive electrode. Gel electrophoresis is run for 25 to 30 minutes and the machine is switched off when the dye molecule has reached near the bottom of the gel.

### 2.11.4 Protein transfer to membrane

The gel is placed on top of a PVDF membrane (Trans-Blot Turbo Transfer Pack 0.2 µm PVDF, BioRad USA) and these are sandwiched between paper that has been wetted with transfer buffer, removing air bubbles before and after. They are placed directly in between the positive and negative electrodes of the BioRad Trans-Blot Turbo Transfer System machine (BioRad, USA). An electric field (negative to positive) at mixed molecular weight setting is applied to transfer the proteins from the gel to adhere onto the membrane, this process takes seven minutes. The membrane is blocked in 5% skimmed milk solution (Marvel, Ireland) with Tris Buffer

Saline with 0.1% Polyoxyethylene 20 solution monolaurate (Tween20, P&R Laboratory Supplies, UK) (TBS-T) buffer for one hour at room temperature with agitation. Blocking the membrane prevents non-specific binding of primary or secondary antibodies to the membrane. The membrane is then washed three times.

#### *2.11.4.1 Immunostaining the blot*

The primary antibody is diluted with 0.1-3% BSA TBS-T solution and added to the membrane ensuring it is completely submerged. This is covered and incubated overnight at 4°C with agitation. The membrane is washed with TBS-T at two minutes per wash for five washes and 5 minutes per wash for two washes in between the two-minute washes in order to remove the residual antibody. The goat anti-species horseradish peroxidase-conjugated IgG secondary antibody (R&D Systems, USA) is diluted in 3% BSA in TBS-T solution or 5% Marvel skimmed milk solution and added to the membrane which is covered and incubated at room temperature or 4°C for one hour with agitation. The membrane is washed with TBS-T seven times as before. The primary antibodies, secondary antibodies and their concentrations used are tabulated in Table 4 below.

**Table 4: Primary and secondary antibodies used in Western blot analysis**

| | IL-17A | IL-17C | IL-17RE | Beta Actin |
|---|---|---|---|---|
| **Primary antibody** | Human IL-17A antibody (R&D Systems, US [MAB3171]) | Anti-IL17C antibody (Abcam, UK [ab67250]) | Anti-IL17RE antibody (Abcam, UK [ab77527]) | Monoclonal Anti-β-Actin Clone AC-15 (Sigma-Aldrich, USA [A5441]) |
| **Concentration** | 1:2000 in 0.1% BSA in TBS-T | 1:2000 in 0.1% BSA in TBS-T | 1:250 in 0.1% BSA in TBS-T | 1:40000 in 3% BSA in TBS-T |
| **Secondary antibody** | Anti-mouse IgG-HRP Conjugate (HAF007) | Anti-mouse IgG-HRP Conjugate (HAF007) | Anti-rabbit IgG-HRP Conjugate (HAF008) | Anti-mouse IgG-HRP Conjugate (HAF007) |
| **Concentration** | 1:8000 in 3% BSA in TBS-T | 1:2500 in 5% Marvel solution | 1:3000 in 5% Marvel solution | 1:10000 in 3% BSA in TBS-T |

### 2.11.5 Developing the Western blot

2.05 mL of ECL Plus Western Blotting Detection Agents (50 µl of solution B in 2 mL of solution A) (GE Healthcare, UK) is pipetted onto the membrane for five minutes. The membrane is wrapped in cling film and taped to a film cassette. In a dark room, an x-ray film is secured in the film cassette next to the membrane for 1-10 minutes, depending on the samples. The film is then developed for one minute in 300 ml of 1:5 replenisher developer G150 (Agfa Healthcare Inv, Belgium), rinsed in distilled water, then one minute in 400 mL of 1:3 fixing developer G354 (Agfa Healthcare Inv, Belgium) and rinsed in distilled water. An example of a Western blot film is shown in Figure 9 below. The film is left to dry prior to analysis. Densitometry of bands on Western blot films were quantified at inverted scale using Adobe Photoshop CS3 Extended software.

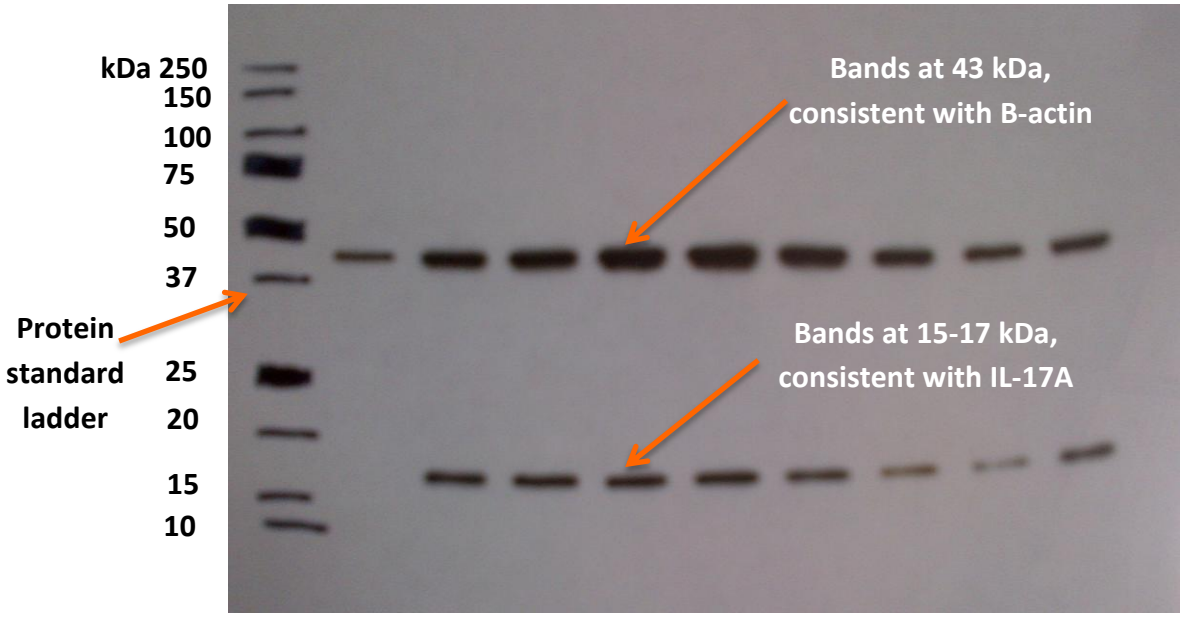



**Figure 9: Example of Western blot film**

**Developed film of a Western blot analysis of JSLE, healthy control and JIA patient $CD4^+$ samples which was conducted according to the protocol detailed in section 2.11. The blot was first stained for IL-17A and subsequently stained for B-actin expression as an internal control. Bands compared against the protein standard ladder drawn show bands at 15-17 kDa, which is consistent with the molecular weight of IL-17A, and bands at 43 kDa, which is consistent with the molecular weight of B-actin.**

## 2.12 Comparing Neutrophil Isolation Methods

A previous study examining the efficacy in neutrophil isolation using Polymorphprep found that purity levels were 94% (151). However, we wanted to determine purity levels (and therefore ability to detect IL-17A protein expression) could be improved by increasing still further the purity of isolation. We therefore decided to compare, by Western blot analysis described in section 2.11, IL-17A protein expression in Polymorphprep-isolated PBMCs and neutrophils as well as neutrophils that had been further purified to see if detection of IL-17A protein expression in neutrophils could be improved further. PBMCs and neutrophils from two healthy adult volunteers were isolated using Polymorphprep, an aliquot of each neutrophil sample was set aside and the remaining neutrophil samples were further purified using the EasySep® Human Neutrophil Enrichment Kit (StemCell Technologies Inc, Canada) which have a reported purity rate of 98-99%, the procedure for which is briefly discussed in 2.12.1 below.

### 2.12.1 EasySep® Human Neutrophil Enrichment

Neutrophils from two healthy adult volunteers were initially isolated using the Polymorphprep™ procedure described in 2.2. The neutrophil samples were pelleted and resuspended in RoboSep® Buffer (StemCell Technologies Inc, Canada) at a concentration of 5 x $10^7$ cells/mL in a 5 ml polystyrene tube. EasySep® Human Neutrophil Enrichment Cocktail was added at 50 µL per mL of cells and incubated at room temperature for 10 minutes. EasySep® Nanoparticles were then added at 100 µL per ml of cells and incubated for another 10 minutes, after which more RoboSep® Buffer was topped up to bring the total volume to 2.5 mL. The tube is placed into a Purple EasySep® magnet and set aside for 5 minutes, after which the negatively selected neutrophil component of the solution is poured into a new polystyrene tube, leaving the unwanted magnetically labelled cells in the original tube. The new tube is placed in the magnet and the process is repeated leaving the desired enriched neutrophils in a new 5 mL polystyrene tube.

### 2.12.2 Determining purity in samples by flow cytometry

Flow cytometry is a powerful technique, which measures and characterises individual particles according to their physical and chemical properties. It works by passing large numbers of cells in a fluid stream through a beam of light, usually a laser. The sample is injected through a central channel, which is encased by a sheath of fluid that is fast-flowing. This sheath fluid focuses the particles haemodynamically to create a single stream of cells. A beam of light is directed at this single stream of cells, and as each cell hits the ray, it scatters light in all directions. The degree to which this light scattering occurs is largely subject to the cell membrane, its shape, nucleus and granular contents as well as any fluorescent material on the cell which may be excited into giving off a light emission. Forward scatter (FSC) detectors are in line with the light beam and roughly equates to the cell size while side scatter (SSC) is collected at 90° to the beam and measures the internal granularity of the cell.

Based on FSC and SSC measurements, different types of cell populations can be determined by size and granularity, this is shown by correlating FSC and SSC measurements in a dot plot as shown in Figure 10. Each dot on a dot plot represents an individual cell that has been analysed. Gating involves setting a boundary based on the pre-determined size of cells of interest, thereby including only those cells in the subsequent analysis. This allows different cell types to be distinguished from each other.

The percentage purities of the fractions obtained from PolymorphPrep-isolated neutrophils (n=2) and EasySep-separated neutrophils (n=2), were calculated by means of gating in flow cytometry FSC/SSC dot plots, following a pre-determined neutrophil protocol. These were then compared with dot plots of PolymorphPrep-isolated PBMCs following a pre-determined PBMC protocol. Flow cytometry was carried out using the MXP software with the Cytomics FC 500 MPL (Beckman Coulter Inc, USA) flow cytometer and plots were analysed with FlowJo software (Tree Star Inc, USA).

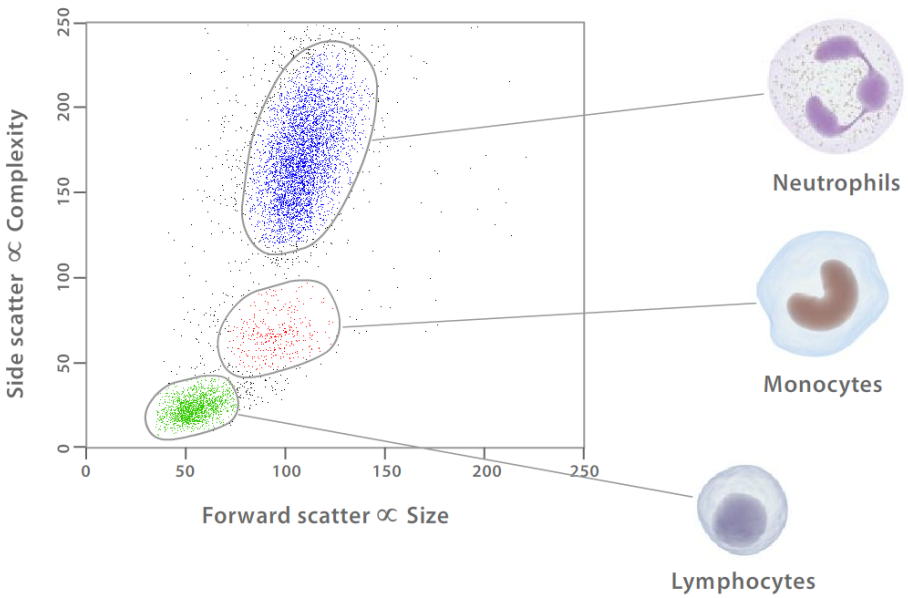



**Figure 10: Cell populations based on FSC/SSC dot plot**

**Neutrophils, monocytes and lymphocytes are amongst the cell types that can be differentiated from a heterogeneous sample by means of an FSC/SSC dot plot. Image adapted from Life Technologies (152).**

## 2.13 Statistical analysis

Statistical analysis of experimental data was conducted using IBM SPSS Statistics 19 software programme, significance level was set at a $p<0.05$ level. Results involving two independent sample groups were plotted onto histograms and assessed for normal distribution. If distribution was not normal, statistical significance was calculated using the Mann-Whitney U non-parametric test. If three or more independent groups were being investigated, presence of statistically significant data amongst all groups was first assessed using the Kruskal-Wallis one-way analysis of variance. If the p value suggested significant ($p<0.05$), the Mann-Whitney U test was then employed to analyse differences between study groups. Data results are expressed as mean ± standard error of mean (SEM).

# Results

## 3.1 Patients

A total of 102 participants were involved in this study, consisting of 31 JSLE patients, 66 paediatric healthy controls, four JIA patients and one patient with lupus nephritis. Samples from JSLE and JIA patients were obtained over multiple episodes, while lupus and healthy control samples were each obtained during one episode. JIA patients were adopted as inflammatory autoimmune controls. The mean age was 15.9 (9-19) in the JSLE group, 13.1 (2-18) in controls, 12.8 (8-16) in the JIA group and the lupus nephritis patient was 17. Female: male ratios in JSLE and control groups were 2.1:1 and 1.4:1 respectively, while all JIA and lupus nephritis patient were female. Ethnicity was predominantly White-British in JSLE, healthy control and JIA groups, apart from one patient in the healthy control group (Chinese 1) and eight JSLE patients (Chinese 1, Indian 3, Bangladeshi 2, Pakistani 2). The lupus nephritis patient was of African ethnic origin.

Mean Global British Isles Lupus Assessment Group (BILAG)-2004 score was 2.7 (0-10) in JSLE patients, while the lupus nephritis patient had a BILAG-2004 score of 13. Out of four JIA patients, two had polyarticular disease, one had systemic-onset JIA and one had oligo-articular disease. Across all JIA classifications, mean number of swollen joints was 1.75 (1-3), while mean number of joints with limited range of movement was also 1.75 (1-3). The mean disease biomarkers measured in the JSLE group are listed in Table 5 and medications JSLE and JIA patients were prescribed at the time of study are listed in Table 6.

Table 5: Disease biomarkers in JSLE patients

| Disease biomarker (normal range) | JSLE patients (n=31) | Lupus nephritis patient (n=1) |
|---|---|---|
| **CRP, mg/litre (0-8.0)** | 7.5 (1-78.9) | NS |
| **ESR, mm/hour (2-8.0)** | 11.0 (1-76) | NS |
| **Total white cell count, X $10^9$/ litre (4.0-11.0)** | 5.8 (2.8-10.2) | 5.4 |
| **Lymphocyte count, X $10^9$/ litre (1.3-3.5)** | 1.6 (0.28-3.31) | 2.1 |
| **Neutrophil count, X $10^9$/ litre (2.0-7.5)** | 3.5 (1.26-9.09) | 2.53 |
| **IgG, g/litre (7.4-14)** | 11.5 (5.29-23.19) | 9.9 |
| **IgM, g/litre (0.5-2.3)** | 1.1 (0.28-2.56) | 0.3 |
| **IgA, g/litre (0.6-3.3)** | 1.5 (0.11-3.56) | 1.8 |

**Values are expressed as mean (range) for each disease biomarker. CRP = C-reactive protein, ESR = erythrocyte sediment rate, NS = not stated**

Table 6: Current medications in JSLE and JIA patients

| Medication | JSLE (n=31) | JIA (n=4) | Lupus nephritis (n=1) |
|---|---|---|---|
| Prednisolone | **12** | **4** | **1** |
| Hydroxychloroquine | **25** | **1** | **1** |
| Methotrexate | **4** | **0** | **0** |
| Azathioprine | **10** | **0** | **1** |
| Mycophenolate Mofetil | **13** | **0** | **0** |
| Cyclophosphamide | **3** | **0** | **0** |
| Biologics | **1** | **2** | **0** |
| NSAIDs | **4** | **3** | **0** |

**List of medications patients from JSLE and JIA study groups have been prescribed. Note patients may be on >1 type of medication. Values are expressed as number of patients per study group.**

## 3.2 Plasma IL-21 expression in JSLE patients and healthy controls

One of the key aims of the study was to investigate the roles of Th-17 related pro-inflammatory cytokines IL-21 and IL-23. In accordance to that, initially an ELISA was conducted measuring IL-21 in JSLE (n=20) and control (n=20) plasma samples using Human IL-21 ELISA MAX$^{TM}$ Deluxe (Biolegend, USA). In JSLE patients compared to control patients, IL-21 concentrations in JSLE plasma (17.2 pg/mL ± 6.2) were higher than those from healthy control patients (7.5 pg/mL ± 1.9), and although not statistically significant, the difference between values was approaching significance (p=0.06), as shown in Figure 11 below.

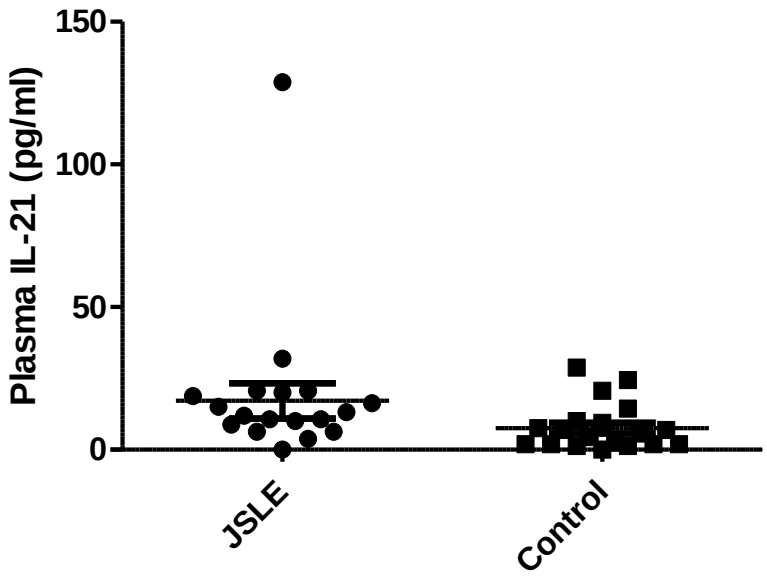



**Figure 11: IL-21 levels in JSLE and control plasma**

**An IL-21 ELISA was conducted examining JSLE (n=20) and control (n=20) plasma samples. Plasma IL-21 levels were not statistically different but the p-value was approaching significance (JSLE 17.2 pg/mL ± 6.2, Control 7.5 pg/mL ± 1.9, p= 0.06). Values are mean ± SEM.**

It was hypothesised that IL-21 expression would be increased in the peripheral blood of JSLE patients compared to healthy controls. However the results obtained from this ELISA experiment did not show a significant difference between JSLE and control groups. Appreciable difficulties in measuring Th17-related cytokines had previously been encountered by other members of our Lupus Research Group. Albeit using commercially validated bio-assays, such as ELISAs and single plex assays, within these experiments, it is well recognised that cytokine interference as described in section 2.4 can significantly impact on one's ability to accurately measure concentrations, especially at low concentrations of the target cytokine and within complex biological environments such as those present in lupus plasma and serum. A series of spike and recovery experiments were therefore conducted in order to validate the Th17-related cytokine ELISA experiments and an IL-17 single plex carried out in this study to determine if this was impacting significantly on these results.

## 3.3 Spike and recovery experiments of Th17-related cytokine bio-assays

Poor recovery of spike protein concentrations has previously been described in the detection of the high mobility box 1 (HMGB1) protein in SLE serum and plasma as reported by Urbonaviciute et al (153). In addition, published data show inconsistencies in relation to IL-17A (19-79 pg/mL) and IL-23 (20.8-377 pg/mL) cytokine concentrations in SLE (88, 111, 133). Validation studies examining different ELISA kits have reported considerable variability in cytokine detection, and even with false positive or false negative results as a result of heterophilic antibodies and other interferents (154, 155).

In view of this, coupled with findings that the results of the IL-21 ELISA experiment mentioned in section 3.2 were not consistent with conclusions from other studies, several spike and recovery experiments were used to validate and assess the assay response of JSLE serum and plasma in Th17-related cytokine detection kits, namely IL-17A, IL-21 and IL-23 assays. As noted previously, these cytokines have been strongly implicated in autoimmunity in previous research, IL-

17A and IL-21 are secreted by Th17 cells and IL-23 expands and maintains Th17 cells. The validation experiments are necessary in order to assess reliability and confidently remove confounding factors from influencing the results obtained.

Spike and recovery percentage results are depicted in Table 7 and Figure 12 below. A spike and recovery experiment using JSLE serum and plasma samples with an IL-17A eBioscience ELISA kit was initially conducted, and this showed poor recovery between 67-80% at each sample dilution factor. However as the dilution factor increased, the mean recovery percentages increased, indicating that increased concentrations of the sample matrix was causing interference in the cytokine recovery potential. At a 1-in-8 dilution the IL-17A kit yielded a recovery of 80% which would have theoretically translated to acceptable recovery. However negative recovery percentages from the unspiked data set revealed that a 1-in-8 dilution effectively diluted out any naturally occurring cytokine, this data is shown in Appendix 4.

The BioLegend IL-21 ELISA kit was then investigated. It showed poor recovery across both JSLE and control plasma samples at every dilution factor ranging from 20-48%. Interestingly, control plasma fared worse than JSLE plasma, the highest recovery being 28% compared to 48% at 1-in-8 dilutions. Once again, the recovery increased as the sample dilution increased, indicating matrix interference.

Subsequently, the IL-23 R&D ELISA kit was investigated. It showed poor recovery across both JSLE and control plasma samples, ranging between 18-53%, out of acceptable recovery range. Recovery did not increase with increased dilution for this assay, showing less marked evidence for matrix interference. The control plasma sample fared worse in recovery compared to the JSLE plasma, yielding 27% recovery compared to 53%.

An IL-17A single plex was validated in order to investigate if an alternative assay would be better at cytokine detection for IL-17A. However, recovery yield was worse compared to the respective ELISA kit and ranged 19-39%. In addition, the recovery increased as the dilution factor increased, once again indicating matrix interference in the samples.

In summary, all four detection kits displayed poor recovery of their target cytokines outside the acceptable spike-and-recovery range of 80-20%. Samples tested in IL-21 ELISA, IL-17A ELISA and IL-17A single plex kits were found to have increased recovery of target cytokines as the samples were diluted down, indicating there may be sample matrix-related interference in IL-17A and IL-21 detection.

**Table 7: Mean recovery percentage of spike-and-recovery single plex and ELISAs**

| Mean Recovery Percentage (%) | | | | | |
|---|---|---|---|---|---|
| | **Neat** | **1 in 2** | **1 in 4** | **1 in 8** | **1 in 16** |
| IL-17 Bio-Plex | - | 19 | 31 | 36 | 39 |
| IL-17 ELISA JSLE | 67 | 76 | 76 | 80 | - |
| IL-21 ELISA JSLE | 33 | 30 | 35 | 48 | - |
| IL-21 ELISA Control | 23 | 20 | 24 | 28 | - |
| IL-23 ELISA JSLE | 53 | 38 | 40 | 48 | - |
| IL-23 ELISA Control | 27 | 18 | 19 | 22 | - |

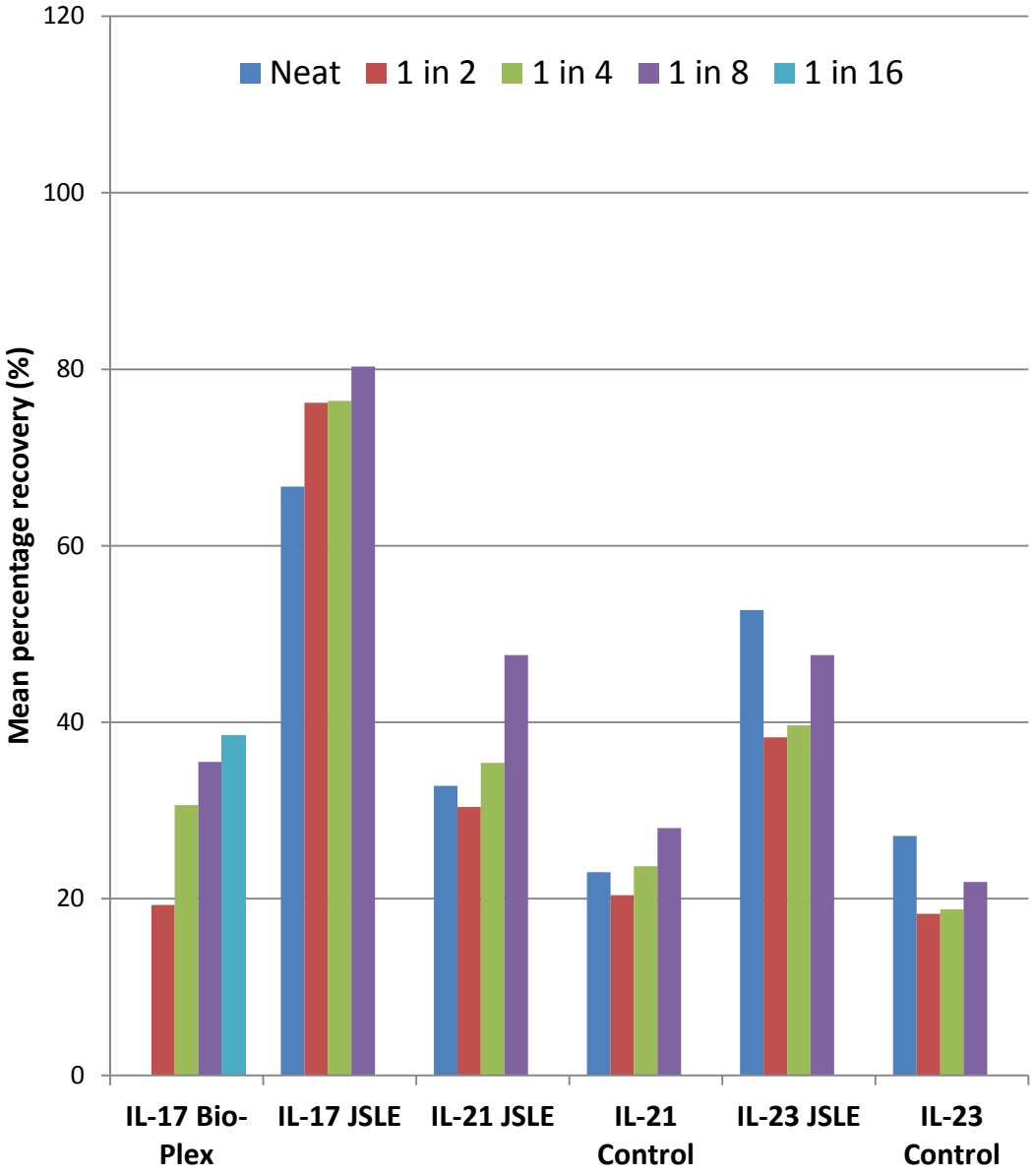



Figure 12: Mean recovery percentage of spike-and-recovery single plex and ELISAS

**Spike and recovery experiments were conducted using cytokine-spiked and unspiked samples in IL-21 ELISA (JSLE plasma n=3, control plasma n=1), IL-23 ELISA (JSLE plasma n=3, control plasma n=1), IL-17 ELISA (JSLE plasma n=2, JSLE serum n=2) and IL-17 single plex (JSLE serum n=5) cytokine detection kits. Recovery percentages were found to be out of adequate 80-120% range in all experiments. Recovery increased with increasing dilution factor in IL-21 and IL-17 assays, indicating matrix interference of the detection in these cytokines in JSLE and control plasma and serum samples.**

## 3.4 IL-17A mRNA Expression in JSLE

Current published literature in relation to the role of Th-17 cells in lupus has focused almost exclusively on IL-17A mRNA expression in adult SLE. However, as we have seen, the control mechanisms of Th-17 cells are complex. In addition, JSLE presents with a significantly more severe disease phenotype and as such provides an important disease model to investigate the role of Th17 cells in lupus. As IL-17A possesses a pro-inflammatory mechanism, we hypothesised it would be upregulated in our patients with JSLE.

#### *3.4.1.1 Housekeeping genes for IL-17A qPCR*

Housekeeping genes are an internal control used in qPCR, in order to normalise against RNA instability, variability in sample loading, and differences in reagent efficiency. Three housekeeping genes, Beta-2 microglobulin (B2M), RPL13A and Beta-actin (B-actin), were evaluated in parallel with IL-17A for use in SYBR Green qPCR of three adult healthy control CD4+ RNA to determine their consistency and efficiency. A previous study by Mane et al determined that B2M and RPL13A were best expressed with little variation in $CD4^+$ T cells (156). Shown in Table 8 below are the mRNA expressions of the housekeeping genes. B-actin demonstrated the smallest degree of variability compared to the other two housekeeping genes and was therefore selected as an internal control for SYBR Green qPCR.

**Table 8: mRNA expression of housekeeping genes**

| Housekeeping Gene | Samples | Copies |
|---|---|---|
| Beta-2 microglobulin | #1 | 4234 |
| | #2 | 3985 |
| | #3 | 4371 |
| RPL13A | #1 | 1963 |
| | #2 | 1776 |
| | #3 | 1849 |
| Beta-actin | #1 | 1412 |
| | #2 | 1416 |
| | #3 | 1742 |

### 3.4.1.3 IL-17A SYBR Green qPCR

JSLE (n=5) and healthy control (n=8) $CD4^+$ RNA samples were reverse transcribed to a 100 ng/µL concentration. Four JSLE $CD4^+$ samples were deemed to be of poor quality due to a freeze/thaw episode and were reverse transcribed to 150 ng/µL concentrations. The four JSLE $CD4^+$ RNA samples were initially analysed for B-actin expression at 100 ng/µL. However they produced inconsistent dissociation peaks and this was coupled with 260/280 ratio values which were out of acceptable range, indicated varying quality. Subsequently, the four 150ng/µL JSLE CD4+ samples were validated for B-actin expression, which showed a reliable uniform dissociation curve as shown in Figure 13 below.

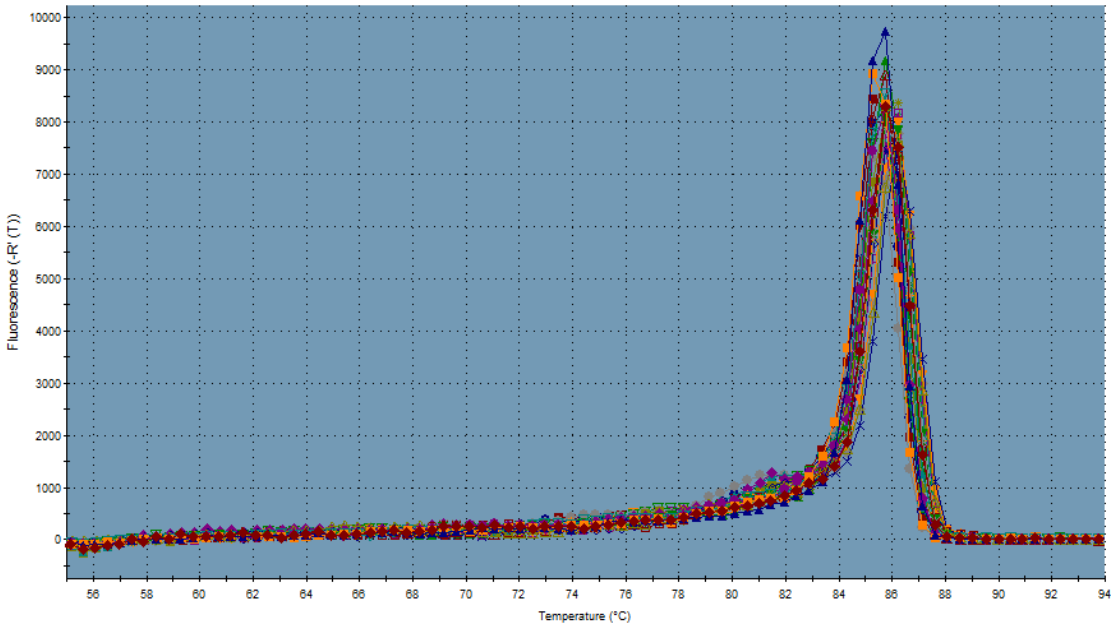



**Figure 13: Dissociation curve of B-actin expression in 150 ng/µL JSLE $CD4^+$ samples**

**JSLE CD4+ RNA samples (n=4) were deemed to be of poor quality at 100 ng/µL concentration and hence were reverse transcribed to 150 ng/µL concentrations and validated for B-actin expression which shows one uniform dissociation curve as shown.**

SYBR Green qPCR was conducted measuring IL-17A and B-actin expression in JSLE (n=9) and control (n=8) $CD4^+$ RNA. No significant difference was found in relative expression of IL-17A against B-actin in JSLE (1.19 ± 0.16) and healthy control (1.43 ± 0.33, p= 0.89) $CD4^+$ T cells as demonstrated in Figure 14 below.

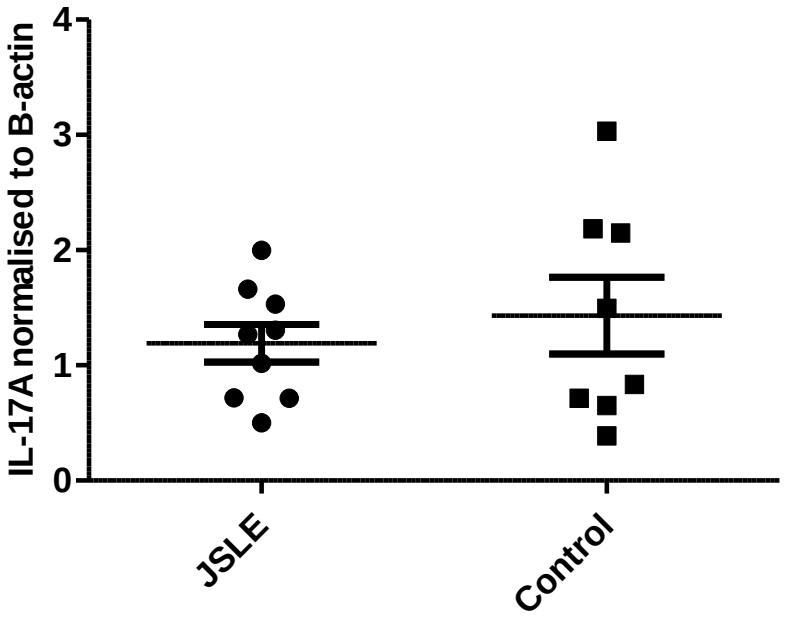



**Figure 14: IL-17A mRNA expression of JSLE and control $CD4^+$ T cells**

**IL-17A expression was investigated in JSLE (n=9) and healthy control (n=8) $CD4^+$ RNA using SYBR Green qPCR as shown. No significant differences were found in the relative expression of IL-17A compared against B-actin between JSLE (1.19 ± 0.16) and control (1.43 ± 0.33, p=0.89). Values are represented as mean relative expression of IL-17A ± SEM.**

## 3.5 IL-17A Protein Expression in Plasma, PBMCs and Neutrophils in JSLE

There is often poor correlation between mRNA expression and its corresponding protein abundance; this can be due to variable mechanisms involved in protein synthesis and degradation (157, 158). In view of finding no significant differences in IL-17A mRNA expression between participant groups, we decided to examine if there were differences in protein expression of IL-17A. Protein expression of IL-17A was assessed by Western blot analysis. IL-17A has a molecular weight of 17 kDa; however it is often seen as 15 kDa on Western Blot films.

### 3.5.1 PBMC IL-17A Protein expression

#### *3.5.1.1 Optimisation of PBMC Western Blot Analysis*

Initially, PBMCs were investigated as we wanted to gain a general overview of IL-17A expression in the blood before moving on to focus on specific cell subsets of interest which would be covered below. In order to optimise the western blot analysis protocol on PBMCs, JSLE (n=4) and healthy control (n=4) PBMC protein samples were run with an IL-17A recombinant protein sample as a positive control and stained for IL-17A. The eight PBMC samples and positive control were prepared with SDS buffer and boiled at 100°C on a heat block.

Figure 15A below shows the resultant film, where it can be seen that the recombinant IL-17A positive control (without reducing agent) ran to a molecular weight of approximately 37 kDa, higher than expected. Three JSLE and four healthy control samples produced bands of approximately 15 kDa as expected.

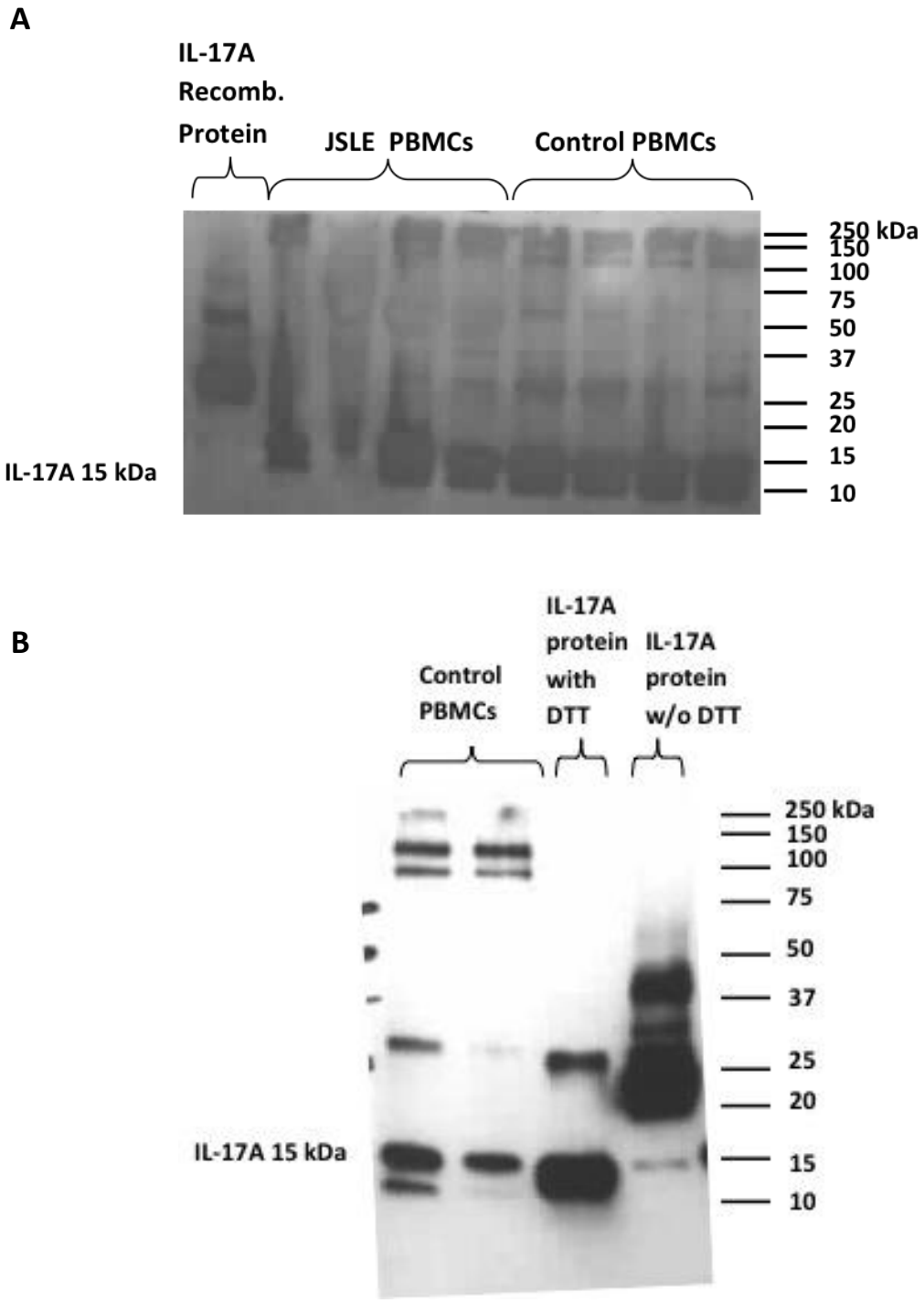



**Figure 15: Optimising Western blots in PBMCs**

**A** Western blot of IL-17A protein expression in JSLE (n=4) and healthy control (n=4) PBMCs compared against an IL-17A recombinant protein without reducing agent as a positive control. The IL-17A recombinant protein did not produce a band at 15 kDa consistent with IL-17A. **B** A subsequent Western blot of IL-17A recombinant protein with and without DTT compared against control PBMC protein (n=2). The IL-17A recombinant protein sample that had been reduced with 10% DTT and 1% protease inhibitor showed a band at 15 kDa that was consistent with IL-17A. Hence DTT and protease inhibitor reduction was employed for all protein samples after this.

It was deduced that the IL-17A recombinant protein had probably formed a IL-17A/IL-17F heterodimer cytokine of molecular weight 37 kDa as reported in a study by Chang et al (159). A subsequent blot was stained for IL-17A where 10 µL samples of two control PBMC protein, the IL-17A recombinant protein boiled with SDS buffer as prepared before, and IL-17A recombinant protein reduced with 1% protease inhibitor and 10% DTT in SDS buffer were run. As can be seen in Figure 15B, the IL-17A recombinant protein when reduced showed a band at 15 kDa, consistent with its theoretical molecular weight as described in previous studies. In addition, the two control samples produced bands at 15 kDa as well. Figure 16 shows an optimized Western blot of four JSLE PBMC protein samples and four controls all showing bands at approximately 15 kDa. It was found that the IL-17A primary antibody could only be used once at a 1 in 2000 dilution to obtain a clear blot showing only the protein of interest.

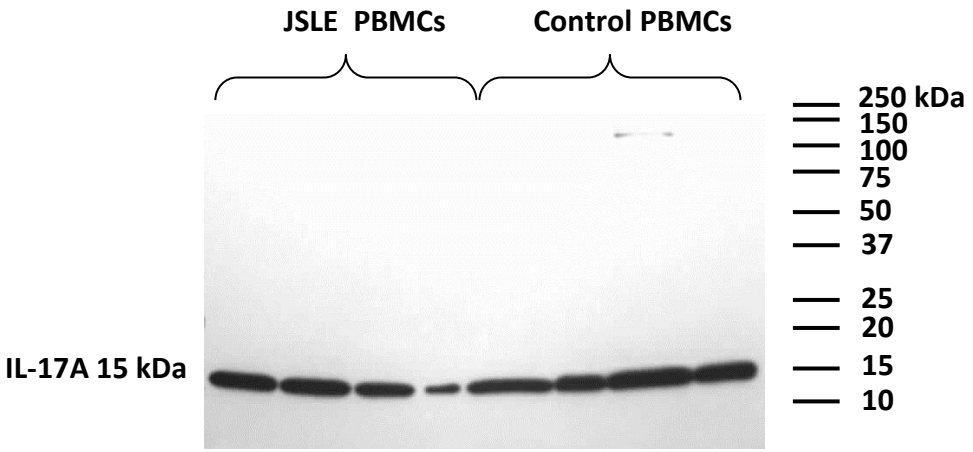



**Figure 16: Optimised IL-17A Western blot analysis in JSLE and Control PBMCs**

**Western blot analysis was conducted examining JSLE (n=4) and control (n=4) PBMCs, which had been reduced using 10% DTT and 1% protease inhibitor SDS buffer at 100°C. The blot was then stained for fresh IL-17A primary antibody made up to 1 in 2000 dilution with 0.1% TBS-T. All samples produced a band at 15 kDa, consistent with IL-17A, indicating that the protocol examining IL-17A expression in PBMC protein was optimised.**

### 3.5.1.2 *Protein expression of IL-17A in PBMCs*

Protein samples of JSLE (n=3), healthy paediatric controls (n=3) and JIA (n=3) PBMCs were run according to the optimized IL-17A Western blot protocol as mentioned in 3.5.1.1 and subsequently stained for B-actin as an internal control, the blot is shown in Figure 17. All samples produced bands at 15 kDa consistent with IL-17A and at 43 kDa consistent with B-actin, however a JIA sample produced a very faint band compared to all the other samples, which may have skewed results. The relative expression of IL-17A compared against B-actin is shown in a graphical representation in Figure 18; no significant differences were found in IL-17A expression amongst all groups (JSLE patients: 0.53 ± 0.079; healthy control: 0.66 ± 0.008; JIA patients: 0.47 ± 0.061, p=0.11). No significant differences were found in IL-17A expression between JSLE patients and healthy controls (p=0.20) or JSLE and JIA patients (p=1.0)

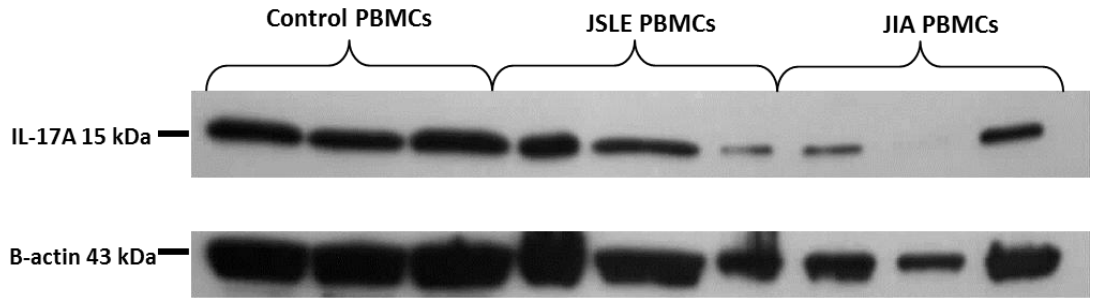



**Figure 17: IL-17A Western Blot analysis of PBMCs**

**Western blot image showing the protein expression of IL-17A which was investigated in JSLE (n=3), healthy control (n=3) and JIA (n=3) PBMCs and compared against B-actin expression.**

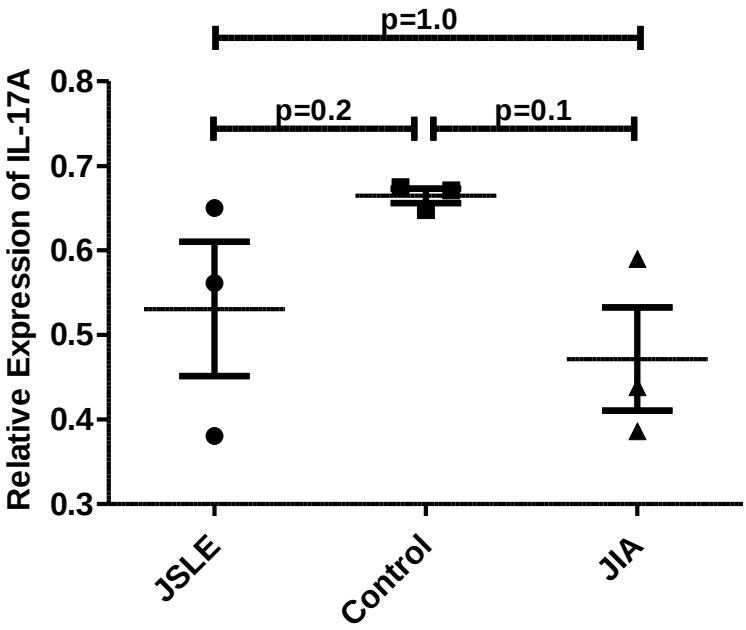



**Figure 18: IL-17A protein expression in JSLE, JIA and control PBMCs**

**The dot plot details the results of IL-17A relative expression in the Western blot image shown in Figure 17, values are mean relative expression of IL-17A to B-actin. Comparison of the relative expression of IL-17A amongst JSLE, healthy control and JIA PBMCs showed no significant differences (p=0.11) present across all groups.**

### 3.5.2 IL-17A protein expression measured by Western blot in $CD4^+$ cells

A Western blot analysis was performed according to the optimised IL-17A protocol mentioned in 3.5.1.1, examining JSLE (n=3) patients compared to healthy control (n=3) and JIA (n=3) patient $CD4^+$ IL-17A protein and then stained for B-actin as an internal control. As shown on Figure 19A below, all samples produced a band at 43 kDa consistent with B-actin. All but one control sample showed visible bands at 15 kDa. The relative expression of IL-17A against B-actin is compared amongst groups in Figure 19B. No significant differences were seen in IL-17A protein expression of $CD4^+$ cells of JSLE patients and healthy controls (p=0.7) or JSLE and JIA patients (p=0.7). No statistically significant differences were found in $CD4^+$ IL-17A protein expression across all groups (JSLE patients: 0.92 ± 0.05; Healthy controls: 0.85 ± 0.14; JIA patients: 0.83 ± 0.13, p= 0.97).

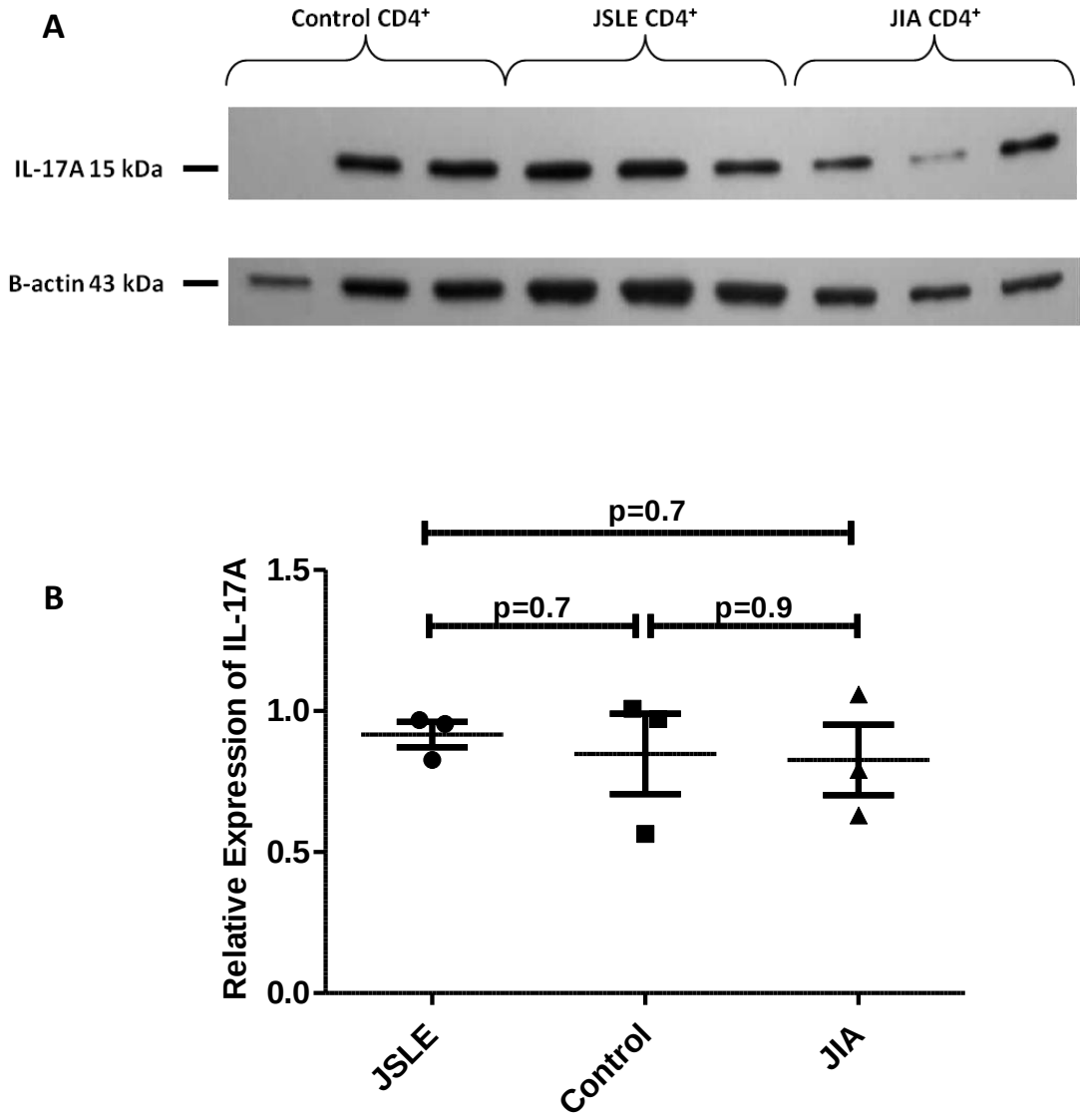



**Figure 19: IL-17A Western blot analysis of control, JSLE and JIA CD4$^+$ protein**

**A** Western blot analysis was performed according to the optimised IL-17A protocol outlined in 3.5.1.1, investigating JSLE (n=3), healthy control (n=3) and JIA (n=3) CD4$^+$ IL-17A protein expression and then compared against B-actin expression. **B** The dot plot depicts the mean relative expression of IL-17A compared against B-actin from the Western blot in **A**. No significant differences were found in CD4+ IL-17A protein expression across all groups (JSLE patients: 0.92 ± 0.05; healthy controls: 0.85 ± 0.14; JIA patients: 0.83 ± 0.13, p= 0.97).

### 3.5.3 IL-17A protein expression in neutrophils extracted from JSLE, Healthy control and JIA patients

Neutrophils, extracted using PolymorphPrep methodology as outlined in section 2.2.1, from JSLE (n=2), control (n=2) and JIA (n=1) patients were run and stained for IL-17A as part of an optimization study using Western blot analysis. As can be seen in Figure 20, all samples yielded a band measuring 15 kDa consistent with the molecular weight of IL-17A following the protocol for PBMC protein as stated in 3.5.1.1.

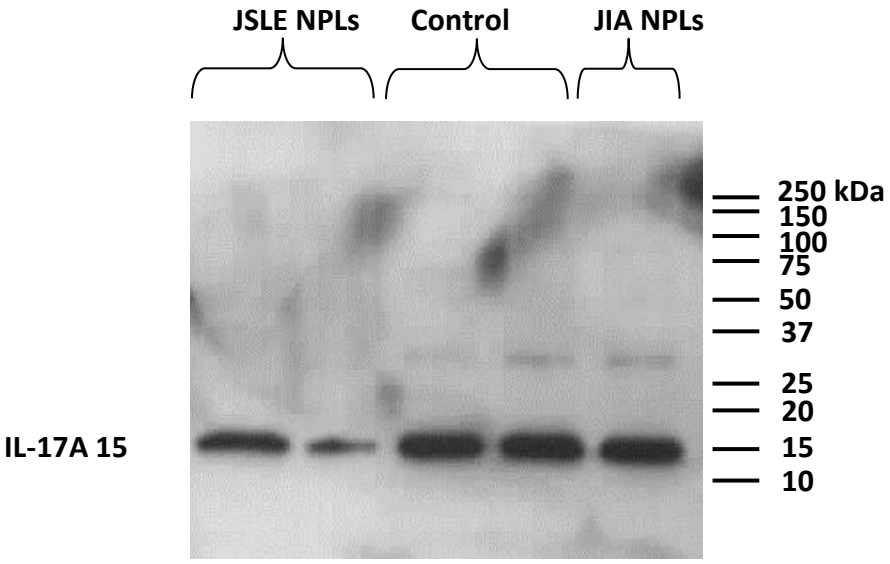



**Figure 20: Optimized Western blot analysis of IL-17A in neutrophil protein extracted from JSLE patients, healthy controls and JIA patients**

**Western blot analysis of IL-17A expression in JSLE (n=2), healthy control ontrol (n=2) and JIA (n=2) neutrophil protein was optimised following the protocol stated for PBMCs in 3.5.1.1. All samples produced bands at 15 kDa consistent with IL-17A expression.**

A subsequent IL-17A analysis was performed using neutrophil protein extracted from JSLE patients (n=4) and healthy controls (n=4) and then stained for B-actin (Figure 21A). 43 kDa B-actin bands were seen in all samples. One JSLE neutrophil sample did not produce a visible band at 15 kDa but was included in the statistical analysis due its B-actin expression. As seen in Figure 21B, expression of IL-17A was significantly decreased in JSLE neutrophil protein (0.63 ± 0.03) compared to healthy control neutrophils (0.87 ± 0.036, p=0.029). If not restricted by time, it would have been preferable to repeat this blot in order to obtain clearer protein bands for B-actin which would have aided in analysis.

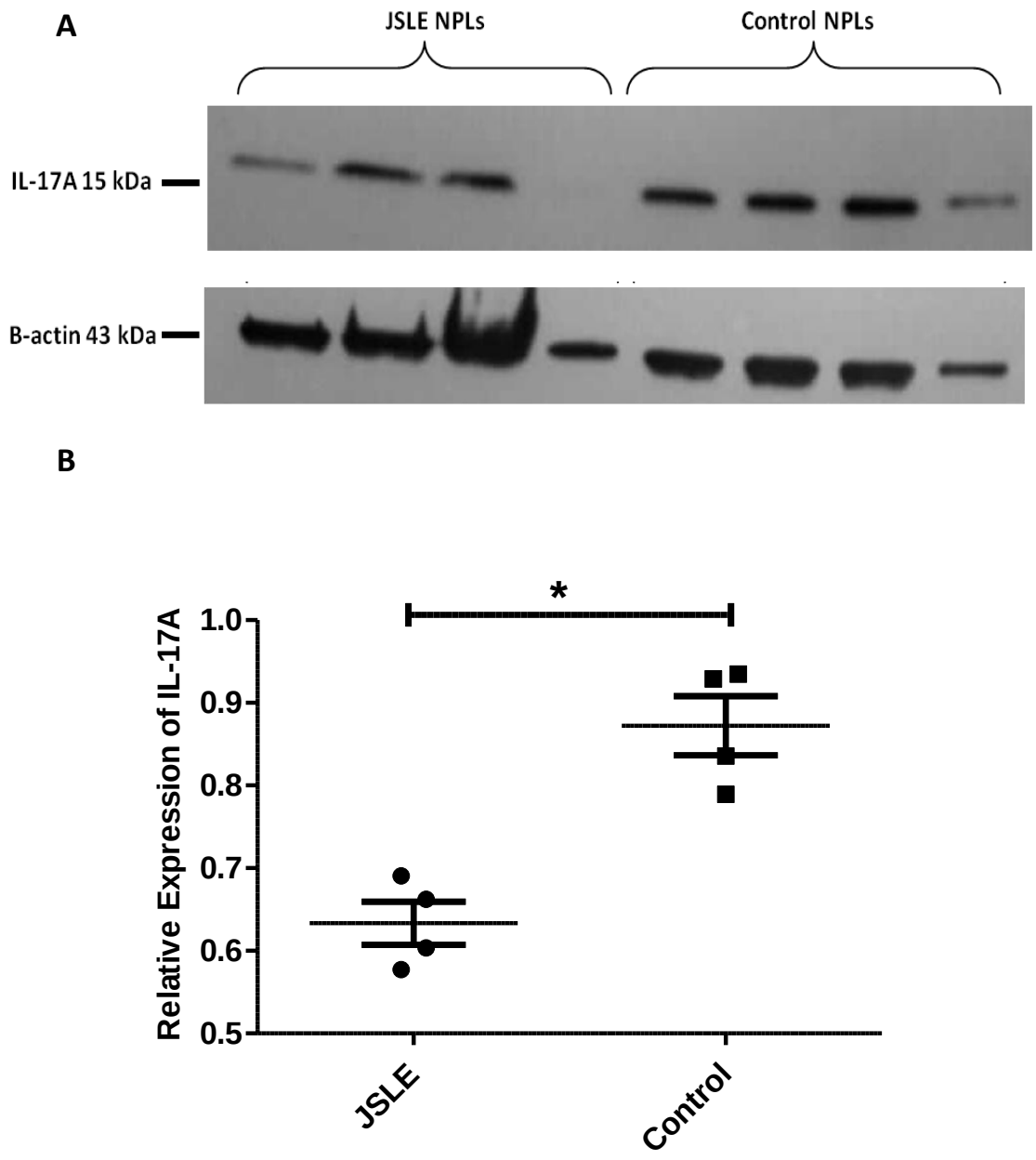



**Figure 21: IL-17A protein expression is significantly decreased in JSLE neutrophils**

**A** Image of Western blot analysis examining IL-17A protein expression in JSLE (n=4) and control (n=4) neutrophils against B-actin expression. **B** The diagram depicts mean IL-17A relative expression against B-actin in the Western blot in **A**. JSLE samples (0.63 ± 0.03, p=0.029) were found to express significantly decreased IL-17A compared to healthy control neutrophils (0.87 ± 0.036). (*) indicates a significant difference between columns.

In order to investigate if neutrophils were indeed expressing IL-17A, neutrophils from healthy adult blood (n=2) were extracted by the PolymorphPrep method described in section 2.2.1 and then magnetically-isolated using the EasySep Human Neutrophil Enrichment method described in 2.12.1. The percentage purities of neutrophils in all samples were determined by gated flow cytometry analysis described in 2.12.2. PolymorphPrep-isolated neutrophil samples demonstrated 91.5% and 94.7% purity (mean 93.1%) in the two healthy adult volunteers and EasySep-purified neutrophil samples had 99.2% and 99.7% purity (mean 99.5%) respectively. This was compared against PBMCs that had been isolated from the same donors by PolymorphPrep. The relevant dot plot diagrams are shown in Figure 22.

A Western blot analysis (Figure 23) was run examining IL-17A expression in 10 µL aliquots of Polymorphprep-isolated PBMCs (n=2), Polymorphprep-isolated neutrophils (n=2), EasySep-separated neutrophils (n=2) and EasySep-separated neutrophils spiked with 10% PBMCs (n= 2). Figure 23 charts the mean relative expression of IL-17A against B-actin in all four types of isolated cells, IL-17A is expressed highest in Polymorphprep-isolated neutrophils and further purified neutrophils are shown to express the smallest amount of IL-17A. The expression of IL-17A is increased in purified neutrophils by the PBMC spike as shown by the fourth column. However, Kruskal-Wallis one-way analysis of variance showed that there were no significant differences amongst all four groups (p=0.083). If repeated, it would be useful to analyse the neutrophil and PBMC cell populations by flow cytomtery in the EasySep neutrophil samples that had been spiked with PBMCs to ensure that 10% of PBMCs were added to each sample.

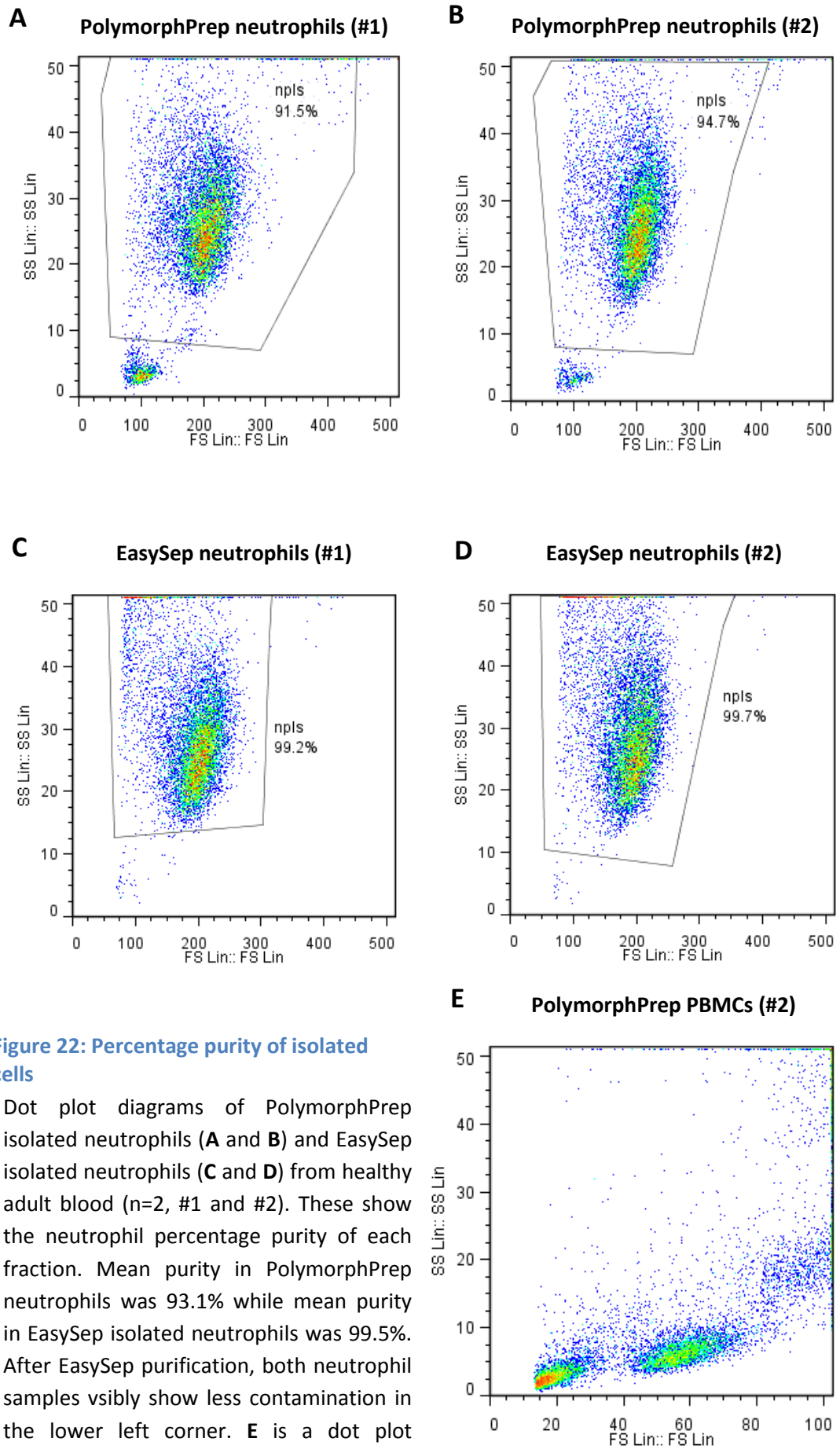



**Figure 22: Percentage purity of isolated cells**

Dot plot diagrams of PolymorphPrep isolated neutrophils (**A** and **B**) and EasySep isolated neutrophils (**C** and **D**) from healthy adult blood (n=2, #1 and #2). These show the neutrophil percentage purity of each fraction. Mean purity in PolymorphPrep neutrophils was 93.1% while mean purity in EasySep isolated neutrophils was 99.5%. After EasySep purification, both neutrophil samples vsibly show less contamination in the lower left corner. **E** is a dot plot diagram of PolymorphPrep isolated PBMCs analysed to a PBMC-specific protocol.

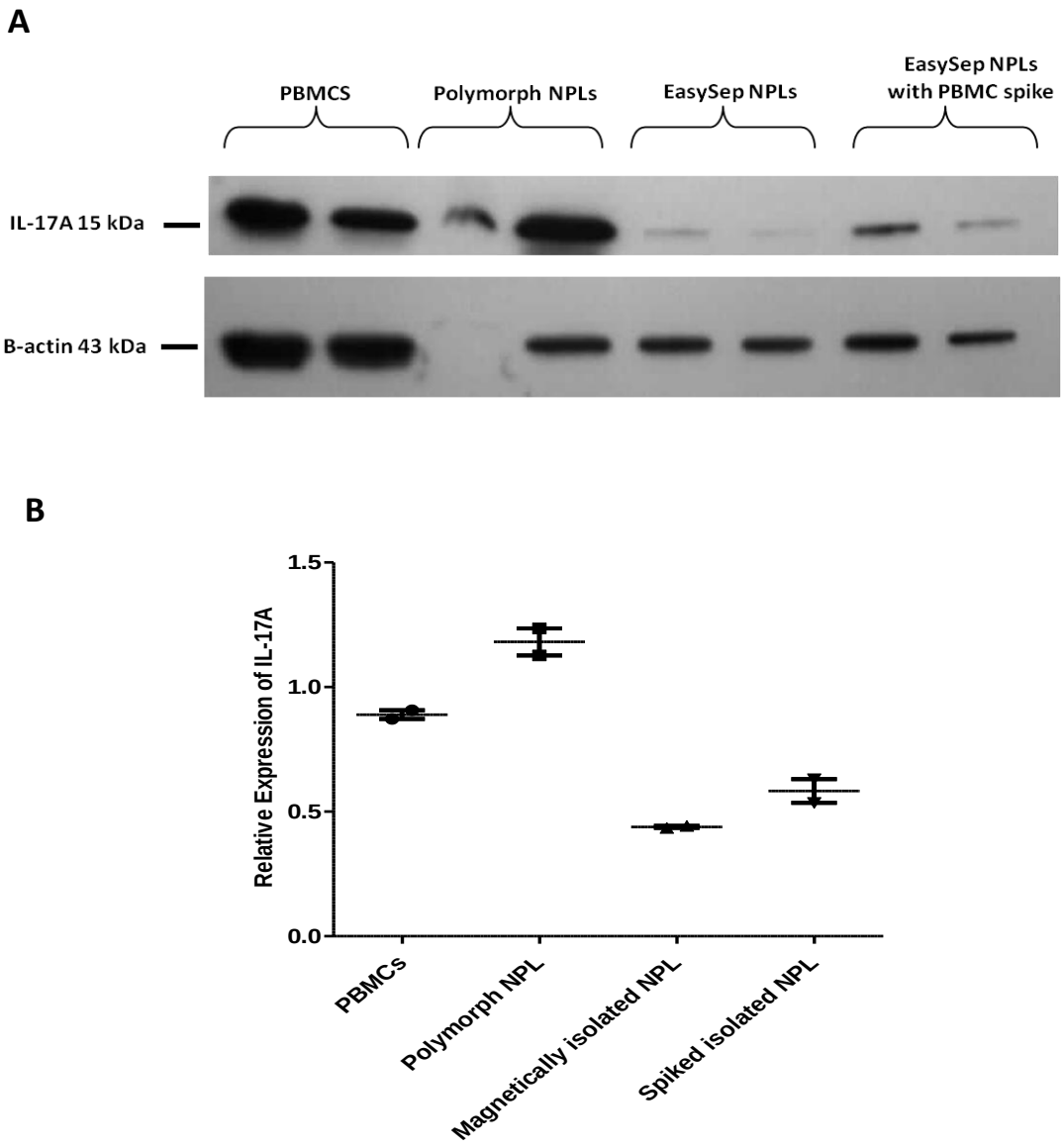



**Figure 23: IL-17A protein expression in different isolated cells from adult healthy controls**

**A** IL-17A protein expression and B-actin protein expression was analysed by Western blot in PBMCs (n=2), neutrophils isolated by Polymorphprep (n=2), neutrophils magnetically isolated by the EasySep Human Neutrophil Enrichment kit (n=2) and EasySep magnetically isolated neutrophils spiked with 10% PBMCs (n=2). All samples were from healthy adult volunteers. **B** The diagram depicts the mean relative expression of IL-17A against B-actin as an internal control in the four different isolated cell groups described in **A**. No significant differences were present amongst all groups (p=0.083), however Polymorphprep isolated neutrophils (1.18 ± 0.055) and PBMCs (0.89 ± 0.0178) had the highest relative expression of IL-17A compared to EasySep magnetically isolated neutrophils (0.44 ± 0.004). A slight increase in IL-17A expression was observed in spiked magnetically isolated neutrophils (0.58 ± 0.048). Values are mean relative expression ± SEM.

### 3.5.4 IL-17A protein expression in plasma using Western blot technique

#### *3.5.4.1 Optimisation of Plasma Western Blot Analysis*

Due to unreliable results shown in the spike and recovery experiments described in section 2.4 using ELISAs and bioplex technology, the protein expression of IL-17A in plasma was investigated using another method. A previous study reported poor correlation between HMGB1 concentrations in SLE and control plasma and serum obtained through ELISA and Western blot assays, where the former showed lower concentrations to those determined by Western blot (153). In view of these data, optimisation of measuring IL-17A in the plasma samples by Western blot analysis was undertaken and shown in the Figure 24, Figure 25, Figure 26 and Figure 27 below.

An initial trial blot was run using JSLE (n= 1), healthy control (n= 1) and lupus nephritis plasma (n=1). As described in section 3.1, the lupus nephritis patient had a BILAG-2004 score of 13, higher than that of the JSLE patients, indicating a more severe disease phenotype. A band was visualised at 15 kDa consistent with IL-17A in the lupus nephritis plasma sample. The protein content from that same lupus nephritis plasma sample was then quantified together with JSLE (n=5) and control (n=4) plasma samples using the protein assay described in section 2.11.1 and it was determined that 1350 µg of protein per plasma sample would be optimal in a Western blot analysis. However, diluting down each plasma sample accordingly to ensure loading of 1350 µg of protein per 10 µl of plasma did not yield conclusive results.

Consequently, we decided to analyse IL-17A expression in JSLE (n= 5) and control (n= 4) plasma protein at varying dilutions of 1:2000, 1:1000, 1:500, 1:100, 1:50, 1:20 and 1:10 dilution factors. Figure 24A and Figure 24B show visible bands at 15 kDa consistent with IL-17A expression in both control and JSLE plasma at 1 in 20 and 1 in 10 dilution factors.

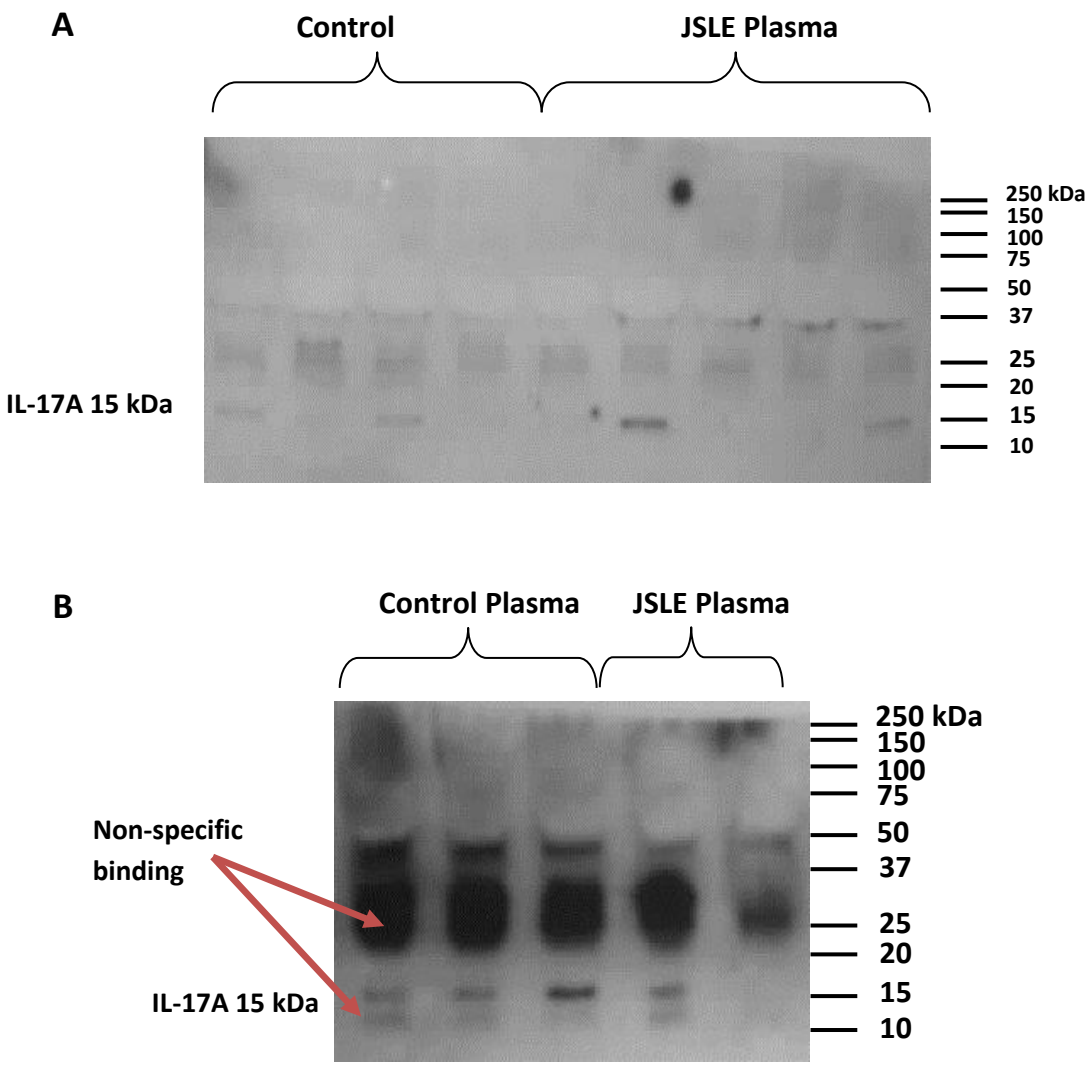



**Figure 24: Western blot of IL-17A in JSLE and control plasma protein at 1:20 and 1:10 dilutions**

Optimisation of measuring IL-17A in JSLE and healthy control plasma by Western blot analysis was carried out in the figures above. **A** Western blot of IL-17A expression in JSLE (n=5) and healthy control (n=4) plasma diluted to 1 in 20. As can be seen in the figure, faint bands were expressed by several control and JSLE samples at 15 kDa consistent with IL-17A expression. **B** JSLE (n=2) and control (n=3) plasma which expressed 15 kDa bands in **A** were then diluted to 1 in 10 and analysed for IL-17A expression to investigate if the dilution factor was optimal however there was strong presence of non-specific binding.

However due to the presence of non-specific binding and IL-17A homodimers or heterodimers seen on the blots, plasma samples were run through Biomax 30 membrane centrifugal filter units (Millipore, USA) that isolated components that were ≤30 kDa in molecular weight. These filtered plasma samples were diluted to 1:10 and 1:20 dilutions and analysed on Western blot both with and without unfiltered plasma samples from the sample patient episodes. However multiple blots examining these filtered plasma samples yielded no bands and this is shown in Figure 25.

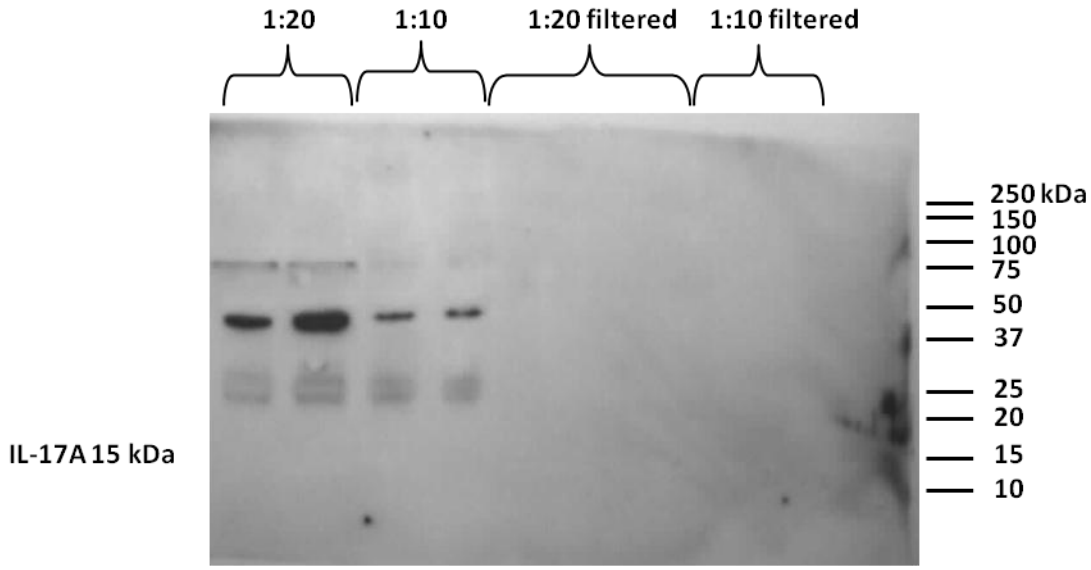



**Figure 25: Control and JSLE plasma compared against same samples passed through filters**

**A Western blot was conducted comparing control and JSLE plasma diluted to 1:20 (healthy control n=1, JSLE n=1) and 1:10 (healthy control n=1, JSLE n=1) against 1:20 (healthy control n=1, JSLE n=2) and 1:10 (healthy control n=1, JSLE n=1) dilutions of the same samples passed through 30 kDa protein centrifugal units. No bands were expressed by filtered samples while 1:20 and 1:10 plasma samples expressed bands but not at 15 kDa.**

A subsequent blot comparing unfiltered 1:10 (JSLE: n=2; control: n=2) and 1:20 (JSLE: n=2; control: n=2) plasma showed that 1:20 dilution is probably optimal for IL-17A detection, however additional optimisation was necessary due to non-specific binding and heterodimer and homodimer cytokines (Figure 26).

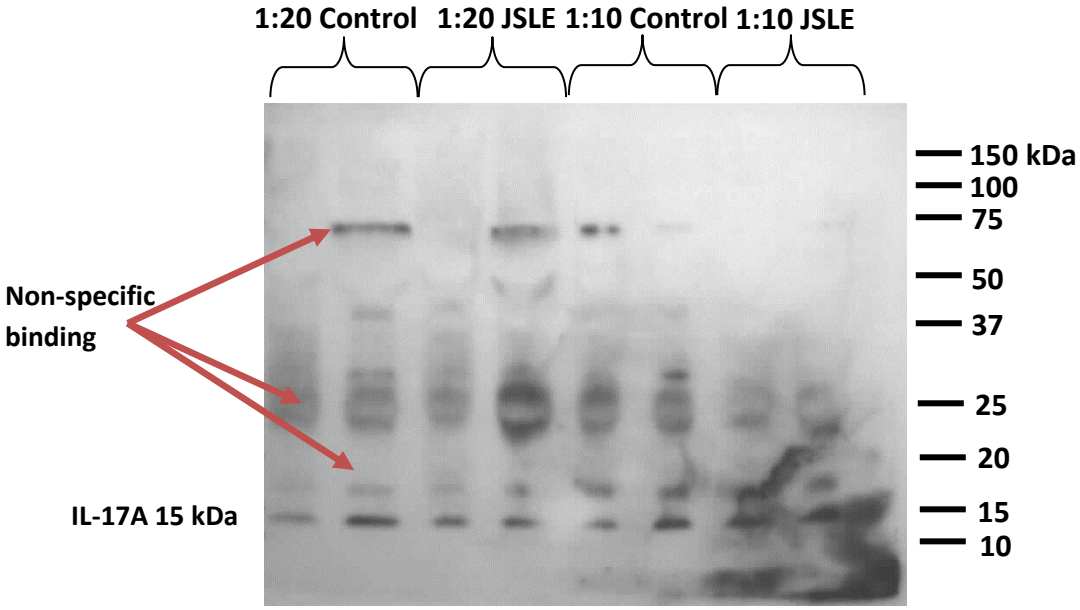



**Figure 26: IL-17A protein expression in control and JSLE plasma at 1:20 and 1:10 dilutions**

**Unfiltered plasma samples from healthy control and JSLE patients were run on Western blot at 1:20 (healthy control n=2, JSLE n=2) and 1:10 (healthy control n=2, JSLE n=2) dilutions and stained for IL-17A. The figure shows that a 1:20 dilution is optimal for IL-17A detection, however additional optimisation is necessary due to the presence of homodimer and heterodimer cytokines and non-specific binding.**

During this time, another member of the Lupus Research Group here in Liverpool validated IL-17A analysis in patient and control plasma using a Human IL-17 Immunoassay Quantikine ELISA kit (R&D Systems, USA) in a spike-and-recovery experiment and found that recovery reached 80-120%, therefore subsequent Western Blot analyses were discontinued at this point.

Following the spike and recovery experiment, JSLE (n=19) and control (n=18) plasma samples were used to measure IL-17 levels. It was found that plasma IL-17A was significantly elevated in JSLE (21.50 ± 5.2 pg/mL) patients compared to controls (7.24 ± 2.5 pg/mL, p=0.028) (Figure 27).

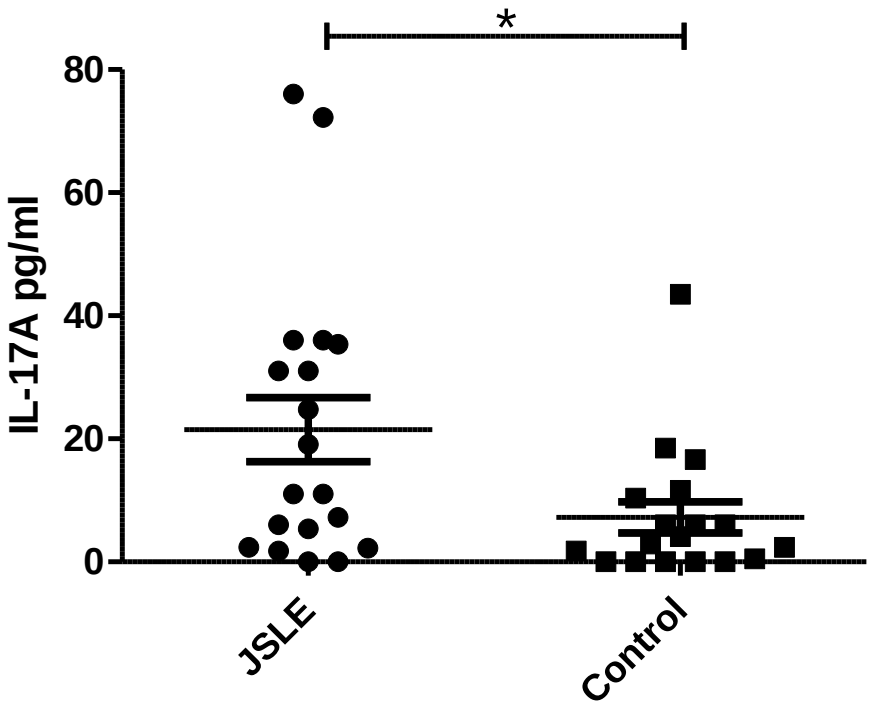



**Figure 27: Plasma IL-17A is higher in JSLE patients when measured with IL-17 Quantikine ELISA**

**Another member of the team used a validated Human IL-17 Immunoassay Quantikine ELISA kit to investigate JSLE (n=19) and healthy control (n=18) plasma IL-17 levels. Plasma IL-17A was significantly elevated in JSLE (21.50 ± 5.2 pg/mL) patients compared to controls (7.24 ± 2.5 pg/mL, p=0.028). Values are represented as mean ± SEM pg/mL. (*) denotes a significant difference between columns (p<0.05).**

## 3.6 mRNA Expression of IL-17B, IL-17C and IL-17RE in JSLE PBMCs compared to controls

Having optimised methods for measuring IL-17A at both RNA and protein level in JSLE and control samples and coupled with the results from plasma IL-17A levels using the IL-17A Quantikine ELISA kit, it was decided that further steps would involve the optimisation of protocols in measuring lesser-known members of the IL-17 family, namely, IL-17B, IL-17C and IL-17RE. This was novel and not in the literature for both JSLE and even adult SLE fields.

### 3.6.1 IL-17B mRNA expression in PBMCs

mRNA expression of IL-17B was quantified in a set of experiments consisting of 100 ng/µL JSLE (n=12) and healthy control (n=12) PBMC cDNA samples. 1.4 µL of each PBMC RNA sample was pipetted to form a single sample mixture that was then diluted with RNAse-free water to 1 in 2, 1 in 5, 1 in 10 and 1 in 20 dilutions. These dilutions and the neat sample were then measured for IL-17B expression using the TaqMan Probe qPCR technique mentioned in section 2.9.3. As shown in Figure 28 below, the combined amplification results from the qPCR experiment demonstrate that there was no mRNA expression of IL-17B in the JSLE and control PBMCs.

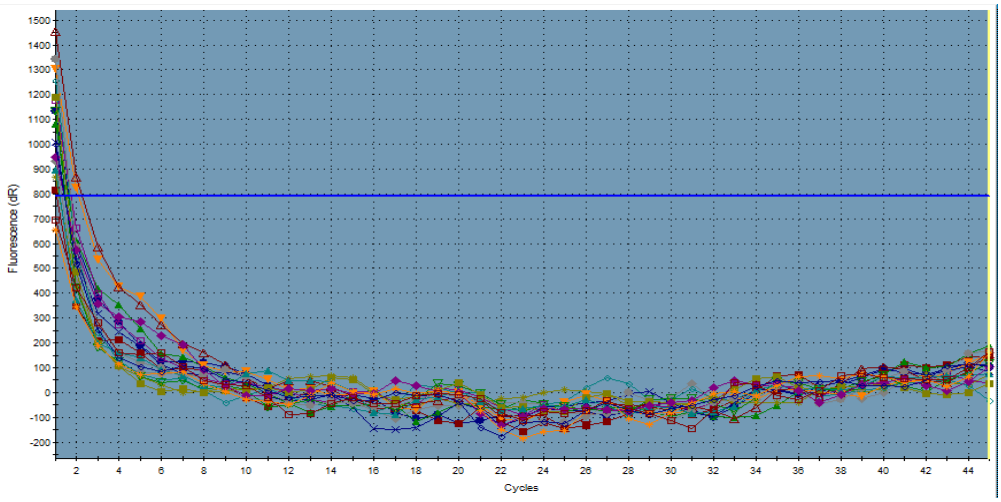



**Figure 28: No mRNA expression of IL-17B in both JSLE and Control PBMCs**

**Taqman Probe qPCR was conducted examining IL-17B expression in JSLE (n=12) and healthy control (n=12) PBMC RNA. The absence of amplification curves, as shown above, demonstrate that there was no mRNA expression of IL-17B in either JSLE or control PBMCs.**

### 3.6.2 IL-17C mRNA expression in PBMCs

#### *3.6.2.1 IL-17C mRNA expression in PBMCs from JSLE patients and healthy controls*

The mRNA expression of IL-17C was investigated in JSLE patients (n=10) and healthy control (n=12) PBMC RNA against 18S. These PBMC samples were not stimulated and serve as a starting point comparison to the CD3/CD28 stimulated PBMCs mentioned in section 3.6.2.2 below. No significant differences (p=0.67) were found in relative expression of either JSLE patients ($2.36X10^{8} \pm 7.73X10^{9}$) or healthy control ($1.77X10^{8} \pm 3.34X10^{9}$) PBMC RNA samples as shown in Figure 29 below.

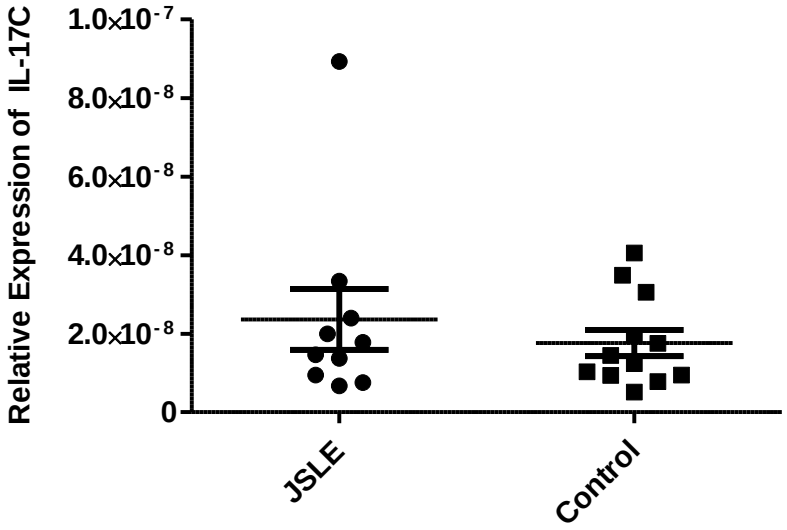



**Figure 29: No significant difference in IL-17C mRNA expression in PBMC RNA**

**Taqman Probe qPCR was used to investigate the expression of IL-17C in JSLE (n=10) and healthy control (n=12) PBMC RNA against 18S as an internal control. No significant differences (p=0.67) were found in the relative expression of IL-17C in either JSLE ($2.36X10^{8} \pm 7.73X10^{9}$) or control ($1.77X10^{8} \pm 3.34X10^{9}$) groups. Values are mean ± SEM.**

#### *3.6.2.2 IL-17C mRNA expression in CD3/CD28 stimulated PBMCs*

ELISA experiments conducted alongside by a fellow member of the Lupus Research Group demonstrated an increase, tending toward significance, in JSLE IL-17A protein production (n=5, 383.9±147.9) compared to controls (n=5, 22.59±7.774, p=0.055) in PBMCs stimulated for 2 days with CD3/CD28 activation beads, this is shown in Figure 30 below. Raw data for this experiment is detailed in Appendix 5.

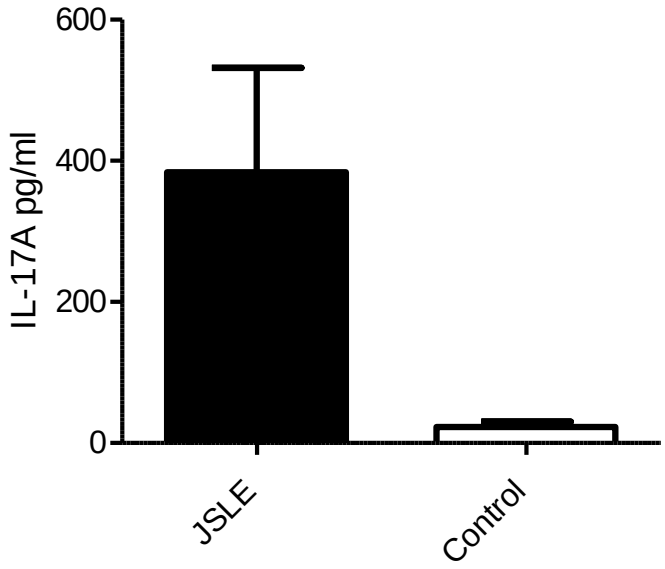


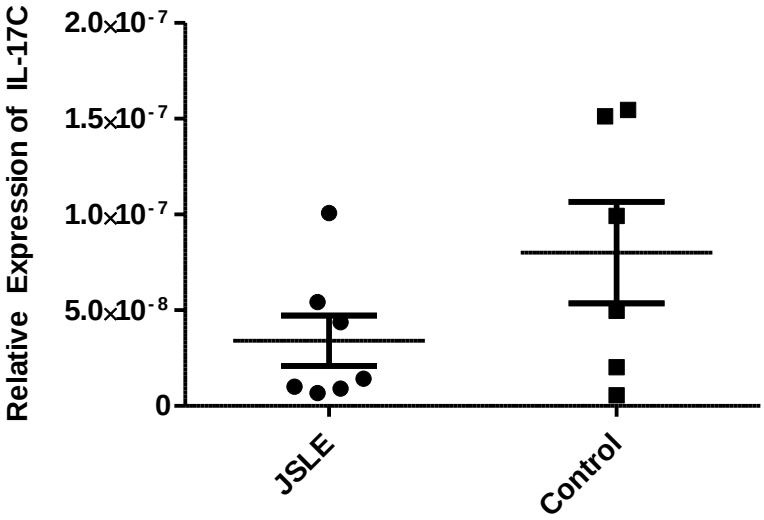

**Figure 30: IL-17A protein expression in CD3/CD28 stimulated PBMCs**

**Protein expression of IL-17A was measured using the IL-17A Quantikine ELISA kit in the CD3/CD28 stimulated PBMCs of JSLE (n=5) and healthy control (n=5) groups. Results reflected a significantly increased expression of IL-17A from JSLE patients (383.9±147.9) compared to controls (22.59±7.774, p=0.055).**

Based on these findings and the absence of positive signal from unstimulated PBMCs, subsequent experiments here investigated expression of CD3/CD28 stimulated PBMCs. The protocol involving T cell activation of PBMCs using CD3 and CD28 beads is detailed in section 2.10.

IL-17C mRNA expression was measured in CD3/CD28-stimulated PBMCs at day 2 in JSLE (n= 7) and healthy control (n=6) patients. The relative expression against housekeeping gene 18S is shown in Figure 31A. There were no significant differences in mRNA IL-17C expression between JSLE (Median $1.42X10^{8}$, SEM $1.32X10^{9}$) and control ($7.46X10^{8}$, SEM $2.65X10^{9}$) PBMCs (p=0.30). Figure 31B depicts the $2^{-\Delta\Delta Ct}$ relative quantification of fold change in JSLE stimulated PBMCs to controls (0.44 [0.1-2.5] to 1). Raw data is detailed in Appendix 6.

**A**




**B**

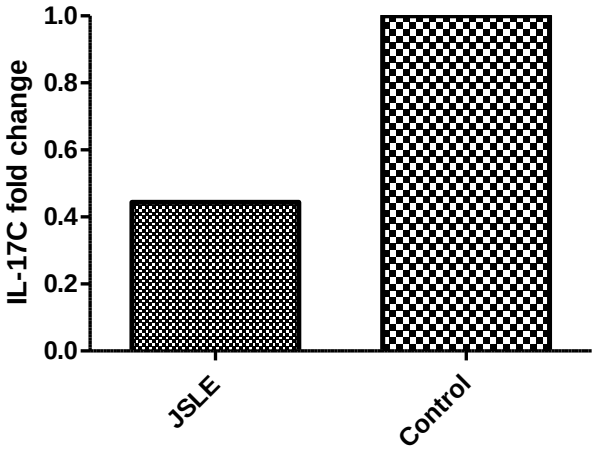



**Figure 31: IL-17C mRNA expression and fold change in CD3/CD28 stimulated PBMCs at Day 2**

IL-17C mRNA expression and fold change of JSLE (n=7) and healthy control (n=6) CD3/CD28 stimulated PBMCs at day 2 were measured using Taqman Probe qPCR. **A** Relative expression of IL-17C against an 18S internal control are expressed as mean relative expression ± SEM. No significant differences were seen between JSLE ($3.41X10^{8}$ ± $1.32X10^{9}$) and control ($8.01X10^{8}$ ± $2.65X10^{9}$, p=0.30) groups. **B** $2^{-\Delta\Delta Ct}$ relative quantification of fold change of IL-17C mRNA expression in JSLE CD3/CD28 stimulated PBMCs were compared to control stimulated PBMCs. JSLE stimulated PBMCs showed a fold decrease of 0.44 to 1 (controls).

### 3.6.3 IL-17RE mRNA expression in PBMCs

#### *3.6.3.1 IL-17RE mRNA expression in PBMCs from JSLE patients and healthy controls*

IL-17RE mRNA expression was measured with Taqman Probe qPCR against 18S in JSLE (n=10) and control (n=12) PBMC RNA. No significant differences (p=0.74) were found in relative expression between JSLE ($1.39X10^{8}$ ± $3.58X10^{9}$) or control ($1.80X10^{8}$ ± $4.65X10^{9}$) samples as shown in Figure 32 below.

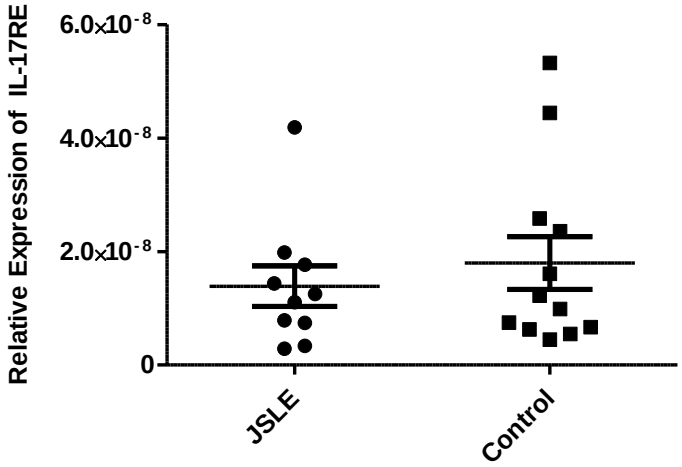



**Figure 32: No significant difference in IL-17RE mRNA expression in PBMC RNA**

**Expression of IL-17RE was investigated using Taqman Probe qPCR in JSLE (n=10) and control (n=12) PBMC RNA against 18S as an internal control. No significant differences (p=0.74) were found in the relative expression of IL-17C between JSLE ($1.39X10^{8}$ ± $3.58X10^{9}$) or control ($1.80X10^{8}$ ± $4.65X10^{9}$) groups.**

#### *3.6.3.2 IL-17RE mRNA expression in CD3/CD28 PBMCs*

mRNA expression of IL-17RE was measured in CD3/CD28 stimulated PBMCs at day 2 of stimulation in JSLE (n=8) and healthy control (n=8) patients. Relative expression against 18S housekeeping gene is shown in Figure 33A below. The relative expression of IL-17RE was significantly decreased (p=0.007) in JSLE PBMCs (Median $2.06x10^{-8}$ ± $7.87x10^{-8}$) compared to control PBMCs (Median $9.35x10^{-8}$ ± $5.09x10^{-8}$). Figure 33B demonstrates the fold decrease in IL-17RE expression in JSLE CD3/CD28 stimulated PBMCs compared to control PBMCs (0.19 [0.03-1.2] to 1). Raw data is detailed in Appendix 7.

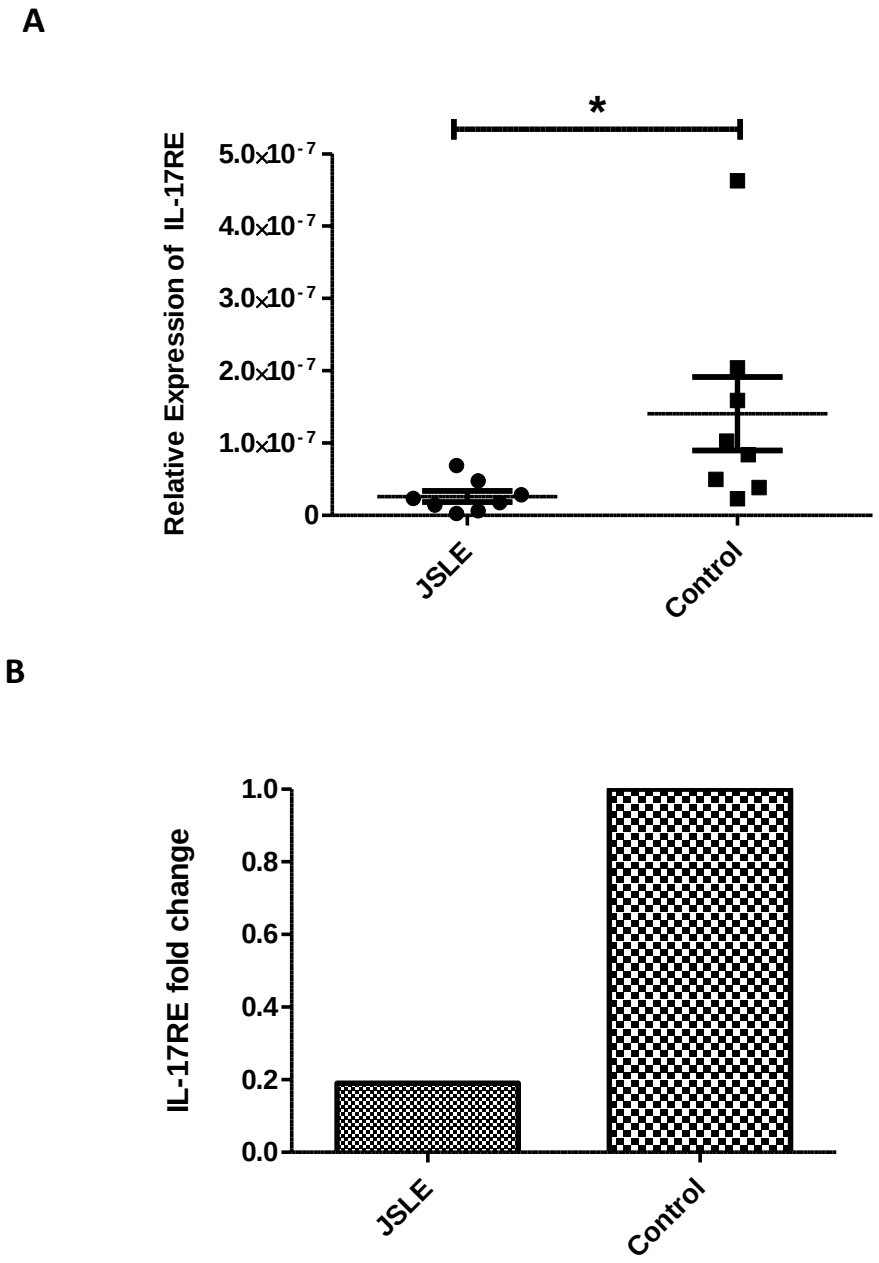



**Figure 33: IL-17RE mRNA expression and fold change in CD3/CD28 stimulated PBMCs at Day 2**

Taqman Probe qPCR was used to measure IL-17RE mRNA expression and fold change of JSLE (n=7) and healthy control (n=6) CD3/CD28 stimulated PBMCs at day 2. **A** Relative expression of IL-17RE against 18S as an internal control are expressed as mean relative expression ± SEM. The JSLE group ($2.62x10^{-8}$ ± $7.87x10^{-8}$, p=0.007) expressed significantly increased levels of IL-17RE compared to the healthy control stimulated PBMCs ($1.41x10^{-7}$ ± $5.09x10^{-8}$). (*) indicates a significant difference between columns. **B** $2^{-\Delta\Delta Ct}$ relative quantification of fold change of IL-17RE mRNA expression in JSLE CD3/CD28 stimulated PBMCs were compared to control stimulated PBMCs. JSLE stimulated PBMCs showed a fold decrease of 0.19 to 1 (healthy controls).

## 3.7 Protein Expression of IL-17C and IL-17RE

### 3.7.1 IL-17C and IL-17RE protein expression in CD4$^+$ cells

Measuring IL-17C and IL-17RE in CD4$^+$ cell protein was optimised using samples from adult healthy volunteers (n= 4). IL-17C has a molecular weight of approximately 20 kDa while IL-17RE has a molecular weight of 71-74 kDa. Initially blot results demonstrated a strong presence of non-specific binding, however it was concluded that using 5% Marvel skimmed milk as a diluent for the secondary antibody and then incubating the secondary antibody for one hour at 4°C in both IL-17C and IL-17RE assays resulted in the clearest bands.

#### *3.7.1.1 IL-17C expression in CD4$^+$ Protein*

JSLE patient (n= 4), healthy control (n= 3) and JIA patient (n= 2) CD4$^+$ samples were measured for IL-17C expression at the protein level. However as seen in the following Figure 34, strong 15 kDa bands were yielded, which indicates IL-17C homology with IL-17A, while three JSLE samples and one control yielded very faint bands at 20 kDa consistent with IL-17C. In addition, one JSLE and one control sample did not express B-actin, which may indicate poor sample quality.

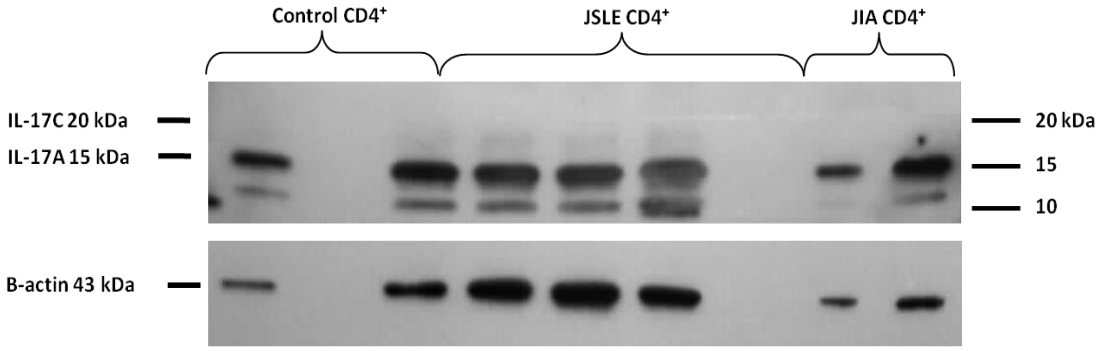



**Figure 34: IL-17C protein expression in CD4$^+$ cells**

**IL-17C protein expression was optimised and measured in JSLE (n=4), healthy control (n=3) and JIA (n=2) CD4$^+$ T cells and compared against B-actin as an internal control using Western blot analysis. Bands were detected in JSLE, healthy control and JIA samples at 15 kDa consistent with IL-17A while faint bands were detected at 20 kDa in healthy control and JSLE CD4$^+$ samples consistent with IL-17C expression.**

### *3.7.1.2 IL-17RE expression in CD4$^+$ Protein*

A Western blot analysis was conducted on JSLE (n= 4), healthy control (n= 3), and JIA (n= 2) CD4$^+$ samples and stained for IL-17RE and B-actin protein expression as an internal control. Figure 35 demonstrates four faint bands at 71-74 kDa consistent with IL-17RE in one control and three JSLE samples. JIA samples did not measure any IL-17RE. In addition, one control and one JSLE sample did not express B-actin and therefore were not included in the final analysis. For both IL-17C and IL-17RE Western blot assays, band densitometry was measured according to the protocol described in section 2.11, however as resultant bands were faintly expressed, calculated values were subject to strong influence from background noise and therefore excluded from the final analysis.

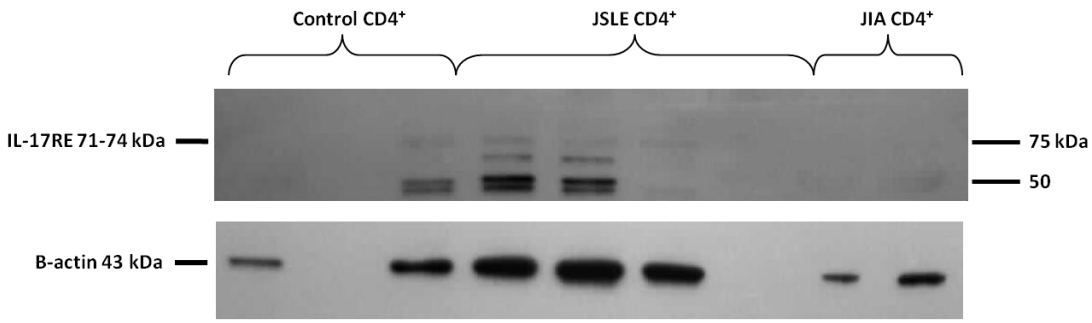



**Figure 35: IL-17RE protein expression in Control, JSLE and JIA CD4$^+$ cells**

**Measurement of IL-17RE protein expression was optimised in JSLE (n=4), healthy control (n=3) and JIA (n=2) CD4$^+$ T cells and compared against B-actin as an internal control using Western blot analysis. Faint bands were detected in healthy control (n=1) and JSLE (n=4) CD4$^+$ samples at 71-74 kDa consistent with IL-17RE expression.**

### 3.7.2 PBMC Protein expression of IL-17C and IL-17RE

Attempts were made to optimise measurement of IL-17C and IL-17RE expression in the protein of JSLE (n=4), control (n=3) and JIA (n=2) PBMCs using Western blots. Although optimised methods for CD4$^+$ protein as mentioned in section 3.7.1 were employed, the Western blots did not yield any clear results on film development. Limitation of time resulted in this being unable to be optimised further at this stage.

## 3.8 Summary of Results

- In summary, 102 patients (JSLE: n=31; healthy controls: n=66; JIA: n=4; lupus nephritis: n=1) were involved in this study, with multiple episode samples from JSLE and JIA patients.

- Poor recovery percentages out of acceptable range and matrix interference were detected in JSLE and healthy control plasma and serum tested by spike-and-recovery validation experiments of an IL-17A single plex and IL-17A, IL-21 and IL-23 ELISAs.

- Methods measuring IL-17A at the mRNA and protein level using qPCR and Western blot analysis were optimised in PBMCs, $CD4^+$ cells and neutrophils of JSLE, healthy control and JIA inflammatory control patients.

- No significant differences were found in IL-17A expression in PBMCs or $CD4^+$ cells between JSLE patients and healthy controls.

- Protein IL-17A expression of neutrophils was significantly decreased in JSLE patients compared to healthy controls ($p=0.03$).

- Neutrophil expression of IL-17A was validated by comparing ultra-purified neutrophils from healthy adult donors to PolymorphPrep-isolated neutrophils.

- IL-17B was found not to be expressed in any JSLE or healthy control PBMC RNA.

- Methods measuring mRNA of IL-17C and IL-17RE were optimised in JSLE and healthy control PBMCs using qPCR. No significant differences were found between JSLE and control $CD4^+$ cells or PBMCs for IL-17C or IL-17RE mRNA expression.

- IL-17RE mRNA expression was significantly decreased in stimulated JSLE PBMCs ($p=0.007$), with fold decreases in IL-17C and IL-17RE of JSLE relative to controls.

- Western blotting of IL-17C and IL-17RE protein expression was optimised in JSLE, healthy control and JIA inflammatory control $CD4^+$ T cells.

# Discussion

The Th17 cell and its related cytokines IL-17A, IL-21 and IL-23 have been shown to have abnormal expression in adult onset SLE while lesser known cytokines of the IL-17 family IL-17B, IL-17C and IL-17RE have been implicated in contributing to autoimmune pathology as discussed in chapter 1. However, in the context of JSLE, existing studies have only measured IL-17A. As JSLE manifests as a much more severe inflammatory disease compared to its adult counterpart, there is an increasing void in current evidence which highlights the need to characterise the profile of Th17 cells and its related cytokines in JSLE.

This study, by applying a scientific and robust approach to the bio-assays employed, has focused on the range of Th17-related cytokines in addition to IL-17A and also the IL-17 family for the first time in the context of JSLE. The study has validated and optimised diverse methodologies in measuring and comparing the expression of these cytokines in various cell subsets such as PBMCs, $CD4^+$ T cells and neutrophils from the peripheral blood of JSLE patients, healthy controls and JIA patients. Such data has never been presented in the published studies reviewed in chapter 1, in relation to the role of Th17 cells in SLE, therefore variability in the data between studies could very likely relate to basic issues in the assays.

## 4.1 Poor recovery in detecting Th17-related cytokines

In the spike-and-recovery experiments involving the IL-17A single plex, IL-17A, IL-21 and IL-23 ELISA assays, recovery percentages in JSLE samples at all dilutions were less than 80%, out of the acceptable recovery range. IL-21 and IL-23 also showed poor recovery in the control plasma samples out of the acceptable range of 80-120% at all 4 dilutions. Interestingly, control plasma samples exhibited a worse recovery outcome compared to the JSLE plasma in both assays, however there is a need to validate these results by increasing the number of control plasma samples. The IL-17A ELISA at a 1-in-8 dilution factor yielded an 80.3% recovery, however this caused IL-17A to be completely undetectable in unspiked samples, hence

minimising interference must be balanced against the risk of diluting out the analyte.

While recovery in the IL-23 ELISA was highest in spiked neat samples, the IL-17A single plex, IL-17A ELISA and IL-21 ELISA experiments showed strong trends of increased recovery as the sample matrix increased in dilution factor. It can be inferred that constituents in the sample matrix may be affecting the detection of these target cytokines. Urbonaviciute et al reported that IgG and IgM antibody titres in SLE and control serum and plasma correlated to the masking of HMGB1 ELISA detection; the study found that adding purified IgG antibodies to plasma and serum samples spiked with HMGB1 resulted in substantial interference in recovery (153). Heterophilic antibodies, weak antibodies produced against a broad spectrum of poorly defined antigens, are thought to interfere with immunoassays by mimicking the target antigen, binding with the antigen or interfering with the binding site between antigen and antibody, giving false positive or false negative results (160-162). Heterophilic antibodies are naturally occurring in 40% of the normal population but may be increased in the form of rheumatoid factor (RF) or other antibodies in autoimmune diseases (163, 164). JSLE patients have been shown to have increased levels of autoantibodies in their peripheral circulation compared to adult SLE patients (165, 166). In addition, autoimmune patients on biological therapeutics such as etanercept may develop anti-animal antibodies that may interfere with immunoassay procedures that are dependent on animal-derived monoclonal antibodies, due to animal cell lines being involved in the production process (161, 163, 167). The ways in which these human anti-animal antibodies interfere with sandwich assays is shown in Figure 36 below.

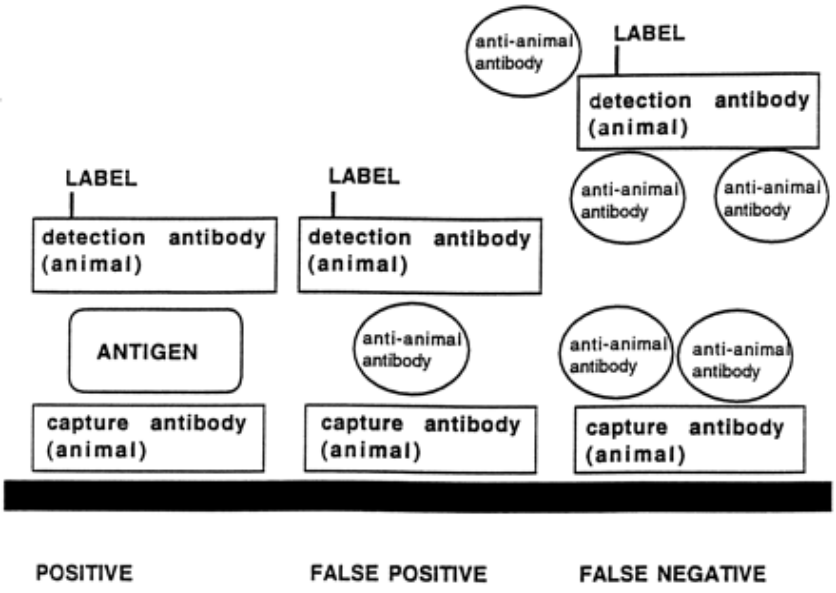



**Figure 36: Interference of human anti-animal antibodies in sandwich immunoassays**

**Human anti-animal antibodies may be increased in autoimmune patients on biological therapies, and may contribute to the false negative results in the spike-and-recovery experiments covered in this thesis. They may bind to the animal capture antibody, or animal detection antibody on sandwich immunoassays, hence reducing the availability of these antibodies to detect naturally occurring cytokines in the sample. Image taken from Kricka 1999 (163).**

It is suggested that all assays should first be validated with a spike-and-recovery experiment with linearity-of-dilution to ensure that results are congruent with an acceptable recovery range (160). Current evidence suggests trying out a range of troubleshooting manoeuvres such as comparing analyte concentrations before and after the use of a commercial heterophile blocking antibody reagent for recovery potential (160, 163). Samples could be treated with polyethylene glycol (PEG) prior to the assay as it depletes all IgG, IgM and 70% of IgA concentrations (168). A series of studies by De Jaeger et al investigating heterophilic antibody interference have reported that incubating test samples with protein-L coated beads prior to the immunoassay was significantly more efficient than PEG at immunoglobulin depletion, they coupled this with the addition of rat and mouse serum to samples in order to block residual non-specific binding (164, 169).

Additionally, the same research team recommended the use of multiplex cytokine assays in preference to conventional ELISAs, due to their ability to analyse multiple cytokine profiles simultaneously with a small amount of sample (170). However as a diverse mixture of animal antibodies from different species are used in the assay, it is more vulnerable to non-specific antibody binding constituents of the assay or other antibodies which would give rise to false results and therefore immunoglobulin depletion must be adopted for such assays (171). In order to minimise interference and increase the robustness of future studies, methods such as using antibody blocking protocols and alternative assays should be considered.

## 4.2 IL-17A expression in cell subsets and plasma

Methods measuring IL-17A in PBMCs, CD4+ cells and neutrophils were optimised at both qPCR and Western blot levels. No significant differences were found in IL-17A expression of CD4+ cells between JSLE, JIA and control groups on both RNA and protein level. Although no significant differences between JSLE patients and controls were found in IL-17A protein expression in PBMCs, working alongside another member of the team found, using a novel and subsequently validated ELISA, an increased protein IL-17A expression tending toward significance in JSLE PBMCs that had been stimulated with CD3/CD28 beads at day 2. No significant differences were found amongst JSLE, JIA and control PBMC protein expression of IL-17A on Western blot analysis. In summary, investigation carried out of IL-17A (mRNA and protein) expression in cell subsets from this study's groups contradict the study hypothesis that upregulated IL-17A expression will be present in the cell subsets of JSLE patients compared to controls.

Consistent with results seen within this study of IL-17A expression in JSLE PBMCs, Kurasawa et al found increased IL-17A expression in the PBMC mRNA and serum of patients with systemic sclerosis but not in that of SLE patients when compared to healthy controls (172). However another study conversely reported higher mRNA IL-17A expression in active SLE PBMCs compared to controls (85). Although many studies have reported raised levels of Th17 cells in SLE, there are

also many that found no significant differences in IL-17A expression in SLE and control CD4+ T cells using flow cytometry analysis, paralleling IL-17A results in $CD4^+$ cells of this study (97, 109, 173, 174).

There are a number of explanations for potential differences between studies and the levels of JSLE IL-17A expression found in this study. Multiple studies have reported that type I IFNs downregulate Th17 differentiation and IL-17A production from PBMCs at the mRNA and protein level both in vitro and in vivo (175-177). Therefore, this may provide an explanation for the low expression of IL-17A seen in $CD4^+$ cells and PBMCs of JSLE patients as raised levels of IFN-α have been detected in the peripheral blood of SLE patients and SLE serum has been shown to induce IFN-α production from PBMCs (178-180).

Levels of IL-17A may have been suppressed by the wide range of immunosuppressive therapies JSLE patients were prescribed, as seen in SLE patients on azathioprine who displayed significantly decreased numbers of IL-17A-expressing $CD4^+$ cells (181). However serum IL-17 levels have been shown to have no association with use of prednisolone, hydroxychloroquine or mycophenolate mofetil in adult SLE patients, in addition a wide-scale study examining hydroxychloroquine use in adult SLE patients found that although pro-inflammatory cytokine levels such as IL-1β, IL-6 and IFN-α levels were decreased, it was not statistically significant (110, 182). Small sample size limitations in this present study precluded appropriate sub-analysis based on patient's medication.

Lupus tends to be more severe in paediatric patients, and so a real difference if present would theoretically be more likely to be seen in the younger age group. One important contributing explanation why this was not the case is that increased IL-17A levels may be concentrated at the target sites of inflammation, such as within the kidney, skin or joints in the patients of this study, while remaining at normal levels in the peripheral circulation. Previously, increased levels of IL-17A expressing $CD4^+$ and double negative T cells have been found in the skin and kidney tissues respectively of SLE patients compared to controls (183-185). IL-17A infiltration into the kidney was found to correlate significantly with disease activity levels. In a

comparison between psoriatic patients and healthy volunteers, IL-17A expression was found to be significantly increased in the dermis of psoriatic patients while there were no significant differences between psoriatic patients and controls when comparing IL-17A levels in T cells isolated from peripheral blood (186). This is supported by a study that reported raised IL-17A expression in rheumatoid arthritis synovial fluid compared to in the peripheral circulation (187, 188). This disparity in IL-17A expression between synovial fluid and peripheral blood is furthered mirrored in the paediatric context of JIA as mentioned previously in section 1.4.1 (128).

Another explanation could be that the pro-inflammatory activity of IL-17A in JSLE could be partly attributed to IL-17A/IL-17A homodimers and IL-17A/IL-17F heterodimers that have been found on ELISAs and Western blots to be expressed by human $CD4^+$ T lymphocytes (122). These findings are congruent with data from this study where blots investigating JSLE and control plasma, neutrophils and PBMCs were stained for IL-17A and resultant bands were consistent with the molecular weight of a disulfide-linked IL-17A/IL-17A homodimer (35 kDa) and IL-17A/IL-17F heterodimer (37 kDa) (122). Chang et al reported that the IL-17A/IL-17F heterodimer was able to work in synergy with TNF-α to regulate pro-inflammatory cytokines in mouse models (159). In rheumatoid arthritis patients, IL-17A-IL-17A has been detected in the synovial fluid and IL-17A-IL-17A and IL-17A-IL-17F expression have been measured in CD3/CD28 stimulated plasma using validated immunoassays (189). Hence it would be useful to quantify and compare the expression of this homodimer and heterodimer cytokines to determine if increased IL-17A is bound in inflammatory dimer cytokines in our subset of patients

Previous research has shown that IL-17A is expressed by neutrophils from the peritoneal fluid of mouse models with systemic vasculitis and from the airway in lipopolysaccharide-induced lung inflammation (190, 191). In this study, JSLE and healthy paediatric control neutrophils were investigated and compared for IL-17A protein expression. Protein from control neutrophil samples isolated using PolymorphPrep showed a significantly higher expression of IL-17A on Western blots compared to JSLE neutrophils. The reason for this is unclear however if repeatable, it would be interesting compare IL-17A expression between JSLE and control

neutrophils that had been purified via EasySep neutrophil enrichment. On further investigation, neutrophils which were isolated using PolymorphPrep and were of lower percentage purity, were found to express a higher amount of IL-17A compared to neutrophils negatively selected using the EasySep method in adult healthy volunteers. The magnetically isolated neutrophils were also spiked with 10% PBMCs from the same donors, and showed increased IL-17A expressed compared to the unspiked samples which may indicate that PBMC contamination rather than the neutrophils were reflecting the IL-17A expression on Western blots.

In contrast, a study detected IL-17A mRNA expression in SLE and control neutrophils which had been purified to ≥95%, however there was no difference between patient groups, it also found significantly increased IL-17A expressing neutrophils in SLE skin biopsies compared to controls (192). Another study found IL-17A at a protein level in healthy control neutrophils on Western blots from neutrophils that had been isolated from peripheral blood to 99.5% purity via flow cytometry, this was validated against a IL-17A recombinant protein and B-actin, although IL-17A expression was weaker when compared to PBMCs from the same donors; this same study also found that neutrophils from psoriatic skin lesions expressed IL-17A (193). The lower levels of IL-17A expression in neutrophils could be due to neutrophils binding to and amassing IL-17A from other cells instead of synthesising it, on the other hand the short half-lives neutrophils have in the circulation (see section 1.1.1.2) may decrease IL-17A transcription and synthesis compared to PBMCs (193). Additional studies examining blots using 95% purified neutrophils of patients with oral inflammation and healthy adult volunteers revealed the presence of IL-17A protein expression, and this was significantly higher in PBMCs compared to neutrophils for healthy adult volunteers (194, 195). These findings are consistent with the data from this study and suggest that human neutrophils may express IL-17A, albeit weakly compared to PBMCs. Conversely, the latter two studies took investigated IL-17A at its homodimer 35 kDa molecular weight which differed from our analysis, therefore the protein expression of IL-17A in neutrophils warrant further investigation.

Working alongside this study, a member of our Lupus Research group demonstrated significantly raised IL-17A in the plasma of JSLE compared to controls in a validated Quantikine ELISA kit experiment. This is consistent with recent findings demonstrating significantly increased serum IL-17A found in Indian JSLE patients which correlated with disease activity scores, this study also found significantly increased levels of IL-23 (196). In addition, the concentration levels measured in JSLE and control plasma with the Quantikine ELISA were congruent with those measured in adult SLE and control plasma in other studies (88, 90). These results indicate that although elusive and difficult to measure, this increased protein expression of IL-17A still very much plays a part in the pathogenesis of JSLE.

## 4.3 The IL-17 family in cell subsets

This is the first time lesser-known IL-17 cytokine members have been investigated in the context of JSLE and even adult SLE. No mRNA expression was detected for IL-17B in both JSLE and control PBMCs in this study, which is consistent with findings by Hwang and Kim in relation to IL-17B mRNA expression in PBMCs and SFMCs of rheumatoid arthritis patients (197). This suggests that IL-17B may not be present in the joints and peripheral blood of humans. However a study by Stamp et al found IL-17B and IL-17C expression in a majority of rheumatoid nodules from 16 patients with rheumatoid arthritis, which may suggest that IL-17 family members may congregate at target tissue organs (198).

In this study IL-17C and IL-17RE protein expression were measured in CD4+ cells whilst IL-17C and IL-17RE mRNA expression was measured using PBMCs and CD3/CD28 stimulated PBMCs. Interestingly IL-17RE expression was found to be significantly increased in the stimulated PBMCs of healthy controls compared to JSLE stimulated PBMCs. There were no significant differences in IL-17C expression between patient groups. JSLE stimulated PBMCs showed a significant fold decrease in both IL-17C and IL-17RE mRNA expression compared to controls. It could be possible that IL-17C and IL-17RE would be found higher numbers at target tissue organs in JSLE patients, similar to IL-17B and IL-17C expression seen in rheumatoid

nodules and IL-17C in psoriatic lesions, thereby depleting these cytokine levels in the peripheral blood (198, 199). It is currently unclear as to why IL-17RE is significantly decreased in stimulated JSLE PBMCs, although this is mirrored in significantly decreased IL-17RE expression in lesional psoriatic skin investigated by Johansen et al (199). However, due to limited quantities detected in the peripheral blood of all patient groups, it is difficult to draw conclusions on how significant a role IL-17C and IL-17RE may play in PBMCs and CD4+ cells. Overall, these results contradict the hypothesis of increased IL-17C and IL-17RE expression in JSLE samples.

Previous studies have shown that IL-17C and IL-17RE are found in very low quantities in fibroblasts and PBMCs of healthy controls, while they are expressed the highest in mucosal tissues such as the lungs and in the colon, these findings are consistent with the low levels of IL-17RE and IL-17C found in this study at the mRNA level in PBMCs (137). Conversely, IL-17C has previously been shown to be present in the PBMCs and SFMCs of rheumatoid arthritis patients and IL-17C expression was increased substantially with IL-15 stimulation of the samples (197). IL-17C and IL-17RE have been shown to share similarities with IL-17A and IL-22 in promoting an inflammatory skin clinical feature in mouse models, where IL-17C and IL-17RE knock-out mouse groups displayed significantly milder skin phenotype with less inflammation, which correlated with decreased inflammatory cytokine profile (137). Adding to this, an increased expression of IL-17C was reported in mRNA and protein expression in psoriatic skin lesions (199). In view of this information, coupled with evidence of IL-17C's local role in inflammatory joints and rheumatoid nodules as discussed in 1.4.3, it would be interesting to examine IL-17C and IL-17RE expression in tissues of target organ damage such as skin lesions, kidney biopsies and synovial fluid of symptomatic JSLE patients.

## 4.4 Implications of findings in the current evidence base

The findings of this study with regards to the ELISA and single plex assays measuring Th17-related cytokines reveal that components in the sample matrix of both control and JSLE serum and plasma are influencing the recovery of target cytokines. This provides a strong explanation for the inconsistencies in the circulating IL-17A and IL-23 concentrations that have observed in adult SLE studies (88, 111, 133). Therefore, existing evidence reporting the measurement of these cytokines within adult-onset SLE, JSLE and control plasma and serum using ELISA and single plex detection kits must be interpreted with caution. In addition, attempts should be made to identify and tackle this interference by means of spike-and-recovery validation, immunoglobulin depletion and blocking non-specific binding with animal serum to ensure that study design is reliable and cytokine measurements are of strong external validity.

In relation to the IL-17 family, this study found that IL-17B may not be present in PBMCs in humans, in contrast to mouse models. Measurement of IL-17C and IL-17RE levels at the protein and mRNA level was optimised and investigated in PBMCs, $CD4^+$ T cells and PBMCs that had undergone *ex vivo* stimulation. Although found at minute, sometimes unquantifiable concentrations, this study showed that IL-17C and IL-17RE are expressed by PBMCs and $CD4^+$ cells in the peripheral blood of human JSLE and control participants for the first time. Currently, there is a paucity of current evidence on role of the lesser known IL-17 cytokine members, particularly IL-17-RE, in human studies and therefore further investigation in human studies is needed into how the IL-17C and IL-17-RE puzzle pieces fit into the IL-17 pro-inflammatory picture.

As described at the beginning of this study in sections 1.3 and 1.4.1, there has been an emergence of studies investigating IL-17A expression in adult SLE patients over the past decade. However, the evidence base examining these cytokines in paediatric patients with autoimmune disease, let alone JSLE patients, is greatly lacking in comparison. Although data from this study contradict the initial hypothesis of increased IL-17A expression in the peripheral blood of JSLE patients, it may be that in contrast to adult studies, IL-17A expression in the peripheral blood of

JSLE patients does not differ from that of controls, and is instead concentrated at target sites of inflammation. This study has involved the optimisation of Western blot analysis methods in examining protein expression of IL-17A in a plethora of cell subsets and has found that neutrophils may also express IL-17A. This is consistent with what current evidence suggests and provides insight into the IL-17A cytokine – that its synthesis may straddle both the innate and adaptive immune system.

### 4.4.1 Therapeutic implications

This research into comprehending Th17-related cytokines and the IL-17 family in pathogenesis of JSLE is aimed at ultimately developing therapeutic interventions that would tackle this life-long disease. A study by Dong et al reported that PBMCs from adult lupus nephritis patients that had been incubated for 24 hours in IL-17A expressed significantly higher protein levels of IL-6, IgG and anti-dsDNA and this was partially blocked by administration of dexamethasone and completely blocked by IL-17A monoclonal antibody mIgG(28) (127). Although not supported by findings from this study, a member of our team found that IL-17A was increased in the plasma of JSLE patients compared to controls. So far, investigation into these pro-inflammatory cytokines has been translated into the commencement of trials involving specific monoclonal antibodies as an interventional treatment in autoimmune disease. A monoclonal antibody to IL-17A, AIN457, was trialled in patients with psoriasis, rheumatoid arthritis and anterior uveitis and generated an improved response in disease activity scores and blood profile, with no difference in severe adverse events between intervention and placebo groups (200). In a double-blind, randomised controlled trial, a significant improvement was observed in disease activity scores in rheumatoid arthritis patients taking LY2439821, another anti-IL-17 monoclonal antibody, as an adjunct to disease-modifying anti-rheumatic drugs and demonstrated good tolerability with only one serious adverse event reported (201).

Targeting IL-23 upstream from IL-17A has also come into play - Briakinumab, a monoclonal antibody directed against the p40 subunit which is present in IL-23, and Ustekinumab, a monoclonal antibody directed against IL-12 and IL-23 cytokines, have been investigated in several phase III randomised controlled trials for

treatment in psoriatic patients. It was found that both drugs were well tolerated and were demonstrated to be vastly superior to etanercept, methotrexate and placebo in treating the disease (202-205). As described previously in section 1.3.3, Tocilizumab is a monoclonal antibody directed against IL-6 receptor and is currently licensed for use in many countries as an adjunct to disease-modifying anti-rheumatic drugs or as a monotherapy in the treatment of rheumatoid arthritis. It has shown promising results and tolerability in several wide-scale randomised controlled trials involving patients with rheumatoid arthritis, JIA and SLE (118, 206, 207).

## 4.5 Limitations of this study

Although the aim was to investigate the role of Th17-related cytokines and IL-17 subsets in JSLE by means of a strong scientific study, this research was constrained by several factors pertaining to the methodology of this project and these have to be considered.

First, many of the JSLE and JIA patients are at varying stages of disease progression and are on a multitude of different immunosuppressive therapeutic regimes. Most of these drugs exert their effect by controlling the immune system, which may inherently influence each individual's cytokine profile at each collection episode, therefore potential acting as confounders to the results obtained.

In addition, the matrix interference discovered in multiple immunoassay kits coupled with the optimisation of qPCR and Western blot techniques for each different cytokine, contributed to the time constraints which restricted to a large extent the full characterisation of IL-17A, IL-21, IL-23 and the IL-17 family in the context of JSLE. Although efforts were put in place to streamline this, results were limited by the semi-quantitative nature of Western blots, which are subject to highly variable image analysis methods and are susceptible to pitfalls at each stage (208, 209).

Finally, SLE, notably JSLE, is an intrinsically rare disease hence the number of patients available locally to this area is small. This is despite Liverpool being the UK's only Centre of Excellence for Childhood Lupus, and one of the largest JSLE cohorts in the country. Patient numbers posed a challenge to the procurement of adequate sample numbers, hence collaborative studies are essential in maximizing study numbers for studies of this nature. Additionally, venous blood samples were only obtained as part of occasional routine clinical protocols and taking into account the sample volumes obtained each episodes, the amount of sample available to be isolated into different cell subsets was limited. These challenges, however, reiterate the need for scientific collaboration in order to facilitate the production of robust scientific studies which would further aid in discovering the inflammatory cytokine profile behind JSLE.

## 4.6 Strengths of study

Despite the obstacles encountered, this study is a piece of scientifically robust research and displays several strengths that will be considered in this section.

Although quantifying Th17-related cytokines IL-17A, IL-21 and IL-23 has been investigated in lupus by previous studies, recovery interference found in the relevant immunoassays has not previously been described in these studies and may probably impact significantly on their findings; the detailed investigation carried out here therefore contributes significantly to this evidence base and understanding of the challenges conducting research in this area. It has been concluded that caution may need to be given when interpreting published data using non-validated cytokine detection kits due to the probability of matrix interference of recovery. Future studies should endeavour to conduct spike-and-recovery validation experiments and take steps to remove interfering constituents from their sample matrix prior to use.

This study has optimised and employed a diverse range of experimental methodologies, qPCR, Western blot analysis and ELISAs to analyse IL-17A expression in the peripheral venous blood samples obtained from routine clinical protocols,

nullifying the need for distressing and invasive sample collections from paediatric patients. Findings do not support data from the majority of other studies investigating IL-17A in SLE, highlighting the importance of future work in this field. This present study additionally focuses in on the IL-17A cytokine profile in the specific subset of a typically severe disease phenotype of patients, namely those with JSLE and compares it to both non-inflammatory paediatric controls, and an autoimmune, inflammatory control (JIA). The results therefore add consistency to the number of studies that report IL-17A expression is not significantly raised in lupus patients in specific cell subsets.

In addition, it examines the protein expression of IL-17A in different cell subsets, notably PBMCs, $CD4^+$ T cells and neutrophils, which have never been done in a paediatric context to date. Supplementing the few adult studies which report this, it was found that neutrophils do express IL-17A at a protein level in JSLE and healthy adult and paediatric control samples, and this was verified against purified neutrophil and PBMCs expression. This provides important information on how neutrophils may express IL-17A weakly compared to PBMCs in humans.

Where existing evidence is rare even in the context of human and mouse models, this study has explored the roles of the lesser known members of the IL-17 family in paediatric controls and JSLE patients, measuring IL-17C and IL-17RE at an mRNA and protein level, which no published studies have done previously.

Finally, exploring both IL-17A and the IL-17 family subsets has never been reported in published research in the rare field of JSLE. The current evidence base is lacking compared to its adult counterpart and the findings from this study contribute greatly to it, however more steps need to be taken in order to further characterise Th17 and IL-17's roles in the development of JSLE.

## 4.7 Future directions

Having addressed the strengths and challenges faced in this study, results from this research form a platform which opens doors to logical further steps that could be taken to fill the gaps in our understanding of the ins and outs of this field. Remedial actions to rectify the obstacles encountered in our methodologies are described below.

First, spike-and-recovery experiments can be executed using the same IL-21, IL-23 and IL-17A ELISA kits following immunoglobulin depletion of the samples with PEG or protein-L and mouse serum blocking to compare the recovery performance using this protocol. Alternatively it would be useful to conduct the recommended multiplex cytokine assay assessing simultaneously the IL-17A, IL-21, IL-23, IL-6 and RORγt profile in JSLE and control patients.

mRNA and protein expression of Th17-related cytokines IL-21, IL-23 and IL-6 as well as RORγt in CD4+ cells can be quantified by means of qPCR and Western blot analysis. In addition, using flow cytometry techniques to measure IL-17A, IL-21, IL-23, IL-17C and IL-17RE-expressing cell populations would be the next step in building up the Th17-related and IL-17 family cytokine profile in the peripheral blood of our subset of patients and controls. Correlating disease activity scores and therapeutic treatment with the resultant IL-17A, IL-21 and IL-23 cytokine expression in JSLE patients and controls at the protein and mRNA levels would identify any association in these variables.

Exploring IL-17A, IL-17C and IL-17RE expression in target organ tissues such as skin and renal biopsies would test the hypothesis that IL-17 expression may be increased at sites of inflammation rather than in the peripheral blood in JSLE. Kwan et al reported an increased in the mRNA expression of Th17-related cytokines IL-17A and IL-23 in the urinary sediment of patients with SLE (87). Stemming from this, investigating urinary sediment in our subset of patients is highly plausible to future researchers as sample volumes collected would be adequate and the protocol for urine RNA extraction, as described by Li et al, is time-efficient (210). JSLE study urine samples are routinely collected during clinical visits at our institute

and this would uphold the principle of minimising invasive procedures to obtain experimental samples from paediatric patients.

Adding to the IL-17 family and the Th17-related cytokines already explored in this study, there have been cytokine and cell subsets which have been identified that would build up the full picture of the inflammatory cytokine climate in JSLE and further steps should be taken to investigate them.

### 4.7.1 IL-17RA

IL-17RA is a significant receptor for several members of the IL-17 cytokine family, namely IL-17A, IL-17F and IL-17E, where IL-17RA preferentially binds to IL-17A which is essential for IL-17A signalling (211-214). In addition, an IL-17RA-IL-17RE heterodimer receptor complex has recently been shown to be required for IL-17C signalling (137). Its expression on cell subsets adopting an ubiquitous nature and is expressed on B and T lymphocytes, fibroblasts, epithelial cells (214, 215). IL-17RA has been strongly implicated as having a role in autoimmune inflammation, mRNA expression of IL-17RA is upregulated in the peripheral blood of rheumatoid arthritis patients and the central nervous system tissues of mice with EAE compared to controls (216, 217). In psoriatic patients, a monoclonal antibody against IL-17RA, Brodalumab, was investigated in a double-blind randomised controlled trial and found that it significantly improved disease activity and physician's global assessment scores compared to placebo (130). IL-17RA expression has been reported to be increased in $CD19^{+}$ B lymphocytes from the peripheral blood of SLE patients compared to controls (218). This suggests that blocking the IL-17A pathway downstream may potentially be of benefit in SLE. It would be interesting to quantify the expression of IL-17RA in the PBMCs of our patient group compared to controls by examining it at the mRNA and protein level. This may explain the normal IL-17A levels detected in JSLE patients, as increased IL-17RA may bind to IL-17A and IL-17C in the blood, causing the formation of receptor-cytokine complexes and an overall depletion of available cytokines for detection.

### 4.7.2 IL-17F

IL-17F, out of all the other IL-17 cytokine subsets, shares the most homology (55%) with IL-17A and is secreted by Th17 cells (219). Similar to the functions of IL-17A, IL-17F is potentiated by TNF-α and IL-1β, and works by mobilising chemokines and neutrophils to exert an inflammatory response (219). Johansen et al have reported significantly increased IL-17F mRNA and protein expression in psoriatic skin lesions compared to non-lesional skin (199). Additionally, the serum concentrations of IL-17F measured in adult SLE and psoriatic patients have been found to be significantly increased compared to controls (183, 220). This existing data suggest that IL-17F may also factor in autoimmune inflammation and would be a useful target to investigate in the context of JSLE. As discussed previously in section 4.3, IL-17F can be expressed as an IL-17A-IL-17F heterodimer cytokine, which has been shown to be potent at chemokine induction for neutrophil aggregation (221). Future research could delve into the correlation of IL-17F expression to IL-17A expression and quantifying IL-17A-IL-17F heterodimers in the peripheral blood to discover if these IL-17 cytokines are being secreted predominantly in heterodimer form.

### 4.7.3 The balance between Th17 and Tregs

On the other face of the peripheral tolerance coin, Tregs (discussed in section 1.1.2.2.1), which suppress T effector function, have been very much researched in the context of SLE. Studies have shown significantly decreased levels of $CD4^+CD25^+$ Tregs and transcription factor Foxp3 expression in active SLE patients compared to inactive SLE patients and healthy controls, and this correlated inversely with disease activity scores (24, 222-225). Disease activity levels were also found to correlate negatively with decreased Treg expressing $CD4^+$ T cell levels in paediatric SLE patients (69). In addition, several studies have demonstrated that SLE patients have lower levels of serum TGF-β1 compared to healthy controls and this correlated with disease activity (226, 227). This evidence indicates that depleted TGF-β1 and Treg cell levels may trigger an autoimmune process. However, studies have shown conflicting results on the expression of Foxp3 and TGF-β1 in SLE (228, 229). These conflicting results may be attributed to wide variation in participants enrolled and the lack of a specific marker for Tregs (174). Pan et al recently reported the absence of correlation between Foxp3 and CD25 expression and instead used CD45RA as a surface marker for Tregs (230). The study found increased expression of $CD45RA^+Foxp3^{low}$ naive suppressive Treg cells and $CD45RA^-Foxp3^{low}$ non-suppressive Treg cells in active SLE patients, where SLE CD45RA+Foxp3low cells demonstrated significantly reduced suppressive ability compared to controls (230).

Several studies have emerged investigating a postulated link between the roles of Th17 and T regulatory cells in SLE. A previous study concluded that there is mutual antagonism between the differentiation of Th17 and Tregs, and that this is dependent on factors IL-6 and TGF-β (231). It is the disequilibrium between the pro-inflammatory effects of Th17 and the inhibitory function of the Tregs that is hypothesized to tip the balance of self-tolerance to manifest as autoimmune disease in SLE.

Three studies investigating lupus prone mice, patients with active SLE and lupus nephritis have demonstrated a significant decrease in Treg cell levels coupled with raised levels of Th17 compared to healthy controls (85, 228, 229). In addition, a strong inverse correlation between Th17 and natural Treg cells has been

demonstrated where the Treg:Th17 ratio was decreased significantly in SLE (85, 173, 225, 229). Strong correlations were found between disease activity (SLEDAI scores) and Th17:Treg ratio and Th17 cell levels, as well as significant increases in Treg cells after the treatment of active SLE patients (85, 173, 229).

These findings mirror Th17/Treg imbalances that have been found in autoimmune diseases such as rheumatoid arthritis, JIA and inflammatory bowel disease (222, 232, 233). The results yielded show strong consistency and substantiate the hypothesis that there is an upregulation of Th17 and downregulation of Treg cells in SLE. Although, Th17 and Tregs originate from the same naive $CD4^+$ T cells, TGF-β alone encourages Treg differentiation while the addition of IL-6 or IL-21 favours Th17 differentiation (94). Their respective transcription factors (RORγt and Foxp3) bind to and inhibit each other's function (234). The cytokine milieu in SLE comprises of low levels of IL-2 and raised levels of IL-21 and IL-6, the low levels of IL-2 seem to contribute to suppression of Tregs and decreased apoptotic clearance of autoreactive T cells, and this appears to provide a conducive environment for Th17 development (23, 94). Therefore, it has been hypothesised that restoring the immune balance by downregulating Th17 cells and promoting Treg cell function would serve as an effective treatment approach to SLE (83).

Th17/Treg imbalances have not yet been investigated in JSLE patients, and would serve as an interesting extension to this study. Treg expression in JSLE $CD4^+$ cells can be measured using flow cytometry, while TGF-β and Foxp3 expression can be quantified using qPCR, ELISA and Western blot analysis at the RNA and protein level. These findings can subsequently be correlated to Th17-related cytokine expression and disease activity in order to establish any association.

# Conclusion

This study has investigated the IL-17 family members IL-17A, IL-17B, IL-17C and IL-17RE, as well as Th17-related cytokines IL-21 and IL-23 in the pathogenesis of JSLE. Data findings conclude that IL-17A is not significantly raised in the peripheral blood subsets of JSLE patients on an mRNA and protein level; however IL-17A was found to be expressed by neutrophils, which collude with a small number of existing studies. Additionally, while IL-17B was absent in the PBMCs of human participants, IL-17C and IL-17RE were found to be expressed by $CD4^+$ cells and PBMCs in small quantities, however they did not show a significantly increased expression in JSLE patients. Previously published research examining IL-17B, IL-17C and IL-17RE in both human and animal models is scarce and this is the first time these cytokine subsets have been examined in paediatric human context. In addition, matrix interference has been found to be present in the recovery of Th17-related cytokines using immunoassay cytokine detection kits, thereby impeding the reliable quantification of these cytokines.

In conclusion, this is a piece of scientific research which embodies a process of rigorous method optimisation over an exponential learning curve. Although the results of this study do not fully adhere to the initial hypothesis, observations drawn from this have added extensively to the limited evidence base available in the fields of JSLE, the Th17 cell and the IL-17 family. These findings translate into future directions aimed at piecing together the elusive puzzle that is the pathogenesis of JSLE, with the ultimate goal of developing targeted therapies that would resolve or even prevent this devastating life-long disease.

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# Appendix 1: Patient information sheet for UK JSLE Cohort Study and Respository

Alder Hey Children's NHS
NHS Foundation Trust

**UK Juvenile Lupus Cohort Study and Repository**

**Information sheet for patients aged 16 years and older**

**(Liverpool only)**

Version 1.3 – 16th October 2009

## 1. Introduction

- We are asking if you would agree to take part in this project.
- It is important to understand why the research is being done and what it will involve
- Please take time to read this leaflet carefully and talk about it with your family and anyone you want to.

*Thank you for reading this*

## 2. Why are we doing this research?

- Lupus can affect people in many different ways. Unfortunately we don't understand what causes it, especially in children. We would like to understand much more about lupus.
- For this reason, we have started a "cohort study" of young people with lupus from across the UK. We want to learn about how lupus affects them, how they are treated, what causes lupus and the roles genes have. We know that white blood cells are important but we don't understand why. We are aware that the kidneys may get affected by lupus and we would like to know more about how to detect this kidney involvement.

## 3. Why have I been asked to take part?

- You have been chosen because you have lupus.
- Almost all the main hospitals in the UK who look after children with lupus are taking part. We hope to study about 500 children and young people with lupus

## 4. Do I have to take part?

- No! It's up to you.
- Your doctor will ask you to sign a form giving your assent and your parent's consent
- You can change your mind at any time during the research without giving a reason. If you decide to stop taking part it will NOT affect the care you receive

**5. What will happen if I take part?**

- Your doctor normally assesses how you are with questions, an examination, and some blood/urine tests.
- We will record this information for the study.
- This is an "observational study" as it involves carefully describing and watching what happens over time.
- We will collect information when you come for routine checkups or if you are poorly, for as long as you are being looked after by doctors taking part in this study

**6. Will any samples be collected?**

- Results of routine tests will be recorded
- On two occasions when you are having your usual tests we will collect a little extra blood (a fifth of a teaspoon). This will be stored to measure your autoantibodies (antibodies the body produces against itself).
- An extra teaspoon full of blood to study white cells when you are well and sick and if you have only just been diagnosed, before you start treatment and at one week, one month and three months after starting treatment
- Once when you are having your usual tests, we will collect an extra teaspoon full of blood. We will invite you to donate this as a gift to the project organisers. It will be stored and in the future used to find out more about the role of genes in lupus.
- At times when you are poorly and when you are well we will take an extra teaspoon full of blood and a urine sample to look for new ways of detecting kidney damage.
- You will not need any extra needles to collect these samples.

**7. What will I be asked to do?**

We are asking your permission:

- To collect all the information your doctor normally collects about your lupus for the purpose of the study
- To record your name and hospital numbers on a list in your doctor's locked office so we know you are part of the study
- To collect a little extra blood and urine when you are having blood tests (see above).
- To let us get in touch with you in the future through your GP and hospital doctors about future studies of lupus to see if you are interested in taking part. We would do this through your hospital records and using your own NHS number.
- To tell your GP that you are in this study

**8. Are there any disadvantages in taking part?**

- No. Taking part in the study will not change how we look after you

- The amount of extra blood and urine we will collect is very small and will only be collected when you are having blood tests anyway

**9. Are there any advantages in taking part?**

- We hope to understand much more about lupus to help us treat patients better in the future.

**10. Will anyone know I'm taking part?**

- Yes – your family and hospital doctors will know you are taking part. Someone involved in the study may check your medical records to make sure the study is being carried out correctly

**11. What will happen to the results of the research study?**

- Everything we discover from the study will be published in medical journals for everyone to see. Results may not be available for several years.

**12. Who is organising and funding the research?**

- It is being organised by a group of doctors and nurses from the hospitals taking part. The group is called the "UK JSLE Study Group."
- It is run from the Alder Hey Children's NHS Foundation Trust, Liverpool.
- The collection of clinical data is not funded. No one, including your doctor, receives any payment for being involved in this study. The Research & Development Department at Alder Hey and the charity Lupus UK are funding the study of white blood cells in lupus. The Alder Hey Renal Fund, Alder Hey Children's NHS Foundation Trust is funding the Renal Biomarkers project.
- Charities will be approached for funding of any other related studies

**13. Who has reviewed the study?**

- The Liverpool Paediatric Research Ethics Committee has given Multicentre Research Ethics approval and your local Research Ethics Committee has also reviewed it

**14. What will happen to the information collected about me?**

- All information and samples collected from you will be strictly confidential and anonymised. This means that no-one will know it belongs to you.
- Forms will be kept in your doctor's office or in the offices of the UK JSLE Study Group (Institute Child Health, University of Liverpool, Alder Hey Children's NHS Foundation Trust). All forms will be stored in locked filing cabinets in rooms that are locked when non-attended.
- All information kept on study computers, kept in the study offices, will only record data using your unique study number and be strictly confidential. Details identifying who you are will not be kept on the study computer. All electronic transfer of data will use codes.
- In your doctor's locked office in a locked cabinet they will keep a list with your name, hospital number and NHS number that registers you as taking part in the study. This will be the only place where your name and the unique study number are linked. No one other than your doctors will have access to this

**15. What will happen to any samples I give?**

- Samples that are collected routinely by your doctor will be tested in the usual way
- Study samples will be stored in your local hospital until they are carefully transferred to the relevant laboratories.
- Autoantibodies will be analysed in the laboratory of Dr N McHugh, Consultant Rheumatologist, Royal National Hospital for Rheumatic Diseases, Bath.
- White blood cells will be tested in the laboratories of Professor S Edwards, School of Biological Sciences, University of Liverpool and the laboratories at the Institute of Child Health, Alder Hey Children's NHS Foundation Trust
- Detecting markers of kidney damage will be tested in the laboratories at the Institute of Child Health, Alder Hey Children's NHS Foundation Trust.
- Samples collected for future genetic studies will be stored in the laboratories of the Institute of Child Health, University of Liverpool, under the care of Dr. M. Beresford, Senior Lecturer and the Manchester Cell Culture laboratory Professor Crow, University of Manchester. These studies will need approval from an Ethics Committee and the UK JSLE Study Group Steering Committee. No genetic results will be fed back to you.

**16. What if I have a problem or would like further information about the study?**

- Please speak to your doctor, your local hospital's complaints department or contact Dr Michael Beresford, Institute of Child Health, Alder Hey Children's NHS Foundation Trust, Liverpool L12 2AP (te. 0151 252 5153 email m.w.beresford@liverpool.ac.uk).

# Appendix 2: Parental consent form for study

**Parental Consent Form (on local centre headed paper)**

**(Liverpool only)**

**UK Juvenile SLE Cohort Study and Repository**

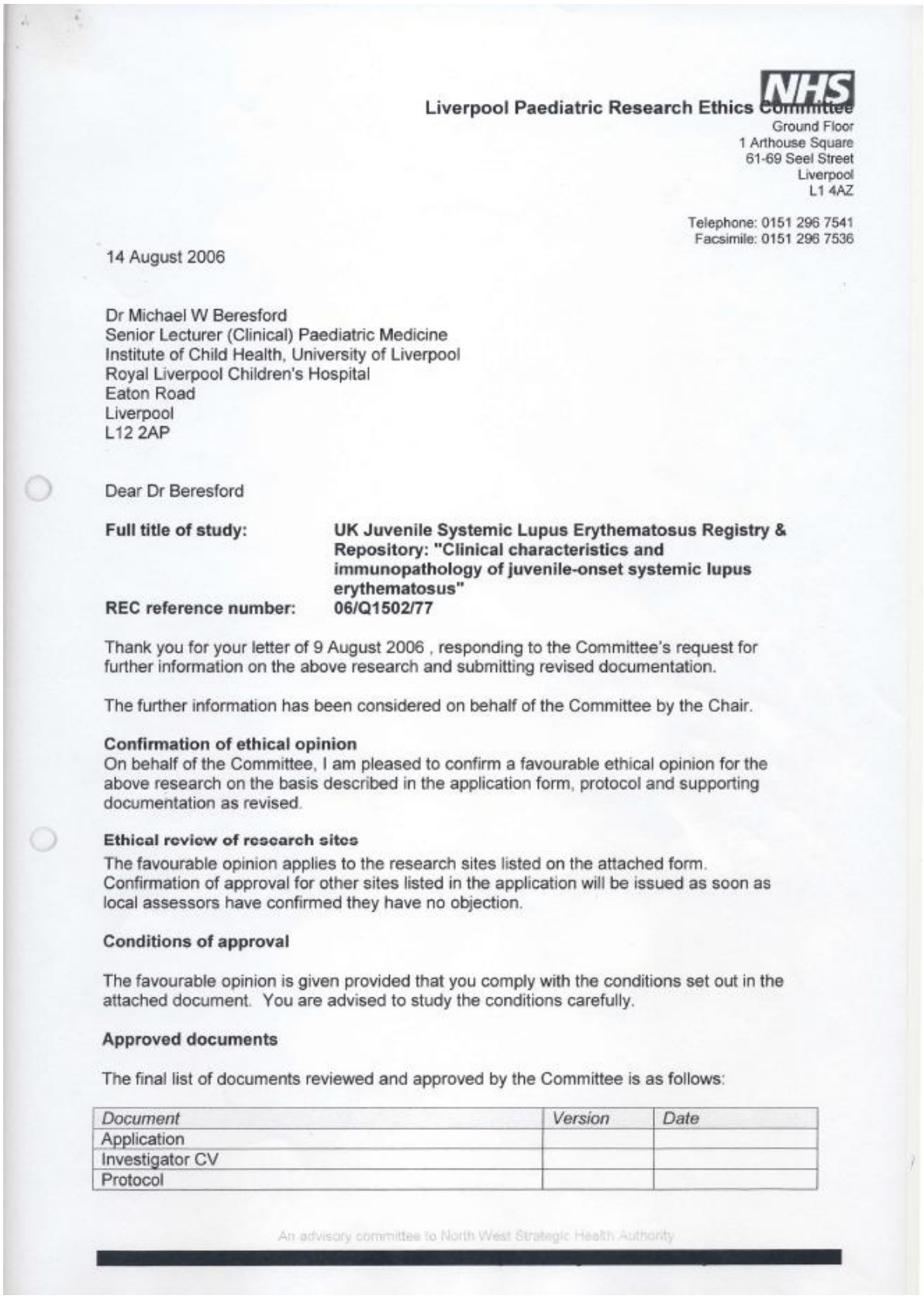
Please INITIAL box

| | | |
|---|---|---|
| 1. | I have read and understand the information sheet (Version 1.3 – 16th October 2009) for the above study and have had the chance to ask questions | |
| 2. | I understand my child's taking part is voluntary and that I am free to withdraw at any time, without giving any reason, without my child's medical care or legal rights being affected | |
| 3. | I understand that relevant sections of my medical notes and data collected during the study may be looked at by responsible individuals from the UK JSLE Cohort Study & Repository research team, from regulatory authorities, or from the NHS Trust where it is relevant to my taking part in this research but understand strict confidentiality will be maintained. I give permission for these individuals to have access to my records. | |
| 4. | I agree that a small amount of my child's blood may be used to investigate white blood cells and also stored and then used to measure their autoantibodies | |
| 5. | I agree that a small amount of my child's blood may be collected and then gifted to the "UK JSLE Study Group." It will be stored for future genetic studies. I understand that no result on my child's genes will be fed back to them or anyone else. | |
| 6. | I agree that a small amount of my blood and urine samples may be used to look into ways of detecting kidney damage in lupus.. | |
| 7. | I agree for my child to take part in the above study | |
| 8. | I agree to allow researchers to make contact with me and my child about other studies or a follow-up of this study through my child's doctors and my child's NHS number | |
| 9. | I give permission for my child's GP to be informed that information about my child is to be held on the study database | |

| | | |
|---|---|---|
| Name of patient | | |
| Name of person with parental responsibility for patient | Date | Signature |
| Name of person taking consent<br>(if different from researcher) | Date | Signature |
| Researcher | Date | Signature |

1 copy for patient and person with parental responsibility, 1 for researcher, 1 to be kept with hospital notes

# Appendix 3: Ethical approval for UK JSLE Registry and Repository



NHS

Liverpool Paediatric Research Ethics Committee

Ground Floor
1 Arthouse Square
61-69 Seel Street
Liverpool
L1 4AZ

Telephone: 0151 296 7541
Facsimile: 0151 296 7536

14 August 2006

Dr Michael W Beresford
Senior Lecturer (Clinical) Paediatric Medicine
Institute of Child Health, University of Liverpool
Royal Liverpool Children's Hospital
Eaton Road
Liverpool
L12 2AP

Dear Dr Beresford

**Full title of study:** **UK Juvenile Systemic Lupus Erythematosus Registry & Repository: "Clinical characteristics and immunopathology of juvenile-onset systemic lupus erythematosus"**

**REC reference number:** **06/Q1502/77**

Thank you for your letter of 9 August 2006 , responding to the Committee's request for further information on the above research and submitting revised documentation.

The further information has been considered on behalf of the Committee by the Chair.

**Confirmation of ethical opinion**
On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised.

**Ethical review of research sites**
The favourable opinion applies to the research sites listed on the attached form. Confirmation of approval for other sites listed in the application will be issued as soon as local assessors have confirmed they have no objection.

**Conditions of approval**

The favourable opinion is given provided that you comply with the conditions set out in the attached document. You are advised to study the conditions carefully.

**Approved documents**

The final list of documents reviewed and approved by the Committee is as follows:

| *Document* | *Version* | *Date* |
|---|---|---|
| Application | | |
| Investigator CV | | |
| Protocol | | |

An advisory committee to North West Strategic Health Authority

| | | |
|---|---|---|
| Covering Letter | | |
| Summary/Synopsis | | |
| Letter from Sponsor | | |
| Peer Review | | |
| Questionnaire: Child health | | |
| Letter of invitation to participant | | |
| GP/Consultant Information Sheets | | |
| Participant Information Sheet: For all groups | 1 | 19 May 2006 |
| Participant Consent Form: For all groups | 1 | 19 May 2006 |
| Response to Request for Further Information | | 09 August 2006 |
| JSLE x 3 | | |
| ACR Criteria annual review | | |
| ACR Criteria at Diagnosis | | |
| JSLE Demographics | | |

**Research governance approval**

The study should not commence at any NHS site until the local Principal Investigator has obtained final research governance approval from the R&D Department for the relevant NHS care organisation.

**Statement of compliance**

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

| | |
|---|---|
| **06/Q1502/77** | **Please quote this number on all correspondence** |

With the Committee's best wishes for the success of this project

Yours sincerely

**Chair**

Email: Ronald.Wall@centralliverpoolpct.nhs.uk

*Enclosures:* *Standard approval conditions*
*Site approval form*

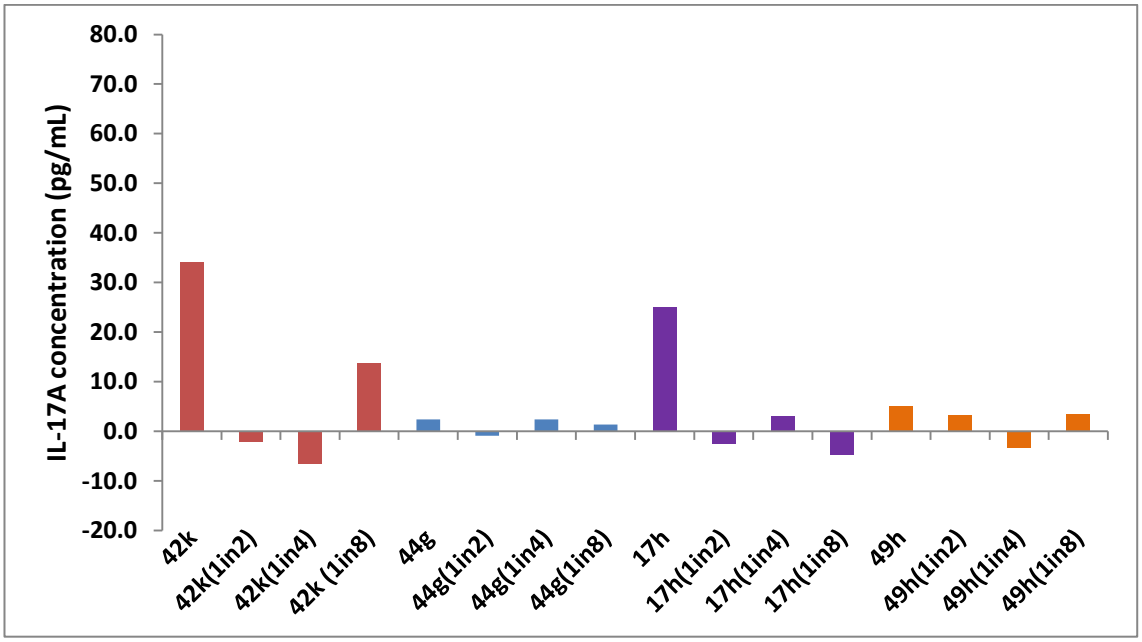
Copy to: G Mitchell, Royal Liverpool Children's Trust Research and Development Department

# Appendix 4: IL-17A protein expression measured by ELISA in JSLE serum and plasma

### IL-17A protein expression in unspiked JSLE patient serum and plasma

| IL-17A concentration (pg/mL) | | | | |
|---|---|---|---|---|
| JSLE sample | Neat | 1 in 2 dilution | 1 in 4 dilution | 1 in 8 dilution |
| L44g | 2.4 | -0.9 | 2.4 | 1.4 |
| L17h | 25.1 | -2.6 | 3.0 | -4.7 |
| L42K | 34.1 | -2.2 | -6.5 | 13.7 |
| L49h | 5.1 | 3.3 | -3.2 | 3.5 |

**The IL-17A protein levels detected in diluted unspiked JSLE patient serum (n=2; #1 and #2) and plasma (n=2; #3 and #4). Values are expressed as mean (pg/mL).**




### Bar chart of IL-17A protein expression in unspiked JSLE patient serum and plasma

**Graphical representation of IL-17A protein concentrations detected in unspiked JSLE patient serum and plasma from section 3.3 using the IL-17A eBioscience ELISA kit. As can be seen, diluting the samples down runs the risk of completely diluting out any naturally occurring IL-17A.**

# Appendix 5: Data table for IL-17A protein expression in CD3/CD28 stimulated PBMCs in JSLE patients and healthy controls over 2 to 4 days

| IL-17A protein expression (pg/mL) | | | | | |
|---|---|---|---|---|---|
| **JSLE** | JSLE day 3 | JSLE day 4 | Control | Control day 3 | Control day 4 |
| **25.7** | 2.4 | 12.9 | 0 | 0 | 0 |
| **0** | 0 | 0 | 38 | 14.3 | 0 |
| **606** | 32.4 | 21.2 | 33.5 | 0 | 0 |
| **38.4** | 0 | 21.3 | 43.93 | 4.36 | 5.82 |
| **513.36** | 202.76 | 38.96 | 160.76 | 36.89 | 61.96 |
| **735.96** | 105.82 | 94.62 | 33.68 | 0 | 0 |
| | | | 7.79 | 0 | 0 |

Raw data from Figure 30: IL-17A protein expression in CD3/CD28 stimulated PBMCs

# Appendix 6: Data table for IL-17C fold change of JSLE stimulated PBMCs to healthy controls

| CD3/CD28 stimulated PBMCs | IL-17C (Ct) | 18S (Ct) | IL-17C-18S | ΔΔCt | $2^{-\Delta\Delta Ct}$ |
|---|---|---|---|---|---|
| **L36f** | 40.93666667 | 13.80333333 | 6.79286E-09 | 1.173 | 0.443 |
| **L44f** | 40.81 | 16.67333333 | 5.42175E-08 | | |
| **L49k** | 41.135 | 16.69 | 4.37846E-08 | | |
| **L50i** | 43.44 | 16.72 | 9.04646E-09 | | |
| **L52i** | 41.01 | 14.45666667 | 1.01543E-08 | | |
| **L54d** | 37.76 | 14.51666667 | 1.00707E-07 | | |
| **L57b** | 38.54333333 | 12.47333333 | 1.41954E-08 | | |
| **C141** | 41.07 | 13.63666667 | 5.51752E-09 | 0 | 1 |
| **C147** | 41.45 | 18.18666667 | 9.93205E-08 | | |
| **C158** | 41.065 | 18.44 | 1.54595E-07 | | |
| **C166** | 38.69 | 14.43 | 4.97751E-08 | | |
| **C169** | 40.47 | 17.81333333 | 1.51239E-07 | | |
| **C177** | 42.105 | 16.55333333 | 2.03321E-08 | | |

Raw data from Figure 31: IL-17C mRNA expression and fold change in CD3/CD28 stimulated PBMCs at Day 2.

# Appendix 7: Data table of IL-17RE fold change of JSLE stimulated PBMCs to healthy controls

| CD3/CD28 stimulated PBMCs | IL-17RE | 18S | IL-17RE-18S | ΔΔCt | $2^{-\Delta\Delta Ct}$ |
|---|---|---|---|---|---|
| **L3k** | 38.17666667 | 12.42333333 | 25.75333333 | 2.3925 | 0.190452087 |
| **L36f** | 38.86 | 13.80333333 | 25.05666667 | | |
| **L44f** | 42.74 | 16.67333333 | 26.06666667 | | |
| **L49k** | 40.47666667 | 16.69 | 23.78666667 | | |
| **L50i** | 42.065 | 16.72 | 25.345 | | |
| **L52i** | 38.78 | 14.45666667 | 24.32333333 | | |
| **L54d** | 42.91 | 14.51666667 | 28.39333333 | | |
| **L57b** | 39.69666667 | 6.38205E-09 | 27.22333333 | | |
| **C141** | 37.14 | 8.40992E-08 | 23.50333333 | 0 | 1 |
| **C147** | 42.445 | 4.98327E-08 | 24.25833333 | | |
| **C158** | 39.48333333 | 4.62728E-07 | 21.04333333 | | |
| **C166** | 39.78666667 | 2.32746E-08 | 25.35666667 | | |
| **C167** | 41.27333333 | 1.02823E-07 | 23.21333333 | | |
| **C168** | 39.17666667 | 2.03754E-07 | 22.22666667 | | |
| **C169** | 40.39666667 | 1.59125E-07 | 22.58333333 | | |
| **C177** | 41.17666667 | 3.86935E-08 | 24.62333333 | | |

Raw data from Figure 33: IL-17RE mRNA expression and fold change in CD3/CD28 stimulated PBMCs at Day 2