Investigating phagocytic markers in Juvenile-onset Systemic Lupus Erythematosus (JSLE) monocytes and macrophages

Institute of Child Health, University of Liverpool

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor in Philosophy (or other degree as appropriate) by David Andrew Harris.

August 2012

Acknowledgements

I wish to thank post-doctoral researchers Dr. Lucy Ballantine and Dr. Angela Midgley, and also Clinical Research Fellow Dr. Louise Watson for their help and support throughout the year. I also wish to thank Professor Michael Beresford for enabling me to undertake this postgraduate project. It has been a privilege to work within this team and I feel inspired towards a career in academic medicine. I would also like to thank Graham Jeffers for sharing his extensive laboratory experience and wisdom. I also found invaluable the help and support given by the rest of the Lupus Research Group, and by the members of other research groups at the Institute. The clinical rheumatology team have all been tremendously welcoming and helpful, and fantastic to work with and learn under. Finally, I would like to thank the patients and their families whose contributions enabled this research to take place.

Table of Contents

List of Figures

List of Tables

Abstract

Investigating phagocytic markers in Juvenile-onset Systemic Lupus Erythematosus (JSLE) monocytes and macrophages

David Andrew Harris

Background Juvenile-onset systemic lupus erythematosus (JSLE) is a chronic, severe, multi-systemic autoimmune disease. Impaired phagocytic function has been implicated as an initial step in the pathogenesis of this disease, in relation to the clearance of cells undergoing apoptosis. The apoptotic process has been shown to be dysregulated in neutrophils, and these cells, and others, are thought to represent a burden of autoantigen-presenting apoptotic cells. After a sequence of immune cell activation, the result is the production of autoantibodies forming immune complexes which cause deleterious effects at end-organs. As key phagocytes, the phenotypes of JSLE macrophages, and their precursors monocytes, have been investigated to determine the extent of their role in the development of JSLE.

Aim To elucidate phenotype and phagocytic function of monocytes and macrophages as a potential important contributing factor to the development of JSLE.

Methods Monocytes were separated from processed whole blood of JSLE patients and paediatric healthy controls (HC), and either retained or cultured to macrophages, with the RNA extracted. RNA was converted to cDNA and real-time qPCR performed with primers for CD36, MER, and CR3, with expression standardised against that of β2M. Concentrations of soluble MER (sMER) were measured in JSLE, JIA and HC plasma samples by ELISA. These samples were diluted to a concentration of 1 in 5, following validation and optimisation conducted through spike and recovery. Adult HC monocytes were incubated with pHrodo E. coli for 30 minutes in JSLE and control plasma and serum, and phagocytosis was measured by flow cytometry. Adult HC monocytes were exposed to a number of environments (LPS, IFNα, TNFα, apoptotic neutrophils) for 2 and 6 hours. Supernatant was extracted and tested by human total MER ELISA, and monocyte RNA was extracted and RT-qPCR performed.

Results Increased mRNA levels of CD36 were present in JSLE monocytes ($p=0.025$) but no differences found with MER or CR3 expression compared with HC monocytes, no differences in expression of these markers in macrophages. CR3 was down-regulated in both JSLE monocytes and macrophages, but no other differences were statistically significant. The concentrations of sMER were significantly greater in JSLE patients compared with HC ($p<0.001$) and JIA patients ($p=0.014$). Phagocytosis of E. coli was decreased when adult HC monocytes were incubated in JSLE serum and plasma compared with controls, though differences were not statistically significant. No MER cleavage was detected following exposure of monocytes to test conditions. qPCR results for CD36, MER and CR3 expression in response to monocyte stimulation were equivocal.

Discussion Differences were detected in receptor expression of CD36 between JSLE and control monocytes, an important receptor in apoptotic cell clearance. sMER is present at a much greater concentration in JSLE patients than inflammatory controls and HC, and this can be expected to negatively impact upon apoptotic cell clearance due to reduced cell expression and by competing for ligand. Phagocytosis of bacteria by monocytes appeared to be impaired when exposed to JSLE environments, and this may suggest the involvement of extracellular factors in the proposed failure of effective apoptotic cell clearance.

Conclusion This study has identified a number of important distinguishing features concerning the phagocytic markers of JSLE monocytes and macrophages and those of HC. It has also identified a number of extracellular features, including increased presence of sMER, which may significantly influence their function, potentially reducing the capacity for apoptotic cell clearance to occur, resulting in autoantigen presentation.

Abbreviations

β2M	Beta-2-microglobulin
BILAG	British Isles Lupus Assessment Group (disease activity index)
CD	Cluster of differentiation
cDNA	Complementary DNA
CRP	C-reactive protein
CS	Corticosteroid
DMARD	Disease-modifying anti-rheumatic drug
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked Immunosorbant Assay
ESR	Erythrocyte sedimentation rate
FCS	Foetal calf serum
HC	Healthy Control
HCQ	Hydroxychloroquine
IFNα	Interferon alpha
IL	Interleukin
JIA	Juvenile idiopathic Arthritis
JSLE	Juvenile-onset Systemic lupus erythematosus
MHC	Major histocompatibility complex
mRNA	Messenger ribonucleic acid
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PS	Phosphatidylserine
qPCR	Quantitative polymerase chain reaction
RNA	Ribonucleic acid
SLE	Systemic lupus erythematosus
sMER	Soluble MER
TNFα	Tumour necrosis factor alpha

1 Introduction

The subject of this thesis is the autoimmune disease juvenile-onset systemic lupus erythematosus (JSLE). The content is, in particular, focused upon monocytes and macrophages in this disease and their potential role in its associated development and process. A failure of apoptotic cell clearance is believed to be a key early step in the pathogenesis of adult-onset SLE, and as key phagocytes these cells have been investigated as to their potential involvement in this.

1.1 Cells of the innate immune system

Phagocytosis is crucial to the appropriate removal of foreign agents and dying cells. Two of the cells of the immune system most responsible for this process are monocytes and macrophages.

1.1.1 Monocytes

Monocytes are generated from myelocytic lineage, deriving from the myeloblast (see Figure 1-1), also the common haemopoetic stem cell of neutrophils, eosinophils and basophils (1). Formed in the bone marrow (2), monocytes are stored both there and in the spleen (3) until required. They typically circulate for between one and three days (4), roaming in case their phagocytic functions may be required, after which point they tend to differentiate into macrophages in the tissues (1). Monocytes are also the precursors of the dendritic cell (5) (see Figure 1-1), another phagocyte, but whose main function is considered to be the presentation of antigens to T-lymphocytes, and as such acts as a link between the innate and adaptive immune system (6). The development of monocytes and

their subsequent differentiation into macrophages or dendritic cells is illustrated in Figure 1-1. The monocyte's involvement in immunity is considered to be most importantly microbial phagocytosis (7), but also includes phagocytosis of dying cells, antigen presentation and immunomodulation (through the secretion of cytokines) (2).

Figure 1-1 Monocyte, Macrophage and Dendritic Cell developmental lineage (8)

CDP, common DC progenitor; CMP, common myeloid progenitor; GMP, granulocyte and macrophage progenitor; HSC, haematopoietic stem cell; MDP, macrophage and DC progenitor.

Monocytes are broadly divisible by their cluster of differentiation (CD) marker expression into $CD14^{+}CD16^{-}$ and $CD14^{+}CD16^{+}$ populations (8). The former is referred to as "classical" and comprises around 90% of monocytes, while the latter is a minority of "non-classical" monocytes first identified over 20 years ago (9), and this group can be further divided according to CD14 expression, i.e. $CD14^{dim}CD16^{+}$ and $CD14^{high}CD16^{+}$. CD14 is a lipopolysaccharide (LPS) receptor complex (10), with LPS typically found on bacteria, and CD16 is the FcγR III immunoglobulin receptor (11). $CD14^{dim}CD16^{+}$-monocytes are considered to be pro-inflammatory monocytes, due to observed increased numbers in infection(12), with toll-like receptor (TLR) stimulation manifesting increased tumour necrosis factor (TNF-α) (13) and decreased interleukin (IL)-10 secretion (13). The other $CD16^{+}$ subset, $CD14^{high}CD16^{+}$, is considered anti-inflammatory due to its propensity to secrete large quantities of IL-10 (14).

1.1.2 Macrophages

The end-stage development of monocytes, macrophages are often described as professional phagocytes as they are able to engulf larger particles and persist to phagocytose numerous organisms, cells, particles and agents and continue to function for weeks or months. Differentiation of monocytes in the tissues results in distinct types of macrophages: a group that are able to mobilise where needed, but also a fixed group that remain localised for months or years, performing the required functions in-situ. Examples of the latter include the Kupffer cells in the liver, mesangial cells in the kidneys, and alveolar macrophages in the lungs (15), see Figure 1-2. The dynamic system of circulating monocytes and free and fixed macrophages is known as the reticuloendothelial, or, more contemporarily, the mononuclear phagocyte system (1).

Figure 1-2 Homeostatic functions of tissue macrophages (15)

Populations of macrophages are located around the body with context-appropriate roles including phagocytosis, antigen presentation and immune suppression.

Macrophages clear around 2 x 10^{11} red blood cells every day, and in doing so accept a huge burden without which the human body could not hope to survive (1). They also clear cellular debris generated in tissue remodelling and are responsible for the removal of cells undergoing apoptosis (1) (programmed cell death, see section 1.2). In the case of apoptotic cell removal, associated-receptors either fail to induce pro-inflammatory signals, or act to induce anti-inflammatory signals (1). This is in stark contrast to the macrophage response

to pathogens or necrosis, which is pro-inflammatory by necessity, thereby activating the wider immune system.

Macrophages have been historically categorised into 2 groups, M1 and M2 (1). M1 macrophages have been described as “classically activated” and are responsible for defence against pathogens and in anti-tumour immunity (15). M2 macrophages indicate “alternative activation” and are mostly concerned with anti-inflammatory and regulating wound healing (15). Over time, the M2 group has been attributed to increasingly-diverse roles (including induction by apoptotic cells), and this traditional perspective is no longer universally supported (16) as macrophages are now believed to occupy a fairly broad phenotypic spectrum (1). However, what these macrophages do have in common is the ability to either induce host defence, anti-tumour immunity and pro-inflammatory responses or the ability to suppress those functions (17).

1.2 Apoptosis

Apoptosis, as first described by Kerr et al (18) in 1972, is a process of controlled cell death designed to minimise damage to the surrounding cells. The alternative method of cell death is necrosis, occurring when a cell can no longer maintain membrane integrity (19), leading to the release of intracellular proteins and enzymes and the induction of a strong inflammatory response. There are three main ways that the apoptosis process may be initiated: by the extrinsic, intrinsic or granzyme B pathways (20) (see Figure 1-3). Ultimately, these pathways result in the activation of the foremost effectors, the caspases: caspase-3, caspase-6, and caspase-7 (20), which are responsible for proteolysis. If not appropriately dealt with, apoptosing cells are allowed to subsequently undergo secondary necrosis (19), through which they may activate the Fcγ receptors or TLRs on macrophages leading to a pro-inflammatory response, or eventually rupture, exposing the immediate environment to their toxic elements.

Figure 1-3 Caspase activation pathways (extrinsic, intrinsic and granzyme B) for the induction of apoptosis (20)

All three pathways result in the activation of caspases, which are responsible for the proteolysis associated with cell death. APAF1, apoptotic protease-activating factor-1; BAD, BCL-2 antagonist of cell death; BAK, BCL-2-antagonist/killer-1; BAX, BCL-2-associated X protein; BCL-2, B-cell lymphoma-2; BID, BH3-interacting domain death agonist; BIK, BCL-2-interacting killer, BIM, BCL-2-like-11; BMF, BCL-2 modifying factor; FADD, Fas-associated death domain protein; FasL, Fas ligand; HRK, harakiri (or death protein-5); PUMA, BCL-2 binding component-3; tBID, truncated BID; TNFα, tumour necrosis factor alpha.

The extrinsic pathway is activated by a process in which ligands, such as TNF-α or Fas ligand (FasL), bind to a cell's death receptors, stimulating adaptor proteins such as the Fas-associated death domain protein (21). This recruits and activates caspase-8, commencing a caspase cascade (see Figure 1-3).

In the intrinsic pathway, intracellular stimuli (e.g. DNA damage) activate BH3-only proteins. There must be sufficient activation in order to overcome the inhibitory effects of the B-cell lymphoma-2 family, to then go on to promote BAK-BAX oligomer production (within the mitochondria) (20). This causes cytochrome c release into the cytosol, triggering the formation of an apoptosome, a large quaternary protein which recruits pro-caspase 9 (22), converting it into its activated form, caspase 9 (see Figure 1-3).

Through the granzyme B pathway, cytotoxic T lymphocytes or natural killer cells release granules of granzymes and perforin (23). Perforin oligomerises in the cell membrane, forming a channel to facilitate the passage of the granzymes into the cell. Granzyme B can then activate caspases 3 and 7, and BH3-interacting domain death agonist (23) (so can also act upon part of the intrinsic pathway as well as through caspases).

During apoptosis, inside the cell the nucleus condenses and the DNA hydrolyses and fragments (20). Fragmentation of the Golgi complex, endoplasmic reticulum and mitochondria also occurs, and proteins are released from the inter-membrane space of the mitochondria, one of which, cytochrome c, forms part of an aforementioned mechanism of apoptosis (20).

Externally, an apoptosing cell retracts from those surrounding it and becomes rounded, and plasma membrane blebbing occurs, in which the cytoskeleton separates from the

plasma membrane. Blebs are vesicles, of which some burst whilst others break-off as apoptotic bodies (24). Importantly, from a homeostatic point of view, the changes that occur visibly separate these apoptotic cells from their non-apoptotic counterparts, alerting the phagocytes to the impending need to clear them (25). Apoptotic body formation is thought to confer an advantage to the efficiency of phagocytosis, with the suggestion that these bodies are produced in order to facilitate the clearance of the apoptotic cell (20).

Communication is important in cell death; dying cells secrete a “find me” signal, which enables macrophages to seek out these apoptosing cells by chemotaxis (26). Much is still unknown about these secretions, but proposed molecules include lysophosphatidylcholine, S19 (a ribosomal protein), and animoacyl-tRNA synthetases (20).

Macrophages and other phagocytes need to be able to differentiate an apoptosing cell from a non-apoptosing cell, and in order that the process may occur before progression to secondary necrosis, recognition needs to take place early in apoptosis. This is achieved by the “eat me” receptors expressed on the surface of the dying cell, enabling recognition of these cells. The most distinctive external elements of an apoptosing cell is the loss of phospholipid asymmetry and exposure of phosphatidylserine (PS), and it is this transformation that is imperative for phagocytosis to occur (27). It is asserted that, as PS is often found to collect externally, these may comprise large recognition domains on membrane blebs (27, 28).

The external changes of the apoptotic cell are imperative so that non-apoptosing cells are spared from phagocytosis; that there is instead a response targeted exclusively at apoptotic cells. Although much remains unknown about the exact nature of many of the precise changes that occur, and their potential interactions with the phagocyte, there are a

number of identified ligands with evidence for their role in this process. PS, normally a constituent of the inner-part of the phospholipid cell membrane, redistributes to the outside in the apoptosing cell (29). The asymmetrical cell membrane is so-called due to the usual presence of the vast majority of phospholipids with attachments including PS and phosphatidylethanolamine, located on the cytoplasmic side of the cell membrane (29). This PS *status quo* is maintained by an ATP-dependent aminophospholipid translocase that transports PS from the outside to the inside in normal, non-apoptotic cells (29). Through various apoptosis-associated mechanisms, those which previously maintained it as internal become inactive, allowing PS to become concentrated on the outside (30). A study has indicated that PS externalisation is the result of its oxidation secondary to cytochrome c (31), and that this oxidation may contribute to the attraction of opsonins. External changes such as those outlined are often described as “eat me” signals, encouraging the phagocytosis of the apoptotic cell.

With regards to PS, research has shown that a phagocyte requires at least one receptor to tether and another to internalise the apoptotic cell (32). The integrins (e.g. αvβ3 and αvβ5) and integrin-associated proteins (e.g. CD36) are generally associated with the tethering process, and the tyrosine kinase receptor MER is one receptor considered important in apoptotic cell internalisation. Of these, αvβ5 and MER are suggested to act in concert, and together enable efficient apoptotic cell uptake to occur. These and other receptors are discussed in further detail in section 0.

1.2.1 Apoptosis and disease

In humans, every second, around 100,000 cells are produced by mitosis, and around the same amount die by apoptosis (33). It is imperative that this is well-regulated to maintain the equilibrium (34). Broadly speaking, aberrant apoptosis can be subdivided into two

opposing groups; either reduced or excessive apoptosis. It has been estimated that dysregulated apoptosis is contributory towards around half of diseases currently inadequately treated (35). Apoptotic failure is an important step in the development of cancer, whilst the occurrence of excessive apoptosis has been observed in the disease processes of neurodegenerative conditions, heart failure, and AIDS (acquired immune deficiency syndrome) (34). Autoimmune diseases, meanwhile, have been implicated with both excessive and reduced apoptosis (34). Apoptosis is a vitally important process which must be well-regulated, and if not can result in the manifestation of disease. Increased rate of apoptosis is postulated to be an important early step in the pathogenesis of systemic lupus erythematosus (SLE) (36). In health, the apoptotic cells are cleared effectively by the body's immune system, so it is important to consider this process in detail.

1.2.2 Apoptosis and SLE

In SLE, the rate of apoptosis of lymphocytes has been shown to be increased *in vitro*, compared to both healthy controls (HC) and rheumatoid arthritis controls (37). The rate of apoptosis was also found to correlate with disease activity. Ren et al investigated the rate of apoptosis in neutrophils, finding it to be significantly greater in SLE patients than controls. The introduction of SLE serum also induced a greater rate of apoptosis in these neutrophils (38). These results have been mirrored in JSLE neutrophils, with the cells themselves showing increased tendency to apoptose, but also with contribution from their (serum) environment (39). The apoptosis-inducing serum of JSLE has also been demonstrated in adult-onset SLE (40). Investigation of the blebs expressed during neutrophil apoptosis also found them to contain nuclear antigen, suggesting a potential source of autoantigen (41); against which an immune response would be mounted in the event of loss of self-tolerance.

1.3 Phagocytosis and the clearance of apoptotic cells

Phagocytosis is the process of cellular ingestion, occurring through initial recognition and binding, resulting in internalisation and degradation (42). It is vitally important that the body may clear the senescent cells and infectious agents that would otherwise be free to accumulate or even proliferate (16). The capacity for a phagocyte to accomplish this is dependent on three main factors (43). First, a rougher surface increases a particle's susceptibility to phagocytosis. Secondly, dead tissues and foreign particles lack protein coats which afford protection against phagocytes. Finally, the likelihood of phagocytosis can be increased by the involvement of other components of the immune system: antibodies, which adhere to the bacterial membrane, and complement, in particular C3, which is important in opsonisation (44).

The process of phagocytosis varies depending on the specific particle in question. In general, internalisation is initiated by receptors on the phagocyte surface interacting with ligands on the particle. Polymerisation of actin occurs and the particle is internalised into a phagosome, which matures and the particle can be subsequently destroyed (1) through fusion with a cell lysosome, traditionally referred to as a "suicide bag" (45) for its role in protein and macromolecule turnover.

It is clear that there are different mechanisms that macrophages can employ to internalise a dying cell. There is, however, no consensus as to whether there is one absolute method for each type of dying cell to be phagocytosed. The two main mechanisms described are illustrated in Figure 1-4. In the zipper mechanism, a sequential ligation of receptors form around the cell, engulfing it (46). In macropinocytosis, membrane ruffling occurs, encircling the cell and extracellular fluid into a pinosome (a fluid-filled vesicle) (47). Evidence suggests

that apoptotic cell uptake occurs through the zipper mechanism and it is necrotic (including secondary necrotic) cells that are internalised through macropinocytosis (48).

Figure 1-4 The physical mechanisms of phagocytosis (46)

The figure shows the two best-described methods of phagocytosis. In the zipper mechanism, sequential ligation of receptors leads to the engulfment of cells, whereas in stimulated macropinocytosis engulfment is achieved by membrane ruffling, allowing part of the phagocyte to encircle the cell and surrounding extracellular fluid.

Considered more broadly, there are two distinct patterns to phagocytosis. When a microbial organism is engulfed, a pro-inflammatory process is engaged, activating the acquired immune response (49). This is important to instigate the recruitment of other elements of the immune system in a full scale response. In contrast, the hallmark of "normal" apoptotic cell phagocytosis is the absence of pro-inflammatory cytokine release, with instead the secretion of anti-inflammatory cytokines, actively preventing the activation of the innate and acquired immune systems (50). These anti-inflammatory secretions are often summarised by a process known as "tolerate me", ensuring the apoptotic cell is dealt with in a self-limiting manner. The three stages of apoptotic cell clearance discussed in this section are graphically summarised in Figure 1-5.

This text box is where the unabridged thesis included the following third party copyrighted material:

Munoz LE, Lauber K, Schiller M, Manfredi AA, Herrmann M. The role of defective clearance of apoptotic cells in systemic autoimmunity. Nature reviews Rheumatology. 2010;6(5):280-9.

Figure 1-5 The stages of apoptotic cell clearance (adapted from (51))

The figures show the three broad processes associated with the normal uptake of a dying cell. (a) 'Find me', in which the apoptotic cell releases chemotactic factors to induce the migration of phagocytes; (b) 'eat me', in which surface molecules including PS (PS) are expressed by the apoptotic cell to encourage phagocytosis; (c) 'tolerate me', in which the phagocyte secretes anti-inflammatory agents to preclude immune system activation. IL-10, interleukin-10; TGF-β, tissue growth factor beta; PGE_2, prostaglandin E_2.

1.3.1 Apoptotic cell clearance and disease

Apoptotic cells that remain non-phagocytosed are left to undergo secondary necrosis. Subsequent phagocytosis of a secondarily-necrotic cell results in a pro-inflammatory response, or continued failure of phagocytosis inevitably results in cell rupture with the exposure of the cell's toxic elements to the body. In the case of autoimmunity, cellular rupture will result in the release of intracellular antigens, against which the immune system may mount an inappropriate response. The relationship between ineffective apoptotic cell clearance and autoimmunity is now well-established (52). In respiratory disease, increased levels of apoptotic cells have been observed in the sputum and lung tissue of patients with diseases such as asthma and cystic fibrosis (53), but there is still uncertainty as to whether this is due to increased apoptosis or decreased clearance (50), though research has presented evidence of associated defective phagocytes (50). Further, aberrant clearance has been implicated in the development of pathologies included atherosclerosis and neurodegenerative disease, among others (50).

1.3.2 Apoptotic cell clearance and SLE

Impaired phagocytosis has had a long-standing historical association with SLE (54), reproduced more recently in the macrophage (55). Today, the hypothesised pathogenesis

of this complex, all-encompassing immune disorder positions this impairment as a first step in a series of subsequent downstream immunological dysfunctions. This, combined with a considered pro-apoptotic environment in SLE contributes to an accumulation of apoptotic cells.

Impaired phagocyte function has an established and longstanding association with SLE, with published research dating back to 1969 (56). However, this impairment may be more a symptom of the disease than a causative element of the disease process. In more recent times, a number of papers have described the impairment of apoptotic cell phagocytosis in SLE.

In research by Herrmann et al (55), phagocytosis of apoptotic cells by monocyte-derived macrophages was observed to be greater in control than in SLE cells. This seemed to suggest an intrinsic phagocyte defect of apoptotic cell clearance. Ren et al (38) also observed a decreased in the phagocytosis of apoptotic cells by SLE monocyte-derived macrophages. They also observed a marked increase of phagocytic performance in macrophages incubated in control serum, and a marked decrease in control macrophages incubated in SLE serum. The results suggested a combination of both the intrinsic, cellular factors and the extrinsic, serum factors. Tas et al (57) investigated phagocytosis by looking separately at binding and ingestion, finding that there was no difference between the ability of SLE and control monocyte-derived macrophages to bind apoptotic cells, but a decreased capability for SLE monocyte-derived macrophages to internalise apoptotic cells. This suggests a nuanced defect, or defects, underlying this difference. Evidence as to the role of cells versus the environment in SLE has been conflicting.

Bijl et al (58) investigated phagocytosis of apoptotic cells by monocyte-derived macrophages from SLE patients and HC, incubated in SLE or HC serum. There were no detected differences between the phagocytic performance of control and SLE monocyte-derived macrophages in control serum. The difference arose when control monocyte-derived macrophages were incubated with apoptotic cells in SLE serum, which was decreased compared with control serum, and active disease serum exerted a greater negative effect on phagocytosis than inactive disease serum. The research reported therefore suggests that it is SLE environmental factors, rather than intrinsic phagocyte failings which may impact upon apoptotic cell clearance.

Other work has also investigated the potential role of complement in apoptotic cell clearance. Grevink et al (59) divided SLE patients according to their measured classical pathway haemolytic complement activity (deemed normal or low) and compared in vitro phagocytosis by control monocyte-derived macrophages of apoptotic cells in these control and SLE sera samples. There was no difference in detected phagocytosis between control and SLE, and nor did the level of complement activity appear to influence this.

Baumann et al (60) identified, from SLE and control lymph node biopsy samples, a subgroup of SLE patients in whose germinal centres resided an excess of apoptotic cells and a relative deficiency of tingible body macrophages. They suggest that in this subgroup the accumulation of dying cells is caused by either this deficiency of macrophages or an impairment of clearance. The apoptotic cells disintegrate exposing autoantigens which ensure the survival of autoreactive B cells, as a potential source of SLE-associated autoantibodies.

1.4 Juvenile-onset Systemic Lupus Erythematosus

Systemic lupus erythematosus (SLE) is a chronic, multi-systemic autoimmune disease. It is sometimes described as the prototypal autoimmune disease, as all the main elements of the immune system have been implicated in its pathogenesis (61). The course and severity of disease varies widely, with an often unpredictable pattern of relapse and remission.

1.4.1 Epidemiology

Juvenile-onset SLE (JSLE), meanwhile, is SLE diagnosed in a person before the age of 17 (though there is a lack of consensus at which age JSLE should be distinguished from SLE). UK incidence has been estimated at 0.73 per 100,000 per year (62) (albeit JSLE was defined as SLE in those under 19 years – evidence of the grey area that is SLE age-segregation), and JSLE cases make up around 10-20% of all SLE cases. . There is a female to male preponderance in JSLE of around 4-5:1 (63), but this difference in gender is actually greater, at around 10:1 in adult-onset SLE. This predominance means that female hormones are generally considered to have an important role in the development of JSLE/SLE. A trial in females with SLE has shown those who received hormone replacement therapy to be at an increased risk of disease flare (64), but much of this apparent hormonal role is not understood. Nonetheless, the hypothesis does appear to be supported by JSLE epidemiology, with under-10s seeming to be at a ratio 1:1, females to males (65), suggesting a role for hormonal change in puberty. Both JSLE and adult-onset SLE have shown to be more common in non-Caucasians, especially affecting the Asian, Hispanic, African American, Afro-Caribbean, and Native American populations (63), with such groups affected with a five-times increased risk compared with Caucasians. JSLE is a disease of significant morbidity, owing mainly to the associated severe organ damage and drug

toxicity (66). There has been a dramatic increase in the survival of JSLE and SLE patients over the last decades, however the JSLE 10-year survival rate in the West may be less than 90% (63).

1.4.2 Pathogenesis

Increased and dysregulated apoptosis (section 1.2.2) and the failure of apoptotic cell clearance (section 1.3.2) are believed to combine, generating a setting of accumulating apoptotic cells (51). A loss of self-tolerance develops (67), resulting in a mounted immune response against autoantigens. Antigen-presenting cells activate T lymphocytes (68), which in turn stimulate B lymphocytes. The B lymphocytes produce autoantibodies which, along with their autoantigens, form immune complexes that cause damage at the end-organs (36) (see Figure 1-6). Other effects include the generation of a pro-inflammatory environment, causing an activated immune response and aberrant cytokine production. The end stage of organ damage can actually serve to create and maintain a vicious circle by causing cell death, creating a potentially continuous source of autoantigens (51). Much remains unknown in the SLE disease process, but especially that of JSLE, in which there is a relative paucity of data.

Figure 1-6 The pathogenesis of SLE (36)

Prolonged exposure of autoantigens is thought to be the result of a combination of dysregulated (increased) apoptosis and the relative failure of apoptotic cell clearance. The autoantigens are then taken up by antigen-presenting cells which activate T cells, and in turn B cells. The result of this is the production of autoantibodies which form immune complexes and result in end-organ damage.

1.4.3 Cytokines associated with SLE

Particular consideration has also been given to tumour necrosis factor alpha (TNFα) and interferon alpha (IFNα), two cytokines particularly associated with SLE.

1.4.3.1 Tumour necrosis factor alpha (TNFα)

Tumour necrosis factor alpha (TNFα) is a pleiotropic cytokine with both pro-apoptotic and anti-apoptotic effects (depending on environmental context), that controls the activation and action of various cells and is an essential part of the inflammatory response (69). It is, in itself, a pyrogen, but can also precipitate sepsis by stimulating hepatocytes to manifest an acute phase response (through IL-1 and IL-6 secretion). The nature of TNFα's action cannot be easily classified, however, and this is neatly illustrated in the SLE murine model and gives indication of the caution that should be afforded given its spectrum of effects. Contrasting studies have shown SLE-disease-esque NZB/W mice with reduced TNF-α levels, in which disease improved with TNFα administration (70), compared with the MRL/lpr/lpr mouse-line which has also demonstrated SLE-esque autoimmunity, but which has been found to have raised levels of TNFα in serum, with improvement from anti-TNFα therapy. To add to the difficulty of summarising the role of TNFα in SLE definitively, TNFα given to NZB/W mice in the later stages of disease actually expedited renal damage (71). Human studies in SLE have shown raised levels of TNFα compared with controls (72, 73) and raised levels of sTNFR1 and sTNFR2 (73) , soluble TNFα receptors shed from the cell surface in part due to stimulation by the cytokine. In JSLE, however, the opposite has been found with TNFα levels significantly lower in JSLE compared with controls (39).

1.4.3.2 Interferon-alpha (IFNα)

IFNα is another cytokine with multiple effects in immune regulation. It may be produced by a number of different cells, but it is the plasmacytoid dendritic cells which are considered

to be the primary secretors of circulating IFNα (74). Increased levels of the cytokine are often observed in SLE patients, perhaps pointing to its involvement in the immune dysregulation of SLE (75). The emergence of IFNα as a treatment for certain malignancies led to the observation that this therapeutic course may have been contributing to the development of SLE-associated autoantibodies and SLE disease manifestation in these patients (75). Further, a number of genes involved in the production of IFNα are associated with increased risk of SLE-onset (76). As if to link these points, studies have shown that families with SLE-sufferers tend to have higher serum IFNα levels than would healthy, unrelated individuals (77). IFNα levels have also shown to be associated with presence of cutaneous and renal disease, and with disease activity (78). IFNα-blocking therapy trials are currently underway, albeit remain at a stage of infancy (75).

1.4.4 Clinical Features

The clinical features of JSLE (and indeed SLE) can be protean and non-specific, especially at presentation when fever, weight loss and lymphadenopathy are commonest (66). This can present difficulty in diagnosis, especially in adolescents in whom symptoms such as fatigue, headaches, weight loss, mood swings and weakness could be considered part of the "normal" teenager. There may be presence of musculoskeletal problems such as arthritis or myalgia, dermatological involvement such as butterfly rash or generalised erythema, or renal signs such as proteinuria. Predominance of symptoms and signs in JSLE can differ from SLE; published data shows statistically increased incidence of renal (proteinuria, glomerulonephritis, urinary cellular casts), neurological (encephalopathy) and haematological (haemolytic anaemia) manifestations in JSLE (79). Less commonly, liver, cardiac and pulmonary systems can also be affected; reflecting a disease process which can affect any part of the body. JSLE patients have also been shown to have more active

disease at presentation, and suffer more disease flares and sustain more organ damage over time (80).

Diagnosis can be made upon satisfaction of 4 of 11 features of the revised American College of Rheumatology criteria (81) (see Table 1-1), a combination of the associated clinical features and laboratory tests. Caution may be required, however, as evolving JSLE may not, at first, meet the diagnostic criteria and early, effective treatment can make a huge difference to disease course.

Malar rash	Non-erosive arthritis	Positive (+ve) Immunoserology
Discoid rash	Encephalopathy	Antibodies to dsDNA
Photosensitivity	Seizures	Antibodies to Sm nuclear antigen
Oral or nasal ulcerations	Psychosis	+ve finding of antiphospholipid antibodies or
Nephritis	Pleuritis or Pericarditis	Lupus anti-coagulant or
Proteinuria > 0.5g/day	Cytopenia	False +ve serologic test for syphilis for >6 months
Cellular casts		Positive antinuclear antibody test

Table 1-1 1997 Revision of the 1982 American College of Rheumatology SLE Diagnostic Criteria (73)

The above table lists the 11 major criteria of which 4 or more must be satisfied for diagnosis of SLE.

There are a number of aids designed to assess disease activity, such as the SLE Disease Activity Index (SLEDAI) and the British Isles Lupus Assessment Group (BILAG) tool, which may be especially useful in the case of disease flare. Organ damage can be assessed and monitored using the Systemic Lupus International Collaborating Clinics/American College of Rheumatology (SLICCS/ACR) damage index (66).

All organ systems need to be considered and screened throughout the course of JSLE, and the investigations undertaken should reflect these considerations accordingly. Useful blood tests include full blood count (anaemia, leukopenia, thrombocytopenia), urea and electrolytes, liver function tests (hypoalbuminaemia, raised transaminases), CRP (C-reactive protein) and ESR (erythrocyte sedimentation rate) (raised inflammatory markers), C3 and C4 (often reduced) and C1q levels and anti-C1q antibodies, among others.

Autoantibodies can be tested for, such as anti-double-stranded DNA, anti-Ro, La and Sm. These autoantibodies, and others, have also been implicated in the SLE disease pathogenesis (82).

1.4.5 Management

The management of JSLE requires a holistic multi-disciplinary approach in order to best support the development of the patient; physically, psychologically and socially. The long-term implications of disease and therapeutics with respect to fertility, cardiovascular involvement and risk of malignancy require careful consideration (66).

Similarly, the need to provide sufficient treatment to suppress the disease process and reduce organ damage is tempered by the immunosuppressive risks of infection and malignancy. Treatment strategy can be divided into that of induction and maintenance therapies.

Induction aims to induce remission of the disease flare, through aggressive treatment to endeavour to prevent organ damage. Corticosteroids are the mainstay of induction, and in mild disease, can be given orally, but in moderate or severe disease, intravenous methylprednisolone should be given in pulses, followed by high doses of oral prednisolone (83). Previously, intravenous cyclophosphamide pulses were part of induction therapy (and still can be), though mycophenolate mofetil is now more commonly used with evidence showing it to be at least as effective as cyclophosphamide for induction (83), and azathioprine is another alternative therapy.

Maintenance therapy may also vary depending upon the JSLE disease severity. Hydroxychloroquine should be given unless contraindicated as it reduces the long-term risk

of flares, is disease-modifying, steroid-sparing, and lipid-lowering (84). Originally an antimalarial, its beneficial effects in SLE appear to be attributable to its reported inhibitory action on the toll-like receptor 9 family of receptors (85). Usually either mycophenolate mofetil or azathioprine will be used in maintenance, in preference to cyclophosphamide which is associated with more severe infections – recent studies have suggested infection is the main cause of death in JSLE (83). Oral prednisolone should be used in conjunction, and gradually withdrawn as disease permits. The regime should be continued for several years to achieve persistent remission, especially crucial during childhood development (66). In the severe stages and manifestations, cyclophosphamide may need to be used but its use is generally constrained to such circumstances (86). Unfortunately, these treatment regimens are not the result of large randomised controlled trials but of a combination of adult studies, small paediatric cohort studies and clinical experience, and this accounts for the variances in pharmacological management across the country, and indeed the world (83). There are, however, a number of new therapies being developed and tested, the majority of which are aimed at B-lymphocyte depletion. Belimumab is one such drug, a human monoclonal antibody aimed at inhibition of B-lymphocyte stimulator/B-lymphocyte activating factor, which has offered good results in trials to date (87). Recently published data from phase 3 trials has continued this trend, with increased odds of positive response to treatment than with standard therapies plus placebo (88). Rituximab has been used in JSLE and associated nephritis to reported good effect, albeit with lingering doubts over its safety profile especially in repeated doses (83). A monoclonal antibody that targets the B lymphocyte transmembrane protein CD20, it causes depletion of these cells by inducing apoptosis as well as through cytotoxicity (89). Ocrelizumab is a fully humanised anti-CD20 monoclonal antibody (90) which thereby avoids some of the concerns surrounding rituximab use in SLE, such as human anti-chimeric antibodies and virus-induced progressive

multifocal leukoencephalopathy (83). There is also a humanised anti-CD22 drug, epratuzumab, well-tolerated in adult-onset SLE with good evidence of clinical benefit (91).

1.5 Receptors associated with apoptotic cell clearance

There are a number of receptors that have been implicated in the process of apoptotic cell clearance. Several of these receptors are demonstrated pictorially in Figure 1-7, a hypothetical phagocyte encircling an apoptosing cell. Some of the receptors shown are associated with the "find me" process, whilst others are associated with the "eat me" process. The sheer number of receptors and the differences in purpose and function highlights the difficulty of attempting to investigate them as potential markers of phagocytosis. However, a few of these receptors have been reviewed in more detail based on their relative importance based upon the currently available literature.

This text box is where the unabridged thesis included the following third party copyrighted material:

Devitt A, Marshall LJ. The innate immune system and the clearance of apoptotic cells. Journal of leukocyte biology. 2011;90(3):447-57.

Figure 1-7 A "superphagocyte" engulfing an apoptotic cell (92)

The figure outlines the various receptors associated with both the apoptosing cell, and the phagocyte responsible for its removal. β2GPI-R, β2-glycoprotein I receptor; ABCA1, ATP-binding cassette transporter 1; aCHO, altered carbohydrate; ASGPR, asialoglycoprotein receptor; BAI-1, brainspecific angiogenesis inhibitor 1; CRT, calreticulin; dCD31, disabled CD31; MFG, milk-fat globule EGF-8; OxLls, oxidized LDL-like site; OxPL, oxidized phospholipid; SHPS-1, Src homology 2 domain-bearing protein tyrosine phosphatase substrate- 1; TBS, TSP-binding site; Tim 1,3,4, T cell Ig and mucin domain-containing molecule 1, 3, or 4.

1.5.1.1 MER and αvβ5 Integrin

MER is a cell-membrane bound receptor tyrosine kinase, part of the TYRO3/AXL/MER receptor family (TAM) first identified in 1991 (93, 94). It is such-named for where it can be found; expressed by monocytes, and in epithelial and reproductive tissue. The principal ligand for MER is considered to be growth arrest-specific protein 6 (Gas6), common to all 3 members of this receptor family, but MER can also bind to the structurally-similar protein

S. It is through their common ~60 amino acid Gla domains that give Gas6 and protein S the capability to in turn bind PS (95) (exposed by apoptosing cells). Interestingly, Gas6 is able to bind-to and activate all three receptors, but with extremely variable affinities, with MER actually showing the least affinity to Gas6 of the three TAM receptors (comparatively lower by 3-10 fold) (96). Upon the binding of Gas6 or protein S to the tyrosine kinase receptor, receptor dimerization and activation of the kinase occurs, resulting in the recruitment, phosphorylation and activation of multiple downstream signalling proteins, modifying cell physiology (97). It was initially shown, in murine studies, that reduced MER expression resulted in reduced apoptotic cell clearance and the manifestation of SLE-like autoimmune disease (98), with just over 50% of MER-knockout mice found to have serum positive for anti-DNA antibodies (compared with 0% of wild-type mice). It was described, by Scott et al, to appear critical for the clearance of apoptotic cells. Macrophages from the MER knockout mice were 83-94% less efficient in apoptotic cell phagocytosis than macrophages from wild-type mice. The authors also demonstrated that macrophages from mice containing a kinase-dead version of MER were able to bind to, but not able to internalise apoptotic cells. Similarly, research has also shown anti-MER antibodies to prevent internalisation but not binding of apoptotic cells (99). Investigation in the murine model has also compared the capacity of this family of receptors for apoptotic cell clearance, elegantly demonstrating that MER knock-out reduced phagocytic capacity by a much greater extent than AXL or TYRO3. The impression of the MER's role as an important receptor for apoptotic cell clearance has been built upon and is now widely-held.

Recent work by a research group from the University of Pennsylvania has served to further increase the MER knowledgebase. Their findings suggest that MER also has a key role in the activation of self-reactive B cells in autoimmunity (100). Mice tangible body macrophages from the germinal centres were found to express MER, and in their MER knockout mice,

they observed greater B lymphocyte populations and greater antibody responses (101). They also report greater B lymphocyte populations in splenic marginal zone of their knockout mice, with some producing anti-dsDNA (102), and that these findings support the suggested role of MER in the regulation of cytokine secretion and antigen presentation. Research published by Galvan et al has linked MER expression to C1q, with results suggesting C1q stimulates expression of MER and Gas6 (103).

Investigation of the role of glucocorticoids, commonly used for the treatment of SLE and JSLE, in apoptotic cell clearance has suggested a novel pharmacological effect of this drug family (104). Their findings indicate that monocyte-derived macrophages incubated with dexamethasone show increased phagocytosis of early apoptotic cells than those cultured without glucocorticoid. The apoptosing cells they used, neutrophils, were specific to protein S, so they suggest that glucocorticoid stimulates associated-recognition. Their results with glucocorticoid-treated macrophages exposed to anti-MER antibodies, which appeared to lose the benefit attained from dexamethasone, suggest that it is MER in particular that is aided in the presence of glucocorticoids. This could indicate a mechanistic basis for the efficacy of steroid treatment in JSLE.

Cleavage of the MER receptor from the cell surface gives rise to its recognised soluble form (sMER), formed by cleavage caused by metalloproteinase exposure. This process has been observed to be expedited in response to the stimuli lipopolysaccharide (LPS) and the protein kinase C activator, Phorbol 12-Myristate 13-Acetate (PMA) (105). PMA activates protein kinase C and in doing so has been observed to rapidly induce apoptosis (106). LPS has also been shown to inhibit the phagocytosis of apoptotic neutrophils through TNFα induction and Gas6 suppression (107). The cleavage site has been recently observed, at proline 485 in mouse macrophages (108). Also described by the authors is the involvement,

specifically, of the ADAM17 metalloproteinase, demonstrating that LPS-induced cleavage required both ADAM17 and disintegrin. Further, they identify the importance of TLR4 (which detects LPS), through TIR-domain-containing adapter-inducing interferon-β (TRIF) adaptor signalling for the cleavage of MER. The soluble form of MER can therefore bind Gas6 (or protein S), blocking activation of MER through the occupation of this ligand, and has been found to thereby inhibit macrophage clearance of apoptotic cells (105). A recent study has shown increased sMER levels in SLE plasma compared with HC, related to both disease activity and SLE nephritis (109). The authors hypothesise that the apoptotic debris present in SLE may be responsible for increased MER expression and shedding.

This tyrosine kinase receptor appeared to be an important receptor for apoptotic cell clearance and with potential significance with regards to autoimmunity. Further, its cleaved form, sMER, has been shown to negatively impact upon the in vitro phagocytosis of apoptotic cells.

1.5.1.2 αvβ5 integrin

αvβ5 is an integrin most closely associated with its cooperation with the AXL/MER/TYRO3 tyrosine kinase family of receptors (110). There is significant synergy shared between receptors; post-translational modification of receptors and recruitment of signal proteins, recruitment of receptors and alterations in ligand-binding affinities (29). It has been shown to bind to PS through the ligand milk fat globule-EGF factor 8 protein (MFG-E8) (111), which is itself an opsonin for phagocytosis secreted by macrophages and dendritic cells. It is postulated that the role of αvβ5 is to bind apoptotic cells (via MFG-E8), increasing the chance of contact and binding of the apoptotic cell to the MER receptor, to internalise the apoptotic cell (29) (see Figure 1-8).

This text box is where the unabridged thesis included the following third party copyrighted material:

Wu Y, Tibrewal N, Birge RB. Phosphatidylserine recognition by phagocytes: a view to a kill. Trends in cell biology. 2006;16(4):189-97.

Figure 1-8 MER and αvβ5 integrin apoptotic cell interaction (29)

The figure illustrates the interaction and cooperation between the MER (labelled Mer-TK on diagram) and αvβ5 integrin receptors in the uptake of apoptotic cells. αvβ5 is thought to be important for tethering cells, and MER for the internalisation process.

1.5.1.3 Scavenger Receptor Class B – CD36 & αvβ3 integrin

The involvement of CD36, and indeed of scavenger receptors themselves, in apoptotic cell clearance was first outlined in 1991, in the recognition of apoptosing neutrophils (112). These CD36 receptors were shown to act in collaboration with the avβ3 vitronectin (CD51/61) receptors, binding either thrombospondin (113) or milk fat globule-EGF factor 8 (MFG-E8) (114), with either of these ligands in-turn bridging to the apoptotic cell (115). CD36 has also been shown to induce an anti-inflammatory response in monocytes with ligation increasing IL-10 whilst decreasing TNF-α secretion (116), but there appears to be an element of variability to the receptor, with this shown not to be the case in the macrophage (117). CD36 expression by monocytes has been investigated in SLE, but the authors report not-significant difference between those of SLE and those of controls (118). This has been corroborated by Yassin et al (119) through similar methods. It has, however, been shown that thrombospondin and avβ3 antibodies inhibited macrophage phagocytosis of apoptotic cells (120), suggesting a role for these synergistic receptors that is currently proving elusive. The avβ3 vitronectin may actually have capacity for multi-function in apoptotic cell clearance, with a recent study presenting findings showing it to be essential in the internalisation of an apoptotic cell, in cooperation with TIM4 as a tethering receptor

(121). As such, the CD36 receptor was targeted for investigation due to its supposed significance as a receptor for apoptotic cell phagocytosis.

1.5.1.4 Complement Receptors CR3 & 4

It has been shown that exposure of PS is involved in complement activation and results in C3bi coating of these apoptotic cells. The macrophage receptors for C3bi, CR3 (CD11b/CD18) and CR4 have been shown to be generally more efficient in apoptotic cell uptake than other receptors (122). Complement component C3 is fundamental to opsonisation (123). Complement is a hugely important part of the immune system and encompasses a number of different proteins with a number of different roles. Complement deficiency is often observed in patients with SLE and the levels are used as a biomarker. It has been observed that a fall in complement levels precedes an SLE disease flare. C1q deficiency is strongly associated with SLE-onset, with more than 90% of C1q-deficient cases developing SLE (124). Around 20% of C2-deficient cases develop SLE (125). A study categorised JSLE patients into three groups, according to C4 protein levels (126). One group had persistently low C4 levels, and a generally mild disease activity. The second group had fluctuating C4 concentrations and were noted to follow a similarly fluctuant disease course. The third group had generally-high levels, with the incidence of low levels coinciding with disease flares. So complement can offer predictive value for disease severity and course, and is important in augmenting phagocytosis. CR3 was selected on the basis of its association with apoptotic cell clearance but also as complement deficiency is often observed in SLE and JSLE patients. It is also, however, a more general phagocytic marker as also important for pathogen uptake.

1.6 Summary

JSLE is a rare but often severe multi-systemic autoimmune disease, diagnosed upon the satisfaction of four or more of eleven diagnostic criteria. It is often more severe than adult-onset SLE, but there remains a relative dearth of research into JSLE. Drugs such as corticosteroids and hydroxychloroquine have dramatically improved symptoms, disease activity and overall survival, but there are no curative treatments and there remains a deficiency of therapeutics targeted at the underlying pathophysiology. The pathogenesis of SLE is believed to be triggered by autoantigens originating from dying cells. The combination of dysregulated cell apoptosis and the failure of clearance of these cells by phagocytosis are thought to result in the exposure of autoantigens. The loss of self-tolerance to these autoantigens sees a mounted immune response against them, with the result of which is the end-organ damage associated with JSLE pathology. A number of receptors are associated with the process of apoptotic cell clearance, such as MER, CD36 and CR3.

Hypothesis

Monocytes and macrophages are key phagocytes and their function in JSLE may be impaired, resulting in the failure of apoptotic cell clearance. This failure may result in the exposure of autoantigens against which an immune response is mounted in JSLE. The study hypothesis is that expression of receptors MER, CD36 and CR3 differ in JSLE monocytes and macrophages compared with HC.

Aim

To analyse the expression of receptors MER, CD36 and CR3 in JSLE monocytes and macrophages compared with HC.

Objectives

- To measure the mRNA expression of receptors MER, CD36 and CR3 in JSLE monocytes and macrophages compared with HC monocytes and macrophages.
- To measure the concentration of cleaved MER, sMER, in JSLE plasma, compared with plasma from JIA and HC patients.
- To compare the phagocytic function of monocytes in JSLE plasma with HC plasma.
- To measure change in expression of CD36, MER and CR3, and loss of sMER, after exposure of monocytes to LPS, IFNα, TNFα, and apoptotic neutrophils.

2 Methods

2.1 Patients

Patients were recruited to this study as part of their participation in the UK JSLE Cohort Study (127), a study with approval from the Liverpool Paediatric Research Ethics Committee (Appendix 1: Research Ethics Approval). Written, informed (Appendix 2: Information sheets for the UK Juvenile Lupus Cohort Study and Repository) consent (Appendix 3: Patient's consent form for the UK Juvenile Lupus Cohort Study and Repository) was sought from all participating patients and their parent or guardian. JSLE patients fulfilled at least 4 of the 11 revised ACR criteria for the diagnosis of SLE before the age of 17 years. Juvenile idiopathic arthritis (JIA) patients, diagnosed according to the modified International League of Associations for Rheumatology for JIA (128), were included as paediatric inflammatory controls. Paediatric "healthy" controls were recruited from those attending hospital for non-inflammatory surgery. In practice, these included procedures such as a dental extraction or pinnaplasty. HC were excluded from recruitment in the case of known inflammatory or autoimmunity disorder, presence of inter-current infection or current drug therapeutic regime. All patients were recruited from outpatient clinics and inpatient wards at Alder Hey Children's NHS Foundation Trust in Liverpool. Sample collection occurred upon clinic attendance at the time of blood requests for disease monitoring. Around 10 mLs of blood was collected with 1-2 mLs for serum and the remaining volume for plasma and cells. Samples were immediately transferred to the laboratory and processed according to protocol. All study samples were anonymised following collection and the investigator was blinded to the corresponding clinical and laboratory data until experiment completion.

2.2 Blood Sample Processing

Blood samples of JSLE, JIA and paediatric and adult HC samples were received and processed in the laboratory. Serum (without anticoagulant) and plasma (heparinised) bottles were used to collect blood; with serum bottles used to process serum alone, and plasma bottles used to process peripheral blood mononuclear cells (PBMCs), neutrophils and plasma. Processing was commenced within an hour of blood collection. All processing was conducted within the sterile field of a tissue culture hood.

Blood (plasma) was layered above an equal volume of Polymorphprep (Axis-Shield PoC AS, Norway) and centrifuged at 530 xg for 30 minutes at room temperature with the brake turned off (Figure 2-1). Polymorphprep needs to be used at around 20^{o}C to allow loss of water from red blood cells to the hyperosmotic medium to occur. If the brake is used to decelerate the rotor, the cell bands can cross-contaminate and the erythrocytes may not settle at the bottom of the tube. Alternatively, if a patient's blood was known to centrifuge poorly upon Polymorphprep (e.g. if white blood cells previously failed to separate from red blood cells), Histopaque-1077 (SIGMA-ALDRICH, UK) wash used in substitution of Polymorphprep.

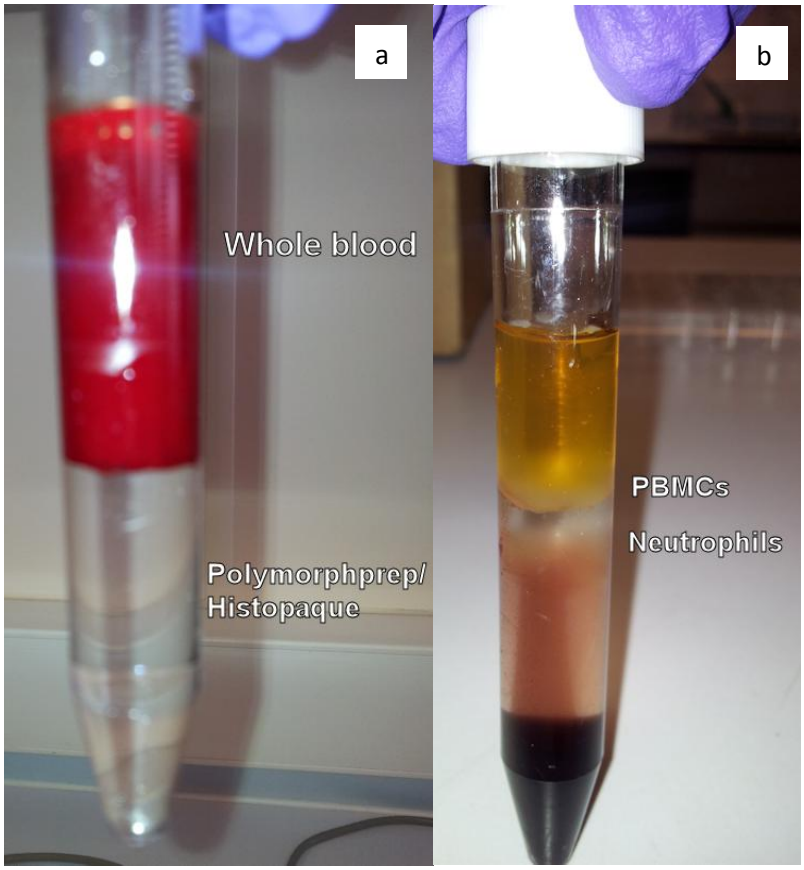


Figure 2-1 Whole blood, before and after centrifugation

Falcon tube with blood layered over Polymorphprep, before (a) centrifugation at 530 xg for 30 minutes at room temperature, and after (b) as the PBMCs and neutrophils separate to form two distinct layers.

A sterile Pasteur pipette was used to transfer PBMCs and neutrophils into separate falcon tubes. These were then washed with RPMI-1640 (LONZA, Belgium) and subsequently centrifuged at 670xg for 10 minutes. The medium was removed and the newly-formed pellets were resuspended in lysis buffer consisting of 9 mLs of Ammonium Chloride 1% and 1 mL RPMI-1640. The solutions lysed for 3 minutes, then centrifuged at 670xg for 5 minutes. By this process, any present red blood cells were lysed leaving a purer remaining population of cells. Medium was again removed and the cell pellets re-suspended.

2.2.1 Storage of PBMCs

1 mL of freezing media (90% Foetal Calf Serum (FCS) [SIGMA-ALDRICH, UK) + 10% Dimethyl sulphoxide (DMSO) [SIGMA-ALDRICH, UK]) was added and mixed with PBMCs, transferred to an insulated cryovial and stored at -70^{o}C for 24 hours, then transferred to storage in liquid nitrogen.

2.3 Monocyte Separation

Processed blood samples were used to separate the CD14^{+} monocyte cell fraction, which was then either used, stored at this point, or cultured into macrophages. The CD14^{+} cell separation was performed according to manufacturer's instructions; MACS Miltenyi Biotec – CD14 MicroBeads Protocol.

Continuing from section 2.2 (following centrifugation at 670xg for 10 minutes) RPMI-1640 was removed leaving the PBMC pellet, and a cell count was performed using a Neubauer haemocytometer (Superior Marienfeld, Germany). During the cell count, cells were re-pelleted by centrifugation at 670xg for 5 minutes.

Buffer was prepared comprising sterile phosphate-buffered saline (PBS) pH 7.2 with 2mM ethylenediaminetetraacetic acid (EDTA) and 0.5% FCS (buffer was kept on ice when not in use). PBS and EDTA solution was passed through a Minisart-Plus filter (SIGMA-ALDRICH, UK) to sterilise, prior to addition of FCS.

RPMI-1640 was removed from sample before adding 80 µl of buffer and 20 µl CD14 MicroBeads (MACS Miltenyi Biotec, Germany) per 10^{7} i.e. 10 million cells, and this solution was incubated at 2-8^{o}C for 15 minutes. The MicroBeads magnetically label the CD14^{+} cells.

The cells were washed in 1-2 mLs of buffer and centrifuged at 670xg for 5 minutes. A magnetic unit was set-up, with MiniMACS Separator (MACS Miltenyi Biotec, Germany) and MACS Separation Column (MACS Miltenyi Biotec, Germany) (Figure 2-2). First, the column filter was wetted with 0.5 mLs of buffer, collected into a falcon tube (for $CD14^-$ cells), and once filtration was finished, centrifuged PBMCs were re-suspended in 0.5 mLs of buffer and transferred onto the column.

Figure 2-2 Apparatus setup for cell separation

Setup for CD14 separation, with the MiniMACS Separator and MiniMACS Separation Column set over a falcon tube (to collect $CD14^-$ cells).

Another 0.5 mLs of buffer was added to PBMC falcon tube to capture any remaining cells and transferred to column, after which a further 0.5 mLs of buffer was added directly to the column, and one final 0.5 mLs of buffer. Once this last quantity of buffer had passed through the magnetic column, the column was transferred onto a clean falcon tube. 1 mL of buffer was added to the column, and cells were flushed through the column by applying

the plunger to the column. At this stage, the $CD14^+$ cells which had been magnetised to the column were expelled into the tube with the buffer.

2.3.1 Macrophage culture

$CD14^+$ cells were washed through centrifugation at 670xg for 5 minutes, resuspended in RPMI, and cell count was performed using a haemocytometer. Cells were cultured at a concentration of $0.5x10^6$ cells in 1 mL of medium per well, in a Nunclon Δ (delta) surface 24 well plate (NUNC, Denmark).

The buffer was removed and the $CD14^+$ cells were resuspended in a culture medium comprising:

- RPMI (LONZA, Belgium)
- 10% FCS (SIGMA-ALDRICH, UK)
- 1% Penicillin Streptomycin (10 mg/mL) (SIGMA-ALDRICH, UK)
- Macrophage colony-stimulating factor (M-CSF) (at a concentration of 50 ng/mL)

Each 1 mL of $CD14^+$ cells in culture medium was transferred into an individual well in the 24 well plate, and the plate was incubated at 37^oC for 6 days.

2.4 RNA Extraction

Culture medium extracted from wells containing monocytes/macrophages, and 350µl of Lysis BME BME (Buffer RLT (QIAGEN, Germany) with 1% 2-mercaptoethanol [SIGMA-ALDRICH, UK]) pipetted into wells and transferred to QIAshredder (QIAGEN, Germany). The QIAshredder was centrifuged at 7527 xg for 2 minutes (at room temperature). The filter was discarded, and 350 µl of 70% ethanol was added to the collection tube, mixed, and all contents were transferred to a RNeasy Mini Spin Column (QIAGEN, Germany) and centrifuged at 7527 xg for 15-20 seconds.

Liquid was discarded from collection tube and 700 µl of Buffer RW1 (QIAGEN, Germany) added to the column, then centrifuged at 7527 xg for 15-20 seconds. The liquid was discarded and 500 µl of Buffer RPE (QIAGEN, Germany) was added to the column, with this process repeated twice - the first centrifuge for 15-20 seconds and the second for 2 minutes. The filter was then dried by transferring it to a fresh collection tube and centrifuged at 7527 xg for 2 minutes.

The filter was transferred onto an eppendorf, 40 µl of RNase-free water (QIAGEN, Germany) was added, and it was left to soak for 30 seconds then centrifuged at 7527 xg for 1 minute. The filter was discarded, the eppendorf (now containing RNA) was retained and stored at -70^{o}C.

2.4.1 RNA Assessment

RNA was assessed for quantity and quality using NanoDrop ND-1000 Spectrophotometer (Thermo SCIENTIFIC, USA) and accompanying computer software. Quantity (ng/mL) was measured and quality was assessed according to the 260/280 value, where a range of 1.8-2.2 was deemed of sufficient quality to be utilised for investigation.

2.5 Quantitative Polymerase Chain Reaction (qPCR)

2.5.1 The principles of PCR

Polymerase Chain Reaction (PCR) is a technique which sees short sequences of DNA amplified by repeated cycles of replication. By increasing and decreasing the temperature DNA was exposed to, it was possible to form duplicates of DNA sequences. The process can be summarised into the following basic steps:

1. Denaturation – the double-stranded DNA helix is heated to 94-98^{o}C, causing it to melt apart into two separate strands.
2. Annealing – the reaction is cooled to 45-65^{o}C, enabling single-stranded primers to "anneal" to the now single-stranded DNA template. At this point, DNA polymerase attaches to the primed template and begins to attach complementary nucleotides.
3. Extension – the temperature is increased to 65-75^{o}C, which enables polymerase to function in an optimised environment, extending the primer sequence through incorporation of complementary nucleotides, yielding a new double-stranded DNA unit. So from each double-stranded DNA helix that is started with, two new DNA helices are formed.

This process can then be repeated for any number of cycles.

2.5.2 The principles of real-time quantitative PCR (qPCR)

Quantitative PCR (qPCR) was a particularly important extension of the PCR principle that enabled measurement of gene expression. By converting RNA to cDNA through reverse-transcription, the cDNA could be subsequently amplified by PCR. This has in turn been improved upon by real-time qPCR, in which amplification of specific nucleic acid sequences is followed by quantitation of the amounts of DNA present after each cycle (129). The

advantages of real-time qPCR over semi-quantitative PCR are reduced non-specific amplification, greater dynamic range and reduced RNA requirement (130).

Real-time qPCR allows quantitative measurement of the products during the course of the reaction. It is performed in a thermocycler that can measure the level of a fluorescent detector molecule, and this these measurements are carried out after every cycle. The fluorescence of such molecules increases in proportion to the increasing amplification of DNA. The use of these molecules reduces the post-processing steps and minimises experimental error (130). Commonly used for qPCR include probe sequences that fluoresce upon hydrolysis (e.g. TaqMan) or hybridisation (e.g. LightCycler), fluorescent hairpins, or intercalating dyes (e.g. SYBR Green) (130).

For the purposes of the experiments conducted, SYBR Green was used. SYBR Green fluoresces upon binding to double-stranded DNA, and does so in a dose-dependent manner (see Figure 2-3), according to the amount of double-stranded DNA present (131). The advantages of SYBR Green are that it is sensitive, relatively inexpensive and simple to use. The main disadvantage of SYBR Green is that as it is unselective in binding double-stranded DNA, it can also bind primer-dimers – where primers bind to one-another – or other reaction products, which may confound results. However, such primer-dimers can be detected through the dissociation curve, in which they can be observed to have a lower temperature of dissociation than target DNA because of their shorter sequences (see 2.5.4).

This text box is where the unabridged thesis included the following third party copyrighted material:

SIGMA-ALDRICH. SYBR Green based qPCR technical animation. SIGMA-ALDRICH; 2012 [cited 2012 8 August]; Available from: http://www.sigmaaldrich.com/sigma-aldrich/areas-of-interest/life-science/molecular-biology/pcr/quantitative-pcr/sybr-green-based-qpcr/syber-green-animation.html.

Figure 2-3 SYBR Green fluorescence in response to double stranded DNA

SYBR Green intercalating dye; showing the direct correlation between amount of double-stranded DNA present and fluorescence: (a) less double stranded DNA and therefore less fluorescence and (b) more double stranded DNA and fluorescence (adapted from (132).

In order that we may realise the full potential of real time qPCR, it is important that good controls are used that will normalise the results, accounting for differences in the quantity and quality of starting material and in reaction efficiency (130). This is achieved by reference or "housekeeping" genes, selected on the basis that their expression is similar in all samples used, resistant to environmental conditions, and that they go through PCR with the same kinetics as the target gene (133) (section 2.6.4).

A number of graphs produced at the end of the real-time qPCR process give indication as to the reliability of results gathered.

2.5.3 Amplification Plot

The Amplification Plot graphs number of cycles on the *x*-axis (n) against the detected fluorescence on the *y*-axis (dR): Figure 2-4 is one such example. The first part of the Amplification Plot is the "exponential" phase, during which every cycle should mark doubling of PCR product as there should be no limiting factors for the reaction. Initially, growth is difficult to detect because the amount of product is small and fluorescence low, but it becomes apparent as the fluorescence increases and passes through the threshold –

the fractional cycle number (C_T). This is the point at which the emitted fluorescence is greater than the background noise. During the "linear" phase, product is continually formed but reaction elements deplete and efficiency decreases. The reaction then enters the plateau phase, as enzymatic activity decreases and substrates limit product formation.

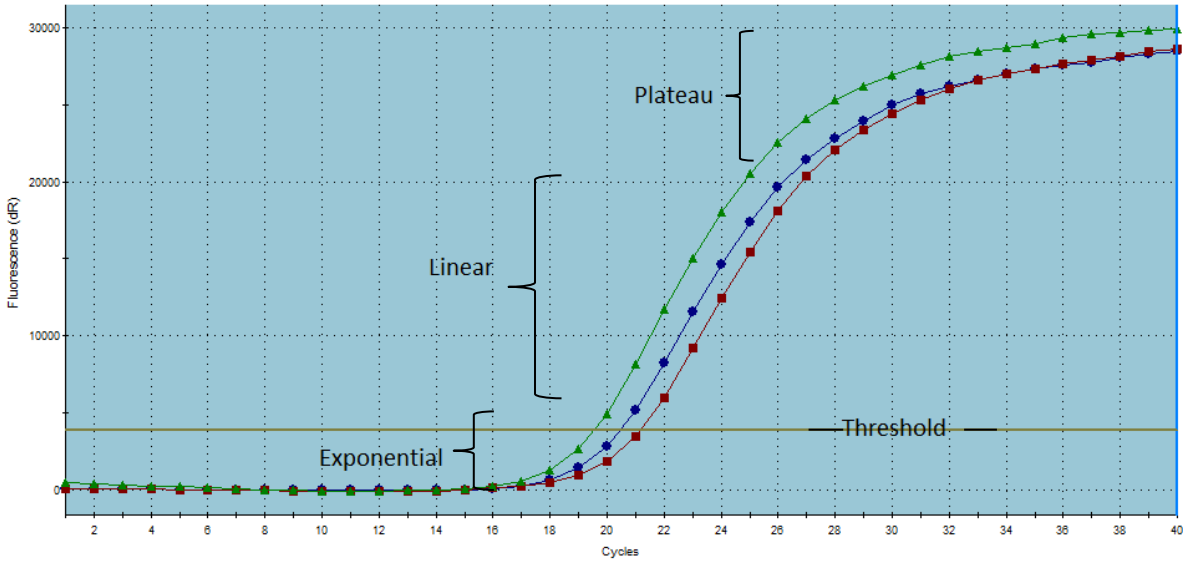


Figure 2-4 Example of a qPCR amplification plot

The graph illustrates the three stages of qPCR: exponential, linear and plateau. Also shown is the threshold, representative of the fractional cycle number (C_T).

2.5.4 Dissociation Curve

The dissociation or melting curve charts the temperature of dissociation of PCR products (see Figure 2-5). Temperature is increased in small increments, and fluorescence measured. At the melting point, the double-stranded DNA will dissociate and fluorescence will decrease. Temperature (^oC, *x*-axis) is plotted against the rate of change of fluorescence ($-R^I(T)$, *y*-axis), and the peak change will occur at the melting point. Performed at the end of the PCR cycles, it means we should be able to observe if there have been any problems with the PCR, for example primer-dimers, mispriming, or contamination. As mentioned earlier, as these other causes will produce smaller products, they will have a lower dissociation temperature, and so produce a distinct peak or distinct peaks.

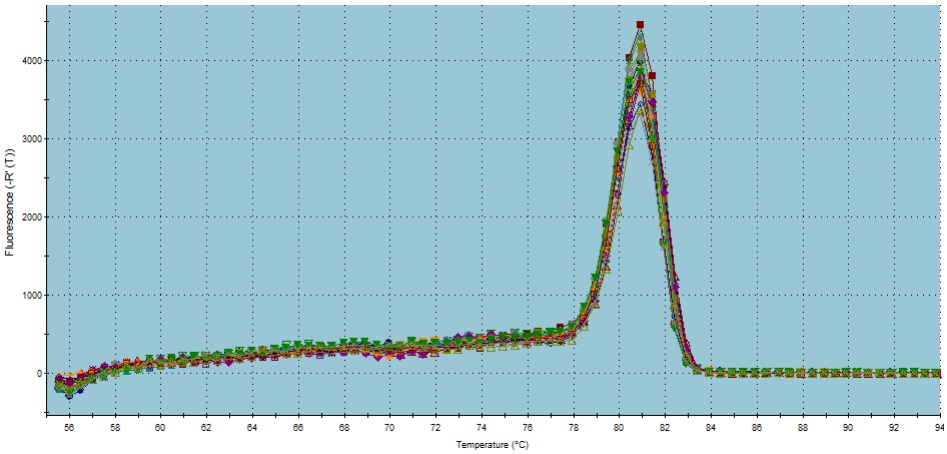


Figure 2-5 Normal Dissociation Curve

This dissociation curve has a uniform peak of negative change of fluorescence at around 81^{o}C indicating the presence of only one product.

Figure 2-6 shows an abnormal dissociation curve, with a peak of certain samples at >80^{o}C, but another, more consistent peak at around 73^{o}C, so a lower-than-expected dissociation demonstrating the presence of more than one product, and suggesting the presence of primer-dimers.

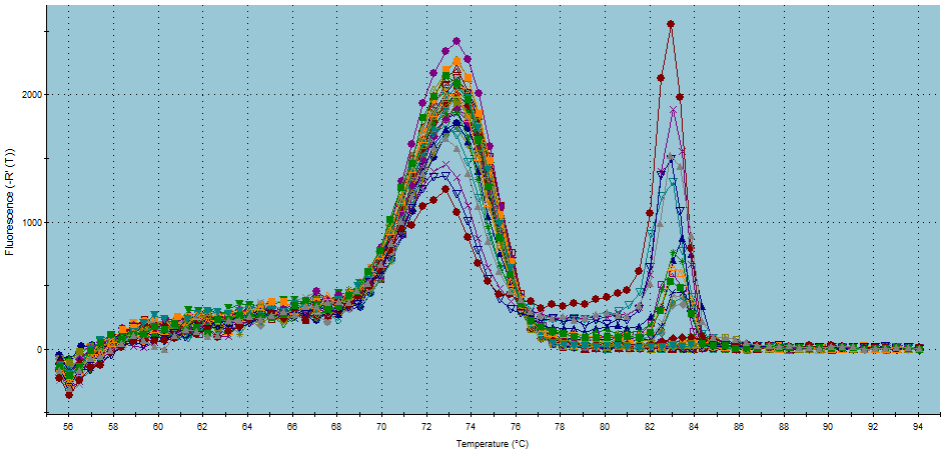


Figure 2-6 Abnormal Dissociation Curve

This dissociation curve has a second peak at a lower temperature indicating the potential occurance of primer-dimerisation.

2.5.5 Standard Curve

The Standard Curve charts the initial quantity (number of copies, *x*-axis) against the C_T; the point at which the fluorescence crosses the threshold (dR, *y*-axis) (see 2.5.3). In the experiments conducted, a 5-point standard curve was used in triplicate, with standards from neat to 1 in 20 dilution, and these 5 points are used for the linear graph. The resultant linear plot is used to work out the amount of DNA initially present in samples based on the measured C_T values (see

Figure 2-7). The consistency of these results may be observed by testing in triplicate.

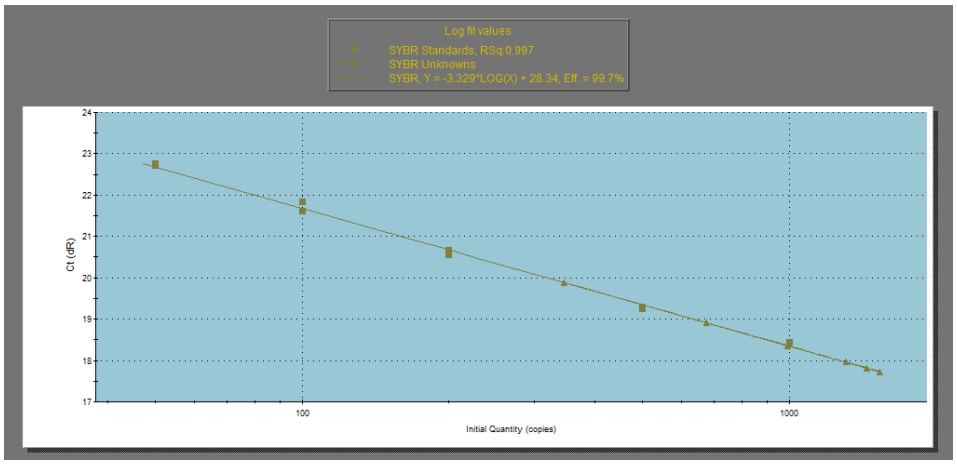


Figure 2-7 An example qPCR Standard Curve

The standard curve has a strong R^2 value (close to 1.0), indicating good reliability.

The Standard Curve should have a good efficiency (as close to 100% as possible), and a R^2 value close to 1.0 so the results may be accepted.

2.6 Reverse Transcription and Real-time qPCR Protocols

2.6.1 Reverse Transcription

RNA (100 ng/µl) was added to RNase-free water (QIAGEN) to a final volume of 14 µl and 1 µl of random primers added, and this mixture was heated at 70^{o}C for 5 minutes. A master mix of reverse transcription was made up consisting of the following, per RNA sample: 5 µl MMLV Reverse Transcriptase 5X Buffer (Promega, USA), 1.25 µl Nucleotides (Promega,

USA), 0.5 µl RNasin RNase inhibitor (Promega, USA), 1 µl MMLV Reverse Transcriptase and 2.25 µl RNase-free water (QIAGEN).

The total 10 µl of master mix was added to each sample of RNA, and samples were heated at 42^{o}C for 1 hour. The cDNA was then diluted to a 1 in 4 solution, and stored at -20^{o}C until required.

2.6.2 Real-time qPCR

Real-time qPCR was carried out using the Mx3005P instrument and MxPro v4.0 software (Strategene, Agilent Technologies, UK). The template was first devised, consisting of a non-template control (RNase-free water)), standards and the samples, all of which were tested in triplicate. To produce the experiment standards, serial dilutions of the pooled standard were made, resulting in 5 standards of varying concentrations (neat, 1 in 2, 1 in 5, 1 in 10, and 1 in 20).

With template samples ready, these were stored on ice whilst a qPCR master mix was made up. This consisted of the following quantities, per well: 0.75 µl Forward Primer (Eurofins MWG Operon, Germany), 0.75 µl Reverse Primer (Eurofins MWG Operon, Germany), 10 µl SYBR Green ([PerfeCTa SYBR Green FastMix Low Rox] Quanta Biosciences, USA) – and 3.5 µl RNase-free water (QIAGEN). 15 µl of master mix was then aliquoted into each well, followed by 5µl of appropriate sample. The wells were sealed with caps and centrifuged for 20seconds at 1200rpm. The following thermal profile was used for real-time qPCR. Figure 2-8 (see below) shows the thermal profiled used for the conducted qPCR experiments. The reasons for the temperatures have been largely explained previously (see 2.5.1), with Segment 2 representing a 40-cycle period of denaturation and annealing, followed by Segment 3 testing for product dissociation.

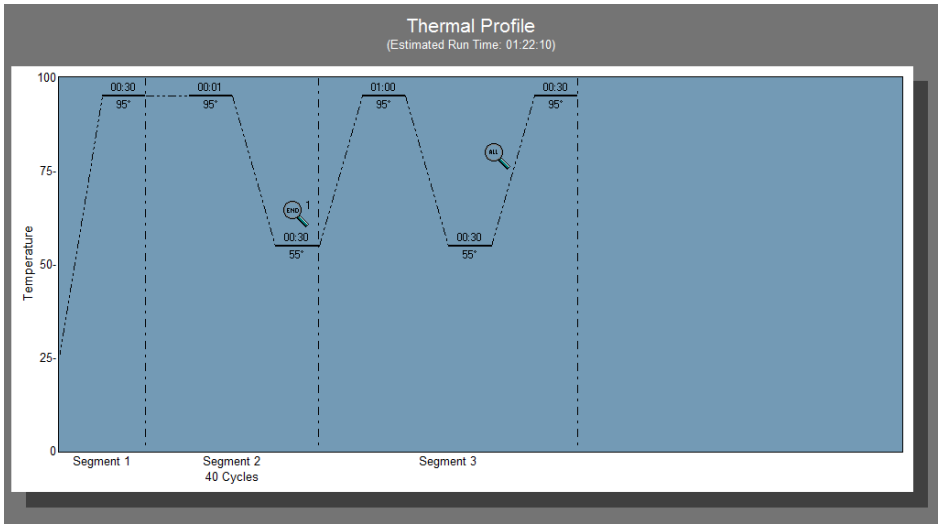


Figure 2-8 Thermal Profile utilised for undertaken qPCR experiments

2.6.3 Data Analysis

Analysis of real-time qPCR data is carried out by one of two methods: absolute levels or relative levels.

Absolute uses the number of copies of RNA. For this, results have been converted into relative expression ratios by:

$$Relative\ expression\ ratio = \frac{Gene\ of\ interest}{Reference\ gene}$$

Where relative quantitation could be carried out, the following formula has been utilised.

$$Fold\ change = 2^{-\Delta\Delta CT}$$

This approach is dependent on the assumptions of 100% efficiency, i.e. doubled product every cycle, and that the reference gene is expressed at a constant level across samples. The resultant fold change is positive, if greater than 1, and negative if between 0 and 1 (in this case, the inverse [i.e. $\frac{1}{x}$] is taken to give fold change reduction).

2.6.4 Reference Gene Selection

Reference genes, as discussed in section 2.5.2, are used to normalise data; in order that we may correct for any differences in starting materials (134). So the resultant quantity found for any gene of interest is compared against the quantity of the selected reference (or "housekeeping") gene. Two reference genes were tested for suitability, beta-2-microglobulin (β2M) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH). β2M is a small polypeptide associated with the major histocompatibility complex (MHC) class 1 heavy chain, found on the surface of almost all nucleated cells (135). GAPDH is an enzyme primarily concerned with the process of glycolysis, commonly used as a reference gene (136). Upon testing with cDNA produced from adult HC macrophage RNA, both genes appeared to be consistently expressed, with β2M appearing superior. Combined with evidence in the literature suggesting GAPDH expression varies in certain experimental settings (137) and can even vary within a cell subpopulation (138), and a study investigating reference gene expression in alveolar macrophages finding it to be unstably expressed (139), β2M was selected as the reference gene to use for the real-time qPCR undertaken.

2.6.5 Primers

Table 2-1 lists the primers used in the real-time qPCR experiments conducted and the forward and reverse nucleotide sequences.

Primer	Forward	Reverse
β2M	5'-TGCCTGCCGTGTGAACCATGT-3'	5'-TGCGGCATCTTCAAACCTCCATGA-3'
CD36	5'-GAGAACTGTTATGGGGCTAT-3'	5'-TTCAACTGGAGAGGCAAAGG-3'
MER	5'-CCGCCTGAGCCCGTCAACATT-3'	5'-CTCCGTCAGGCTGGAACAGT-3'
CR3	5'-AAGGTGTCCACACTCCAGAAC-3'	5'-GAGGAGCAGTTTGTTTCCAAG-3'

Table 2-1 Primer Sequences used for real-time qPCR

2.7 Enzyme-linked Immunosorbent Assay (ELISA)

ELISA is a laboratory assay that enables quantitative measurement of antibodies or antigens. First described in 1971 (140), the ELISA fast became and still remains indispensible across a number of different scientific disciplines. For the experiments performed here, antigens of interest were tested through the use of sandwich ELISA. By this method, reactants are absorbed onto a plastic plate, such that they are immobilised to the surface. The antigen is "sandwiched" between two monoclonal antibodies that bind specifically to two distinct sites on the antigen (150). The amount of antigen is measurable by comparison against a set of standards with pre-determined quantities of antigen. The sandwich ELISA is a sensitive test and has reduced risk of cross-reactivity because of the use of two separate epitopes, or antibodies.

ELISAs undertaken were conducted in 96-well Maxisorp plates (NUNC, Denmark). Figure 2-9 (below) illustrates the principle of the ELISA process occurring in a single well, and corresponds to the outlined methods.

This ELISA was devised from the manuscript published by Wu et al (109). The authors used a kit for detecting MER in cell lysates, instead to measure MER in human plasma (i.e. sMER). The antibodies and standards from the human total MER ELISA kit DYC-891 (R&D Systems, USA) were used by the same methods used for all ELISAs conducted. The kit and methods were first validated and optimised by spike and recovery and linearity of dilution, and fresh plasma samples were then tested accordingly. The reagents used are listed throughout the protocol, and represent the only deviations from the ELISA kit manufacturer's instructions.

The capture antibody was reconstituted to a working concentration of 8 µg/mL in phosphate buffered saline (PBS) [SIGMA-ALDRICH, UK], and added to the wells. Capture antibody has a specific binding site for the antigen of interest. The plate was covered and incubated overnight at room temperature. The plate was then washed three times to remove any excess. This was performed with wash buffer (PBS + 0.05% Tween 20 [SIGMA-ALDRICH, UK]).

Reagent diluent (PBS + 1% bovine serum albumin [SIGMA-ALDRICH, UK]) was used as a block buffer (containing an excess of unrelated protein), and added to the wells to prevent the binding of any remaining well sites not occupied by capture antibody. The plate was incubated at room temperature for 1-2 hours. The plate was washed three times to remove any excess.

Samples containing the antigen of interest were diluted (as appropriate) and added to wells (in duplicate) to bind to the capture antibody. The plate was covered and incubated at room temperature for 2 hours. The plate was then washed three times to remove any excess.

The detection antibody was reconstituted to a working concentration of 200 ng/mL in reagent diluent, and was added to the wells. Biotinylated monoclonal detection antibody recognises and binds to a different site of the antigen of interest. Biotin is a low-molecular weight vitamin B which may, in turn, be bound. The plate was covered and incubated at room temperature for a further 2 hours. The plate was then washed three times to remove any excess.

Streptavidin conjugated with an enzyme, horseradish peroxidase (Streptavidin-HRP), was diluted to a working concentration of 1 in 200 in reagent diluent, and added to the wells. Strepatividin-HRP is a bacterial protein that has a high affinity to bind biotin (141). The plate was covered and incubated for 20 minutes outside of direct light. The plate was then washed three times to remove any excess.

Substrate (1:1 mixture of H_2O_2 and Tetramethylbenzidine (R&D Systems) for the enzyme was added to the wells. The substrate is hydrolysed, producing a colour change (to blue) that is proportional to the amount of antigen that was present. The plate was covered and incubated for up to 20 minutes outside of direct light.

This reaction is stopped using sulphuric acid, which causes a colour change (to yellow), and this colour change is spectrophotometrically analysed using a microplate reader ELX800 (BIotek, USA) and the proprietary software KCJunior. These results were then displayed in Microsoft Excel 2010 and subsequently analysed with IBM SPSS v19 and/or Graphpad Prism v5.

This text box is where the unabridged thesis included the following third party copyrighted material:

Paulie S, Perlmann H, Perlmann P. Enzyme-linked Immunosorbent Assay. eLS: John Wiley & Sons, Ltd; 2001.

Figure 2-9 ELISA principle (142)

The steps are: capture antibody, then block buffer, then samples/standards, then detection antibody, then streptavidin-HRP, with washes between steps. Wells are coated with capture antibody, then block buffer added to occupy any free binding sites. Samples and standards are added to wells, with any antigen of interest intended to bind to capture antibody. Detection antibody is added, sandwiching the antigen. The addition of strepatividin-HRP binds to the biotin of the detection antibody. Substrate solution manifests a colour change proportional to the quantity of antigen of interest, and this reaction is halted using stop solution. The colour change is then measured by spectrophotometer.

2.7.1 Data Interpretation

In order to measure the amount of antigen of interest present, samples are tested alongside a set of standards which contain known-amounts of antigen. The measured colour is compared back to the known amount for all standards, and the resulting standard curve is used to ascertain the quantity of antigen present in samples. The r^2 of the curve indicates the consistency of each duplicate and of the standards. An r^2 of 1 indicates that all points lie on the curve, and an r^2 of greater than or equal to 0.9 was considered acceptable. By plotting a standard curve and ascertaining the equation of the curve, the values returned by the spectrophorometer can be converted into concentrations of antigen found in each unknown sample. The standard curve can also be used to validate the ELISA, as the difference between standards should follow a predictable, consistent pattern.

2.7.2 Spike and Recovery and Linearity of Dilution

Spike and recovery and linearity of dilution are methods of validating the accuracy of ELISA experiments. Serial dilutions of samples are performed and samples are either left un-spiked, or spiked with a known quantity of the antigen of interest. The aim of assay development is to maximise the signal-to-noise ratio whilst achieving identical results for spike alone compared with spiked samples. By performing these tests, it confirms that both the ELISA kit works correctly and excludes the possibility of interference from another antigen found in the samples. The linearity of dilution indicates whether there is a substance present in the samples that is interfering, as in this case it is expected that the interference identified will decrease with increasing dilution.

2.8 Flow Cytometry

2.8.1 The Principles of Flow Cytometry

Flow cytometry is the measurement of the characteristics of cells or particles in a fluid stream as they pass through a light source (143). Sample taken up by the flow cytometer must firstly be ordered into a stream of single particles through the fluidics system. This system consists of a central core, through which the sample is passed, and a surrounding sheath containing sheath fluid (see Figure 2-10). The sheath fluid flows faster than sample and this pressure differential causes a drag effect on the narrowing central core resulting in a parabolic flow; focusing the cells or particles into a single stream through this process of hydrodynamic focusing (143).

Figure 2-10 The principles of hydrodynamic focusing

The sample is channelled down the central core; the faster flow of the surrounding sheath fluid creates a drag effect, focusing the cells into a single stream (144).

At the interrogation point, a beam of monochromatic light is projected upon the cells or particles (145), and collection optics direct the light scatter or fluorescent light of the cells or particles to an electronic network (143). This means that particular wavelength bands are isolated (145). The light signals from each cell or particle is detected by the electronic network which converts these signals to a measure proportional to light intensity. These measurements are recorded by computer to be analysed and interpreted by the user.

Emitted light is measured by fluorescence channels or detectors which are specific for different wavelengths. This is in turn determined by optical filters that may either block or transmit certain wavelengths. The photomultiplier tubes are detectors termed FL- followed by a number to indicate the range of wavelength each may detect (143). The filter FL-4 is used to detect far red fluorescence.

2.8.2 The Principles of pHrodo

Phagocytosis assays have been undertaken with a variety of methods, and a number have been undertaken investigating phagocytosis in adult-onset SLE. However, a number of potential phagocytosis assay protocols could result in a overestimation of phagocytosis with assumed internalisation when a bacterium or cell has instead remained bound to the cell surface (146). The methods described by Miksa et al (146) outline a staining method by which internalisation can be assured. pHrodo (Invitrogen, USA) is a fluorescent dye which emits red light in response to and with increasing intensity in relation to decreasing pH. As described in section 1.3, after internalisation a phagocytosed particle fuses with the lysosome. The lysosome is an acidic sac (45) and the fusion of the phagosome and lysosome therefore exposes the phagocytosed particle to an acidic environment (146). The fluorescence emission can be observed visually (see Figure 2-11) and can be detected and measured by flow cytometry. E. coli particles can be obtained pre-stained with pHrodo, and it is also possible to stain cells independently, used in the aforementioned study to measure apoptotic cell clearance (146).

This text box is where the unabridged thesis included the following third party copyrighted material:

Miksa M, Komura H, Wu R, Shah KG, Wang P. A novel method to determine the engulfment of apoptotic cells by macrophages using pHrodo succinimidyl ester. Journal of immunological methods. 2009;342(1-2):71-7.

Figure 2-11 Images illustrating pHrodo fluorescence in association with apoptotic cell attachment (146)

The above graphics show: (a) there is minimal fluorescence compared with engulfment (b), where there is clear fluorescence. This occurs due to fusion of the phagosome (internalised cell stained with pHrodo) with the cell lysosome, resulting in a drop in pH and resulting in red fluorescence.

2.8.3 Protocol (Monocytes + pHrodo E. coli)

Following on from the monocyte separation protocol (section 2.3), $CD14^+$ cells were re-suspended in RPMI, to a working concentration of 1 x 10^5 cells per 100 µl. 1 x 10^5 cells were added to each flow cytometry tube, and were then washed with uptake buffer (HBSS [Invitrogen, USA] and HEPES 20mM [SIGMA-ALDRICH, UK] at pH 7.4) and centrifuged at 670 xg for 5 minutes. The supernatant was removed, and a total of 50 µl of solution was mixed with the $CD14^+$ cells (consisting of pHrodo E. coli BioParticles conjugates [Invitrogen, USA] or uptake buffer plus 10% fresh serum, fresh plasma plasma or neither [in which case uptake buffer was used in substitution]). These samples were then immediately kept on ice or incubated at 37^{o}C with 5% CO_2 for a pre-determined time course. At this point, samples were washed with uptake buffer and centrifuged at 670 xg for 5 minutes. The supernatant was removed and the cells were resuspended in uptake buffer. The samples were then tested by Flow Cytometer (Cytomics FC 500 MPL [BECKMAN COULTER, UK]). As pHrodo positivity is marked by red light emission, FL-4 was used in detection. The data was recorded by computer using MXP software (BECKMAN COULTER, UK). Analysis of flow cytometry data was performed using FlowJo v7.6.5 (Tree Star, USA).

2.8.4 Data Interpretation and Analysis

Flow Cytometry assesses the characteristics of cells or particles are assessed in a number of ways. In order to analyse a cell population, this population must first be "gated" from any debris or dead cells, for example. A density plot graphs particles according to size, by forward scatter, and cell granularity, by side scatter, and groups can then be gated to return a purer population of cells (see Figure 2-12).

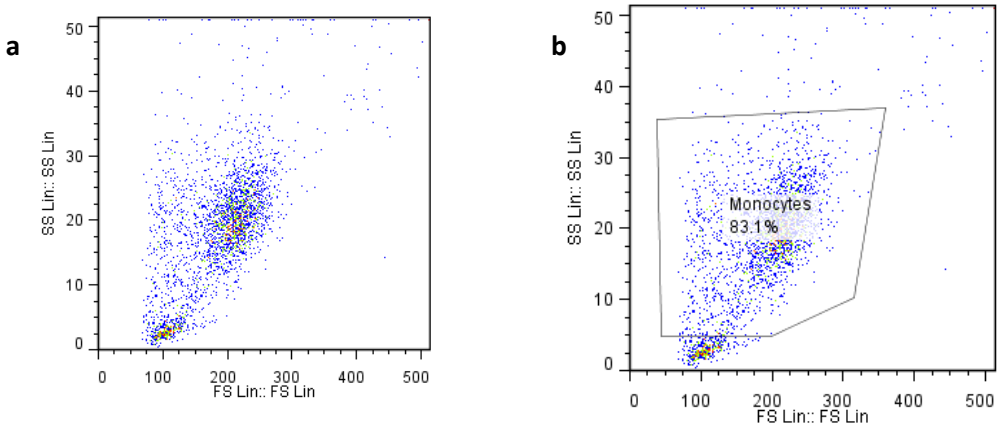


Figure 2-12 Flow cytometry of a population of separated $CD14^+$ cells

The above flow cytometry scatter plots show: (a) post-cell separation $CD14^+$ population; (b) gated monocyte population.

The gated cells are then plotted in a histogram, with the x-axis for fluorescence or light scatter intensity, and the cell count on the y-axis. This will be expected to produce one or more normally-distributed peaks. As pHrodo fluoresces red, and is therefore detected with FL4, pHrodo flow cytometry histograms have been plotted according to FL4 fluorescence. A negative control of monocytes alone was used to determine a relative baseline from which to measure phagocytosis (see Figure 2-13). Further controls were used of samples stored on ice to provide another baseline for comparison with cells incubated at 37°C. Cells stored on ice act as negative controls; so amount of phagocytosis occurring would be limited in

comparison to incubated cells. By deducting determined pHrodo positivity of ice-stored samples from incubator-stored samples, an assessment of the amount of phagocytosis was made.

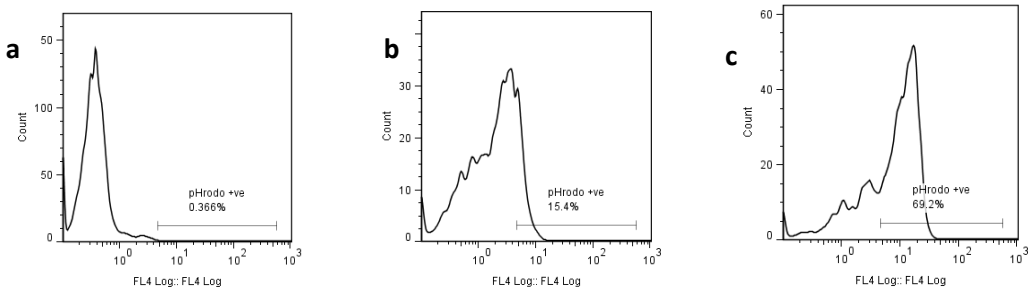


Figure 2-13 Example histograms illustrating pHrodo positivity

The above are examples of histograms comparing the number of cells with pHrodo positivity (FL-4 *x*-axis, cell count *y*-axis). Three test conditions are shown: (a) monocytes alone; (b) monocytes in 10% serum on ice; (c) monocytes in 10% serum, incubated.

2.9 Monocyte Stimulation

An experiment was designed to attempt to ascertain the conditions or environments that may result in the cleavage of MER from the surface of the phagocyte. Adult HC monocytes were exposed to a number of environments associated with SLE/JSLE for a pre-determined length of time. Also of interest was the effect that these same environments may have on the expression of the phagocytic markers of interest: CD36, MER and CR3.

$CD14^+$ cells were separated from PBMCs from adult HC whole blood according to protocols outlined previously (sections 2.2, 2.3). 2 x 10^5 cells in RPMI media were placed into separate wells on a 96-well plate and incubated at 37^{O}C for 2 hours to enable them to adhere to the plate. At this time point, media was removed and replaced with RPMI media containing different stimulators. RPMI media was reapplied for negative controls. LPS was added to RPMI media at a concentration of 50 ng/mL. IFNα was added to RPMI media at concentrations including 100 pg/mL, 500 pg/mL, 1000 pg/mL and 2000 pg/mL. TNFα was added to RPMI media at concentrations including 100 pg/mL, 500 pg/mL and 1000 pg/mL. Monocytes were also exposed to apoptotic neutrophils, at a ratio of 1:1 (apoptotic neutrophils : monocytes), 2:1 and 4:1. The methods for inducing neutrophil apoptosis are outlined below, in section 2.9.1. The monocytes were incubated at 37^{O}C, for either 2 or 6 hours. The supernatant was extracted and stored at -70^{O}C. Any supernatant with neutrophils was first centrifuged at 670 xg for 3 minutes to pellet the neutrophils. RNA extraction was performed on the adherent cells according to protocol, outlined in section 2.4.

Extracted supernatants were measured for presence of sMER by ELISA the assay protocol described previously (section 2.7).

Reverse transcription of RNA was performed (section 2.6.1), and resulting cDNA was tested by real-time qPCR by previously outlined protocols (section 2.6.2) for gene expression levels of β2M, CD36, MER and CR3.

2.9.1 Inducing Neutrophil Apoptosis

Neutrophils were separated according to the previously outlined protocols (section 2.2). Neutrophils were resuspended in RPMI-1640 at 1 x 10^6 cells and apoptosis was induced by adding 10 µg/mL of cyclohexamide, before incubating the cells at 37°C for 2 hours. An aliquot of neutrophils was left unexposed to cyclohexamide so that stimulated and unstimulated neutrophils could be stained with propidium Iodide (PI) and annexin V, to measure the amount of necrosis or apoptosis undergone. This was performed and analysed by flow cytometry. First, the cell profiles were examined for forward and side scatter to confirm the purity of the cell population (Figure 2-14).

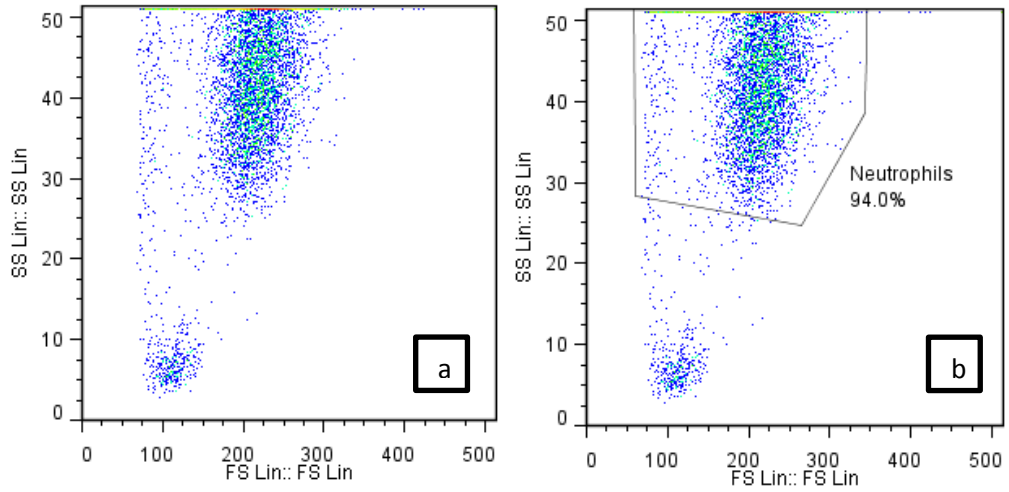


Figure 2-14 Flow cytometry graphs of a population of neutrophils

The above scatter plots are obtained from flow cytometry of a population of neutrophils, presenting forward scatter (*x*-axis) against side scatter (*y*-axis). (a) shows the neutrophil population forward and side scatter profiles, whilst (b) shows a gated neutrophil population.

PI staining is measured by FL-3 and indicates the number of necrotic cells present, whilst annexin V is measured by FL-1 and gives indication as to the number of apoptotic cells.

Figure 2-15 is an example of cells stained with both; and the proportion of apoptotic cells is indicated by the Q3 area which encompasses cells which stained positive for annexin V and negative for PI. To ascertain the percentage of apoptotic cells present, the percentage of annexin V-positive neutrophils at the 0 hour time-point (control) was deducted from the percentage of annexin V-positive neutrophils at the 2 hour time-point, giving a net level of apoptosis. So, in Figure 2-15 for example, net apoptosis would be calculated as 62.8% of cells.

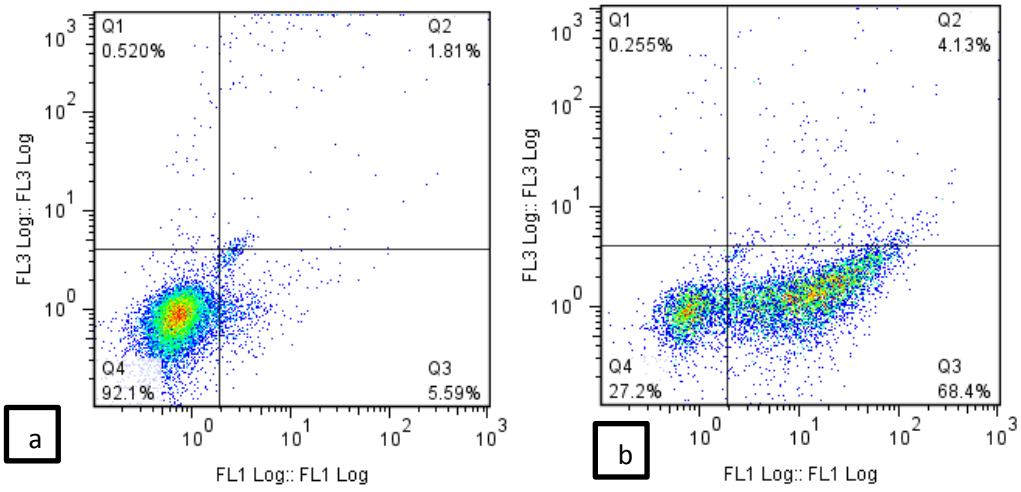


Figure 2-15 Flow Cytometry assessing cells for the percentage of apoptosis in a neutrophil population

The above scatter plots show the annexin V positivity (FL-1, *x*-axis) and PI positivity (FL-3, *y*-axis) in a population of neutrophils induced for apoptosis with cyclohexamide (10 µg/mL): (a) shows the measured fluorescence at the 0 hour time-point (i.e. non-induced); and (b) the 2 hour time-point (apoptosis-induced).

2.10 Statistical Analyses

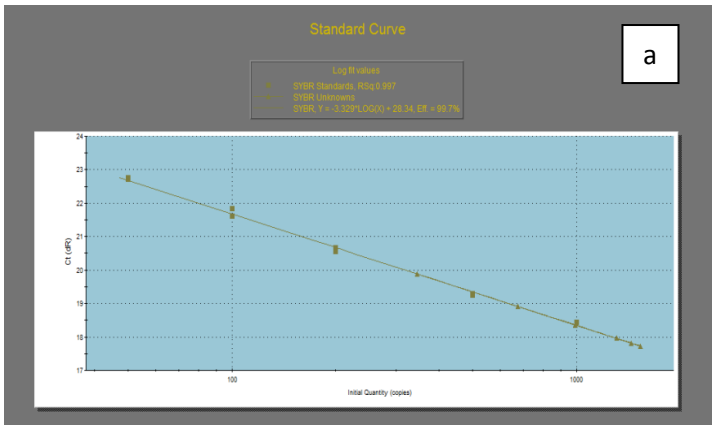
Statistical analyses were performed using IBM SPSS v19.0.0, and all graphical displays produced using either Microsoft Excel 2010 or GraphPad Prism v5. All datasets were assessed for the distribution of data; all were non-parametric. The non-parametric data have thus been displayed in text and graphically as (median [interquartile range]). A 2-tailed Mann-Whitney U test was utilised for analysing statistical significance between groups, with $p < 0.05$ taken to indicate statistical significance. Correlations were evaluated using the Spearman's rank correlation test.

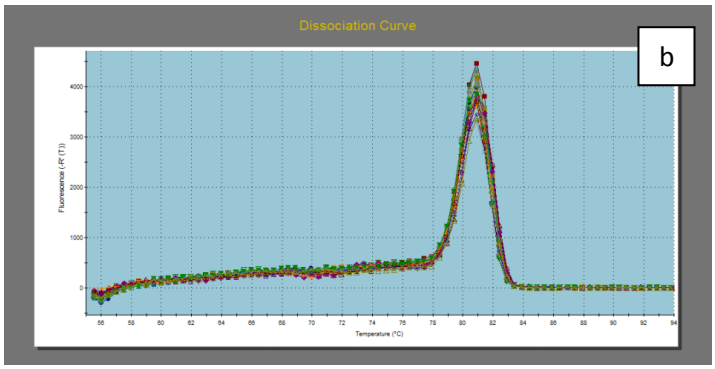
3 Results

3.1 Real-time qPCR of monocytes and macrophages

3.1.1 Validation and Optimisation

First, methods were validated and the experiments optimised using adult HC monocyte and macrophage cDNA. They were considered validated and optimised providing triplicate results showed consistency, the standard curve demonstrated good (80 – 120%) efficiency, and the dissociation curve showed a single peak of fluorescence. β2M was selected as the reference gene after comparison with GAPDH: both appeared to be stably expressed, but GAPDH expression has been reported as variable within cell populations (138) and depending on experimental settings (137). Standard and dissociation curves from initial validation and optimisation experiments are shown in Figure 3-1a, b, c and d.





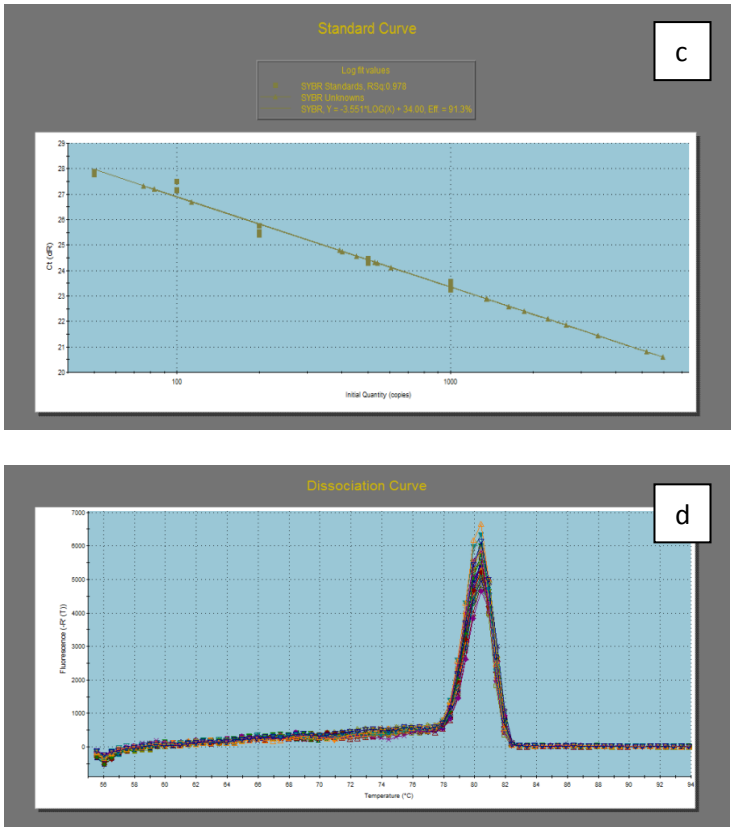


Figure 3-1 Example standard curves and dissociation (melting) curves from initial validation experiments measuring β2M expression (a & b) and CD36 expression (c & d)

The data gathered from these initial experiments are shown in Table 3-1.

	B2M Quantity (copies)	MERKT Quantity (copies)	Relative Expression Ratio	CD36 Quantity (copies)	Relative Expression Ratio	CR3 Quantity (copies)	Relative Expression Ratio
Mϕ 1	1311	970.4	0.74	422.1	0.32	1738	1.33
Mϕ 2	1444	2898	2.01	582.7	0.40	1424	0.99
Mϕ 3	1538	1225	0.80	1606	1.04	1592	1.04
THP-1 Mo	346	17.29	0.05	88.72	0.26	255	0.74
Mϕ 5	992	923.1	0.93	2046	2.06	3675	3.71
Mϕ 6	677	1284	1.90	4979	7.36	4162	6.15

Table 3-1 Data from initial qPCR of mRNA from adult healthy control macrophages (Mϕ) and THP-1 cell line monocytes

The primers were then utilised in a series of experiments testing the gene expression of a number of receptors associated with apoptotic cell clearance in monocytes and macrophages from JSLE and control donors.

3.1.2 mRNA expression of phagocytic markers on JSLE and control monocytes

Real-time qPCR was performed to analyse the gene expression of a number of receptors. These were CD36, MER and CR3 and they were selected on the basis of their associations with the process of apoptotic cell clearance (section 0). Given the hypothesised relationship between failing apoptotic cell clearance and SLE pathogenesis, the gene expression of these receptors was investigated to attempt to identify and analyse differences between the monocytes of a HC and a JSLE patient (see

Objectives). Figure 3-2 presents the data of the mRNA expression of these receptors in monocytes from JSLE patients and controls.

$CD14^+$ cells from paediatric HC and JSLE patients were separated from PBMCs. RNA extraction and reverse transcription of these $CD14^+$ cells was performed and resulting cDNA was tested for expression of three receptors associated with apoptotic cell clearance (CD36, CR3, MER), compared against that of a reference gene (β2M) for relative expression ratios. JSLE monocyte expression (n=6) was compared against HC monocyte expression (n=6).

CD36 expression was found to be significantly higher in JSLE monocytes (0.96 [0.68-1.34]) compared with HC monocytes (0.53 [0.39-0.70]; p=0.025) (Figure 3-2a). There was no difference in expression of MER in JSLE monocytes (0.47 [0.30-0.74]) compared with HC monocytes (0.30 [0.19-0.44]; p=0.200) (Figure 3-2b). In CR3 expression too, no difference was found in JSLE monocytes (0.65 [0.49-0.96]) compared with HC monocytes (0.77 [0.71-1.32]; p=0.150) (Figure 3-2c).

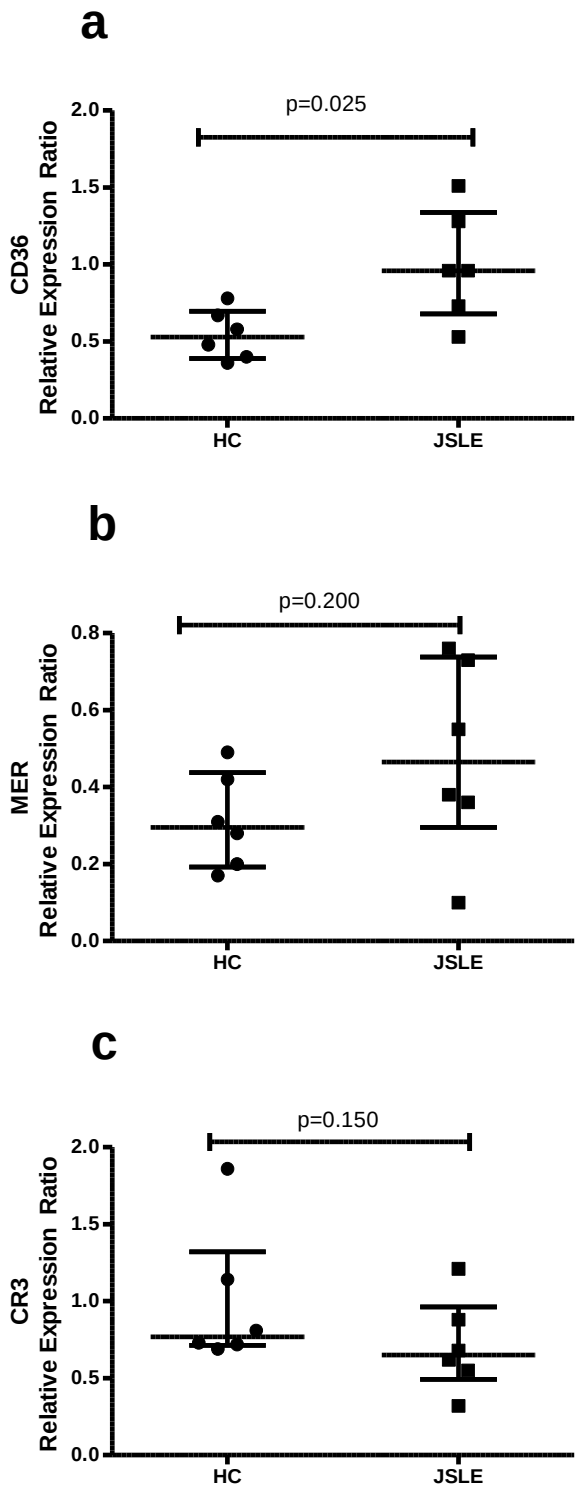


Figure 3-2 mRNA expression of phagocytic markers on JSLE and control monocytes

Real-time qPCR was performed with primers for β2M (reference gene) and: (a) CD36; (b) MER; (c) CR3. These were tested in HC (n=6) and JSLE (n=6) monocytes. Data are shown as median (interquartile range), and p-values were determined by the Mann-Whitney U test.

3.1.3 mRNA expression of phagocytic markers on JSLE and control macrophages

Monocyte derived-macrophages were also analysed using the same primers to investigate gene expression. As a much more capable phagocyte than the monocyte, this would demonstrate whether expression trends were consistent in monocytes and macrophages or whether there were discrepancies. Figure 3-3 shows the data measured from macrophage mRNA of HC and JSLE patients.JSLE macrophages expression (n=3) was compared against HC macrophages expression (n=9).

There was no difference in expression of CD36 by JSLE macrophages (1.02 [0.64-2.18]) compared with HC (0.58 [0.48-0.99]; p=0.166) (see Figure 3-3a). There was also no difference in expression of MER by JSLE macrophages (0.85 [0.74-0.86]) compared with HC macrophages (1.14 [0.78-1.73]; p=0.166) (see Figure 3-3b), and the same was true of CR3 expression, with statistically no difference in JSLE (0.49 [0.26-0.62]) compared with those of HC (1.40 [0.35-2.23]; p=0.229) (see Figure 3-3c).

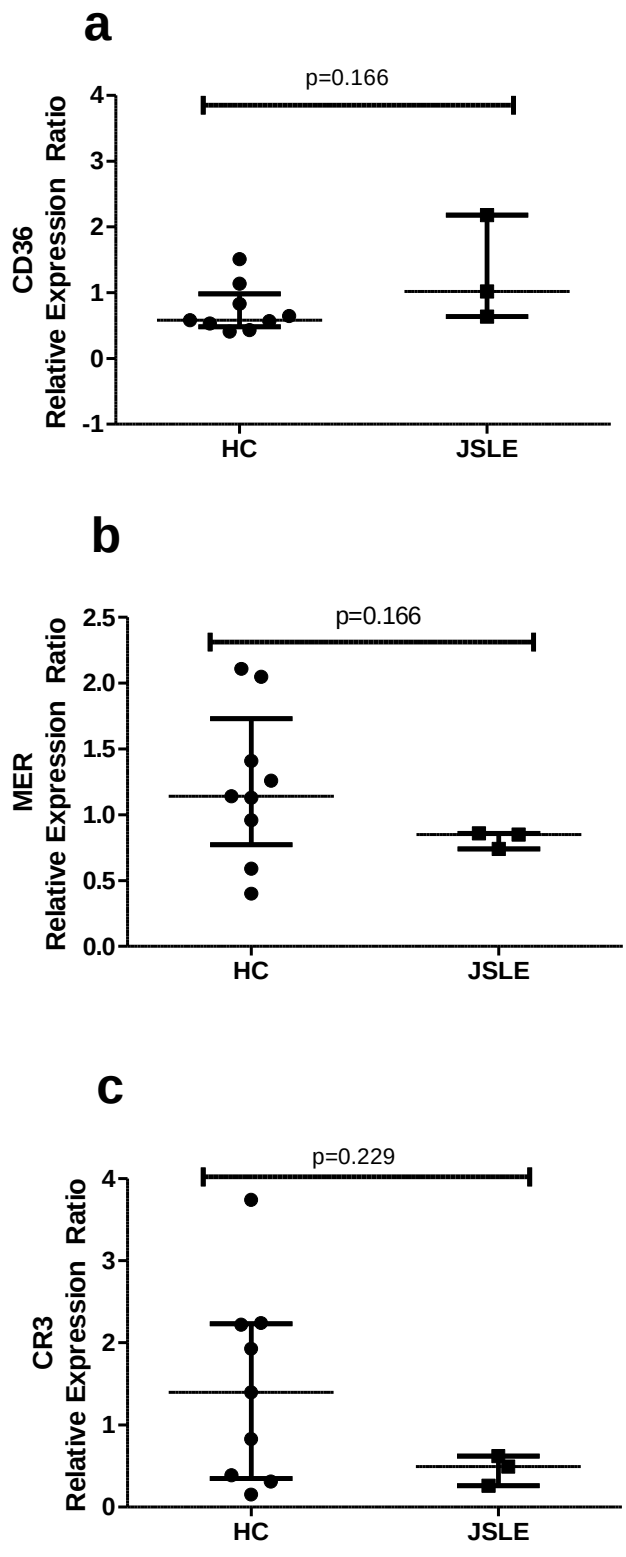


Figure 3-3 mRNA expression of phagocytic markers on JSLE and control macrophages

Real-time qPCR was performed with primers for β2M (reference gene) and: (a) CD36; (b) MER; (c) CR3. These were tested in HC (n=9) and JSLE (n=3) macrophages. Data are shown as median (interquartile range), and p-values were determined by the Mann-Whitney U test.

3.2 Soluble MER (sMER) ELISA

This section shows results of work to measure the amount of sMER in plasma samples of JSLE patients, compared with inflammatory (JIA) and non-inflammatory (HC) controls (see Objectives).

3.2.1 Spike and Recovery and Linearity of Dilution

Conducting a spike and recovery ELISA ensures that the ELISA kit functions correctly, and can be used to identify matrix interference, as the sample may contain components that affect the assay. Research conducted at our laboratory has shown evidence of matrix interference affecting a number of cytokine assays, including TNFα, IL-17, IL-21 and IL-23 (147). As such, it was important to verify the integrity of the ELISA before testing patient samples.

The study investigating sMER presence in adult-onset SLE (109) used plasma samples diluted to a concentration of 1 in 10, so for this experiment dilutions of 1 in 5, 1 in 10, 1 in 20, and 1 in 40 were used to validate the ELISA kit and methods. This was aiming to confirm that adequate recovery of MER was possible irrespective of the dilution use. The standard curve (Figure 3-4) shows good reliability, with an R^2 of 0.9993.

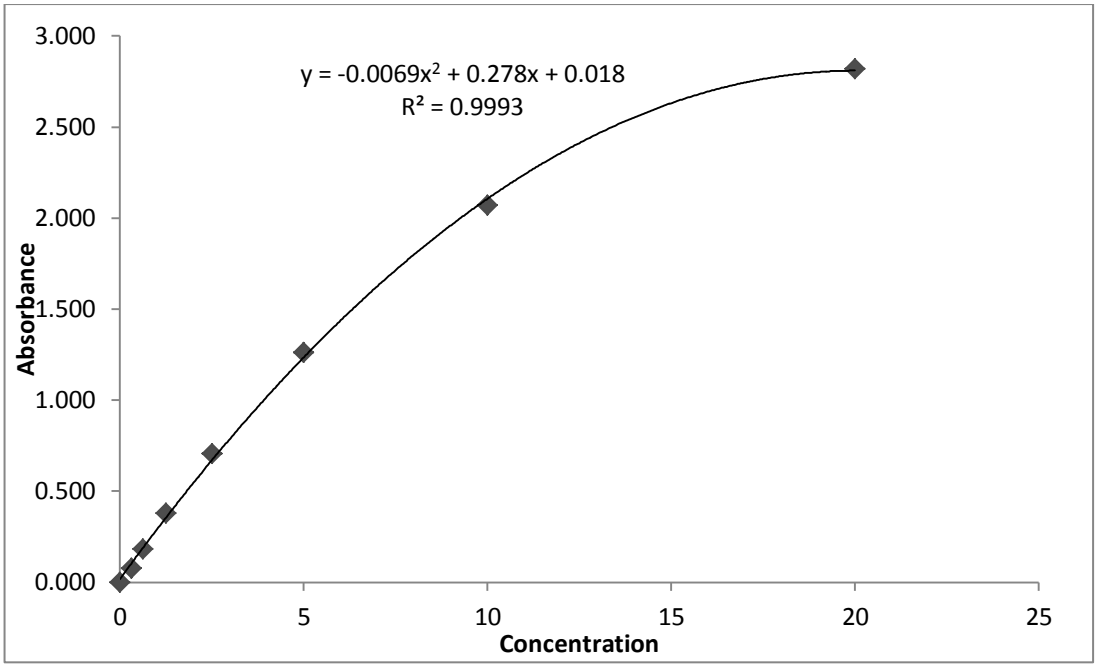


Figure 3-4 sMER Spike and Recovery ELISA Standard Curve

The graph, plotting concentration against absorbance, gives the formula used to calculate amounts of sMER present (in ng/mL), and the R^2 was 0.9993 so the results can be considered reliable.

From this, measured concentrations were ascertained and the amount present in un-spiked samples deducted from the spiked, and recovery calculated by dividing by the expected amount. The results of the spike and recovery experiment are summarised in Figure 3-5.

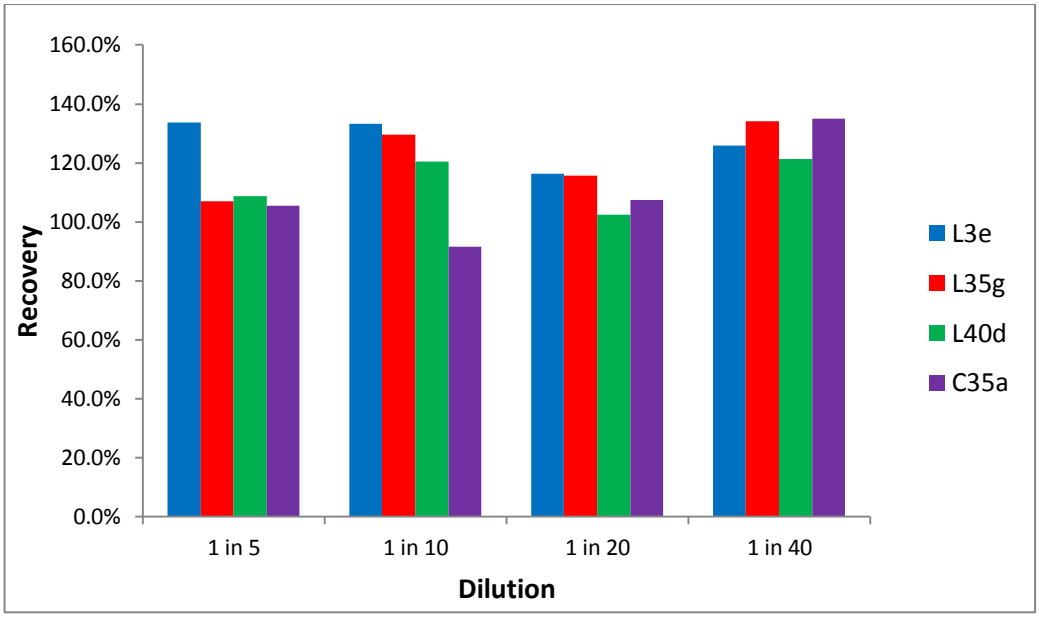


Figure 3-5 sMER Spike and Recovery ELISA results

Recovery percentages are shown (y-axis), with increasing dilutions across the x-axis of 1 in 5, 1 in 10, 1 in 20, and 1 in 40. Three JSLE plasma samples were tested (L3e, L35g, L40d) and one HC (C35a) to confirm absence of matrix interference.

Each coloured bar denotes a different patient and sample: JSLE (L) and control (C), followed by the unique numerical coding used, followed by a lower case letter indicating the clinic visit. Recovery was consistent across all dilutions, but a 1 in 5 dilution was opted for as increasing dilutions decrease the potential accuracy of the results.

3.2.2 sMER ELISA

An ELISA for the measurement of sMER concentrations was undertaken with 1 in 5 dilutions of plasma samples, to compare JSLE against inflammatory controls, Juvenile Idiopathic Arthritis (JIA) and HC.

3.2.2.1 Demographics

Patient samples were age and sex-matched with HC. Table 3-2 shows the demographic data, and the disease and therapeutic profiles of the patient cohort.

	JSLE	JIA	HC
Frequency	15	10	15
Median age [years (range)]	15 (3-18)	10.5 (8-17)	14 (2-17)
Gender, F:M (%)	9:6 (60:40)	8:2 (80:20)	9:6 (60:40)
Active Disease (%)	5 (33)	4 (40)	
Median BILAG Score (range)	2 (0-15)		
Corticosteroids			
Number of patients (%)	10 (66)	3 (30)	
Median dose, mg/day (range)	10 (1-25)	Unknown	
Antimalarials	13 (87)	0 (0)	
DMARD	11 (73)	5 (50)	
Biologics	2 (13)	8 (80)	
Others	3 (20)	3 (30)	
Median ESR (range)	7 (1-50)	3.5 (0-72)	
Median CRP (range)	4 (<4-37.3)		
C3 (range)	1.05 (0.75-1.98)		
C4 (range)	0.16 (0.09-0.42)		

Table 3-2 sMER ELISA Demographic Data

This table presents the demographics, disease and therapeutic profiles, and clinical biomarker results of the JSLE, JIA and HC groups (wherever possible). Any JSLE patients with a BILAG score of A or B for any organ system, and any JIA patients with limited movement in one or more joints at the time of sample collection were considered to have active disease. Patients taking the listed medications at the time of sample collection were included in these groups. Antimalarials –Hydroxychloroquine; DMARD (disease-modifying anti-rheumatic drug) – Mycophenolate Mofetil, Azathioprine, Methotrexate; Biologics – JSLE – Rituximab or Infliximab, JIA – unspecified; Others – JSLE – Cyclophosphamide, IVIG, JIA - Aspirin

3.2.2.2 Increased concentration of sMER in JSLE compared to JIA and HC

The results of this ELISA are shown in Figure 3-6. The concentrations of sMER were significantly greater in JSLE patients (median 15.2ng/mL, [interquartile range 11.1-21.2]) compared with HC (6.2, [5.8-8.6]; p<0.001). Comparing JSLE with JIA patients, the greater concentrations observed in JSLE patients were statistically significant (10.4, [8.2-11.7]; p=0.014). sMER concentrations were also found to be significantly greater in JIA patients compared with HC (p=0.001).

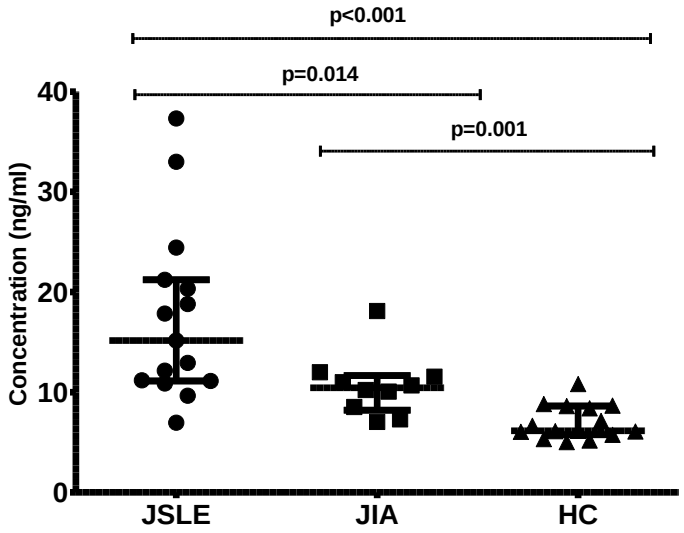


Figure 3-6 sMER ELISA Results – Vertical Scatter Graph

This graph displays the measured concentrations of sMER (ng/mL) of samples in each group, JSLE (n=15), JIA (n=10) and HC (n=15). Data are shown as median (interquartile range), and p-values were determined by the Mann-Whitney U test.

Next, correlation relationships were investigated within the test groups. The scatter plots (Figure 3-7) show the distribution of sMER concentrations against age across the three groups. There is strong inverse correlation between correlation and age in JSLE patients (r=-0.714, p=0.003) (Figure 3-7a). This is not a pattern that is replicated in JIA (r=0.345, p=0.328) (Figure 3-7b) or HC (r=0.048, p=0.864) (Figure 3-7c).

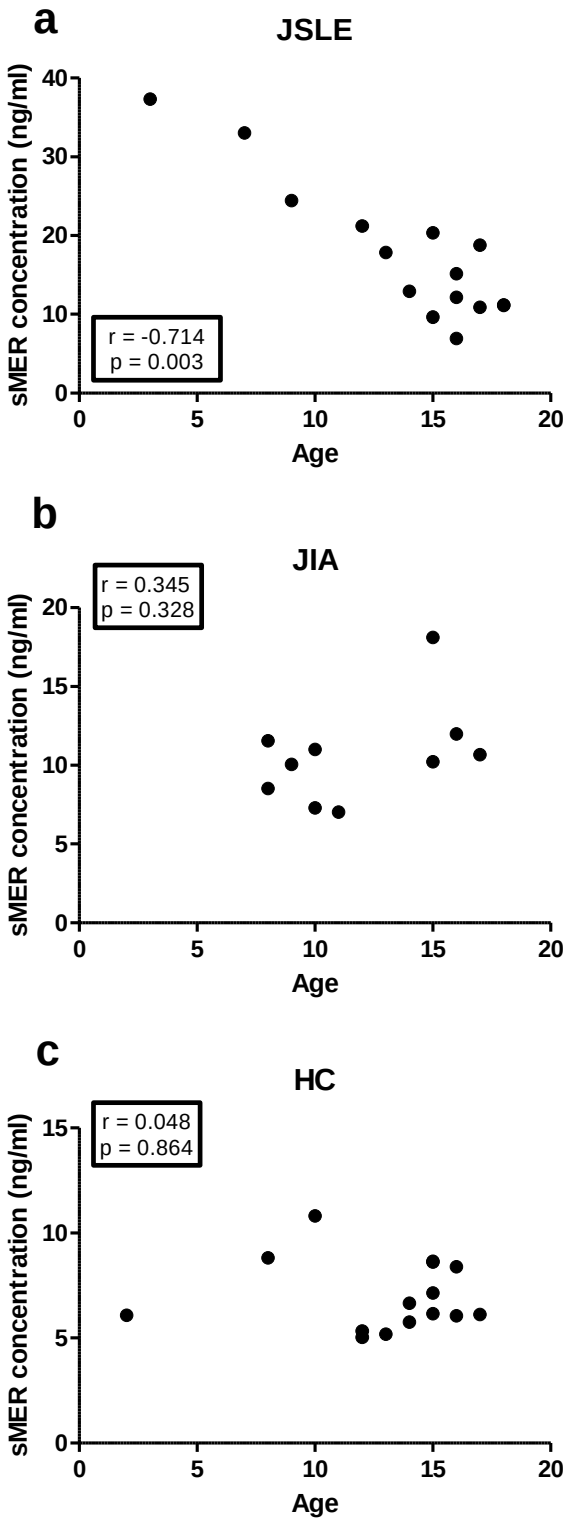


Figure 3-7 sMER-age scatter plots

The scatter plots above chart JSLE (a), JIA (b) and HC (c) sMER concentrations (ng/mL) against patient age. Correlation data (Spearman's rank correlation test) is shown for each group.

Figure 3-8 scatter plots show disease activity and levels of commonly used clinical biomarker of JSLE in the against sMER concentration in the JSLE group. The BILAG disease activity scores suggest a positive correlation with sMER concentration (r=0.472), but this was not statistically significant (p=0.075) (Figure 3-8a). There was no evidence of correlation between ESR and sMER (r=-0.375; p=0.168) (Figure 3-8b), nor between CRP and sMER (r=-0.016, p=0.954) (Figure 3-8c). There was also no evidence of correlation between complement levels and sMER concentration; C3 (r=0.132, p=0.639) (Figure 3-8d), and C4 (r=0.113, p=0.688) (Figure 3-8e).

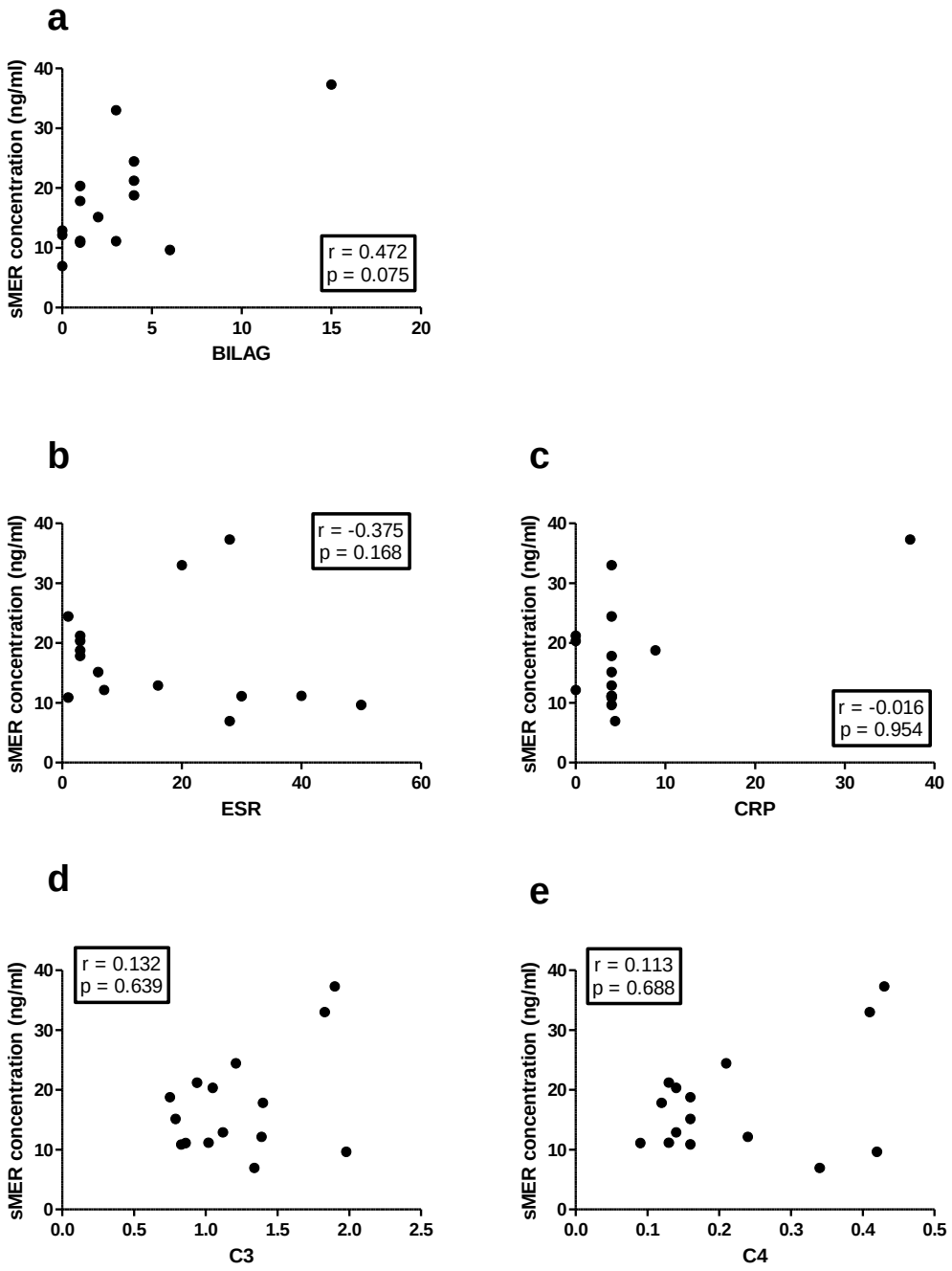


Figure 3-8 Scatter plots of BILAG, ESR, CRP, C3 and C4 against sMER concentration

The scatter plots chart the sMER concentrations of JSLE patients against the BILAG disease activity index (a) and a number of biomarkers: ESR (b), CRP (c), C3 (d) and C4 (e). The correlation data (Spearman's rank correlation test) is shown on each graph.

The JSLE group was divided by gender to investigate for any differences between males and females in sMER concentration (Figure 3-9). In the tested samples, male JSLE patients had greater concentrations of sMER (22.39 [16.28-34.08]) than female patients (12.15 [10.39-

16.50]; p=0.036). This is also reflected by the generally-higher disease activity BILAG scores found in males: (median 3.5, range 1-15), compared with females: (1, 0-6), albeit not a significant difference (p=0.187, Mann-Whitney U test).

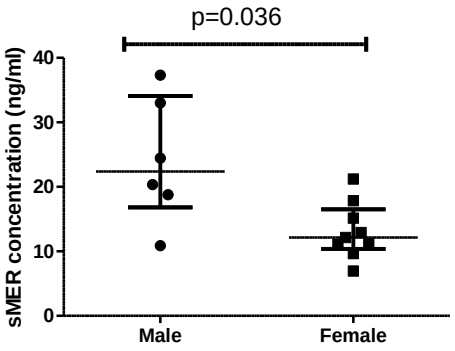


Figure 3-9 Vertical scatter of sMER concentrations in males and females with JSLE

The above graph separates JSLE patients into males (n=6) and females (n=9) to show sMER concentrations (ng/mL) in each. Data are shown as median (interquartile range), and p-values were determined by the Mann-Whitney U test.

The JSLE patients were split according to their therapeutic profiles at the time of sample collection (see Table 3-2). Figure 3-10 separates patients according to whether or not they were receiving hydroxychloroquine, and Figure 3-11 separates patients according to whether or not they were receiving corticosteroids.

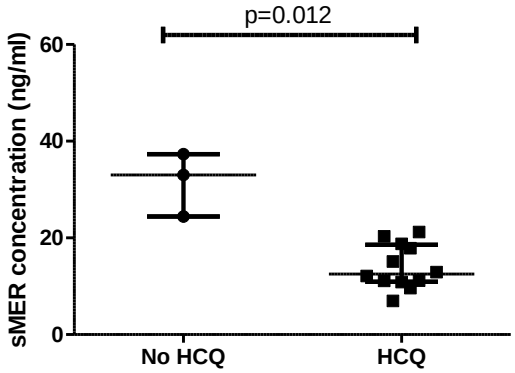


Figure 3-10 sMER concentrations of JSLE patients divided according to current hydroxychloroquine therapy

The vertical scatter separates JSLE patients according to whether or not they were treated with hydroxychloroquine at the time of sample collection: not currently taking hydroxychloroquine therapy (No HCQ, n=3), patients taking hydroxychloroquine therapy (HCQ, n=12). Data are shown as median (interquartile range), and p-values were determined by the Mann-Whitney U test.

Comparing the sMER profile of patients on medications, those taking hydroxychloroquine (12.50 [10.93-18.56]) have significantly higher levels of sMER than those not taking hydroxychloroquine (33.00 [24.44-37.31]; p=0.012).

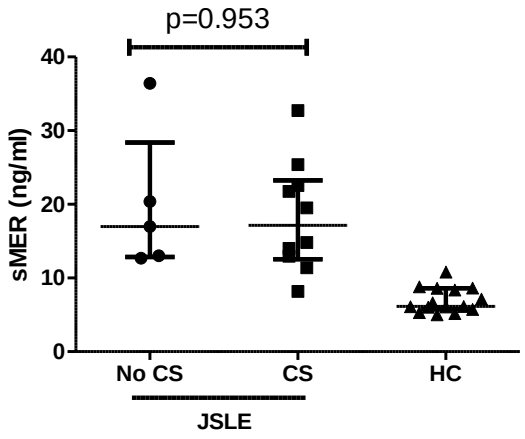


Figure 3-11 sMER concentrations of JSLE patients divided according to current corticosteroid (CS) therapy

The vertical scatter separates JSLE patients according to whether or not they were treated with corticosteroids at the time of sample collection: not currently taking corticosteroids (No CS; n=5) compared with those taking corticosteroids (CS; n=10). Data are shown as median (interquartile range), and p-values were determined by the Mann-Whitney U test.

There was no difference between the sMER concentrations of those on corticosteroid treatment (17.16 [12.56-23.25]) and those not on corticosteroid treatment (16.98 [12.86-28.39]; p=0.953).

3.3 Monocyte Phagocytosis of pHrodo E. coli

This series of experiments was intended to measure phagocytosis by adult healthy control monocytes, comparing function in plasma from JSLE and HC patients (see Objectives).

3.3.1 Validation and Optimisation

To validate and optimise methods, a number of assays were undertaken. Three time-points were used (30, 60, and 90 minutes), to ascertain the levels of phagocytosis at each in order to determine which time-point would be most appropriate, to allow sufficient phagocytosis to take place but not so long as to introduce other limiting factors for phagocytosis. Monocytes were incubated in the absence of pHrodo to establish a baseline at which pHrodo positivity should be around 0%, due to a lack of phagocytosis. Monocytes and pHrodo E. coli were either stored on ice or incubated at 37^{o}C. Monocytes stored on ice were used as negative controls in which there should be very little evidence of phagocytosis occurring. The percentage of pHrodo positive monocytes on ice was therefore deducted from the percentage of pHrodo positive monocytes incubated at 37^{o}C to give a corrected level of phagocytosis. Monocytes and pHrodo E. coli were also tested, with and without serum, to assess for any differences. It was expected that phagocytosis in serum would be greater than without, since serum contains opsonising agents such as complement. The histograms resulting from one set of conducted assays are shown in Figure 3-12, Figure 3-13 and Figure 3-14.

3.3.1.1 30 minutes

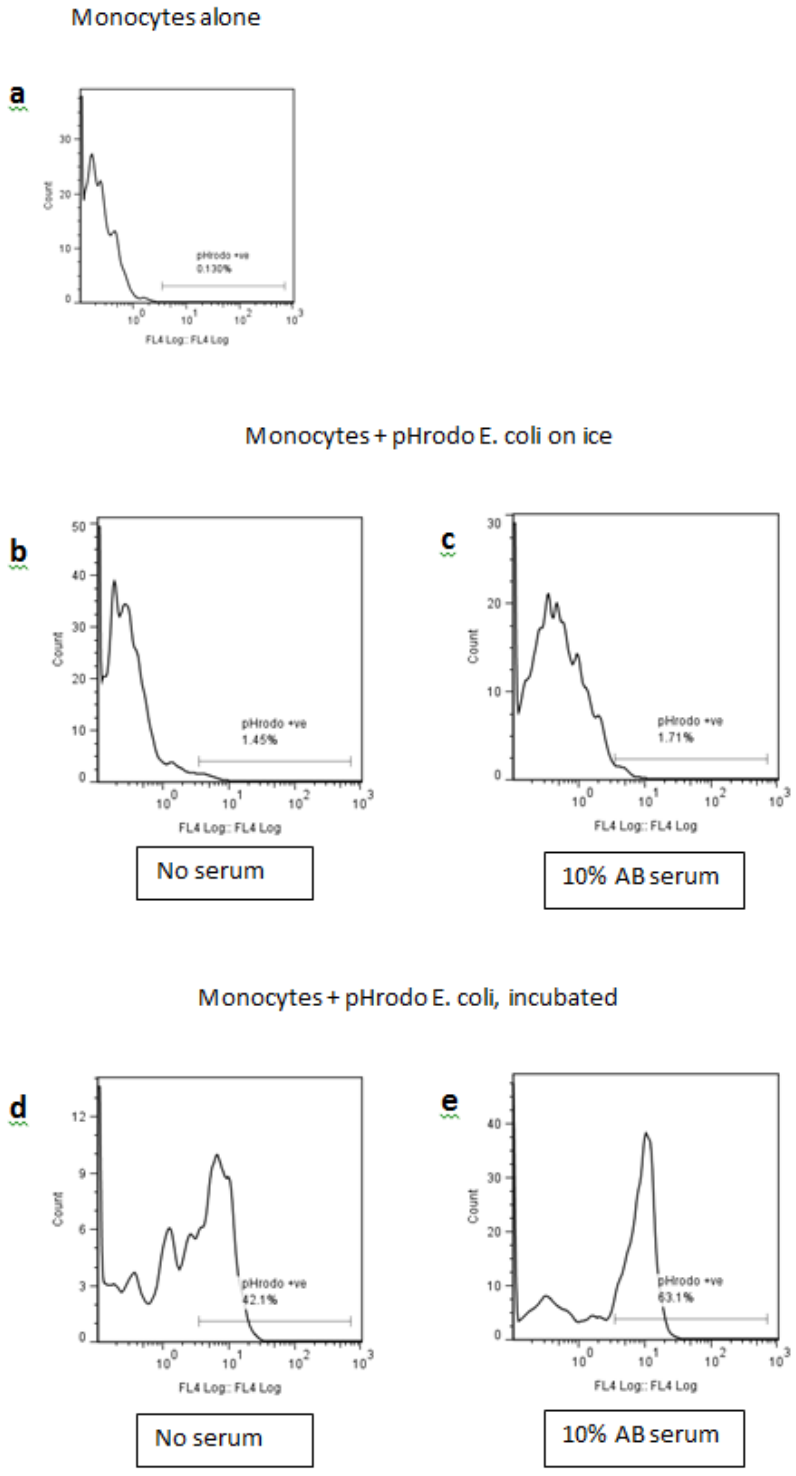


Figure 3-12 Flow Cytometry histograms of adult control monocytes incubated with pHrodo-stained E. coli for 30 minutes

These histograms show levels of pHrodo positivity detected by flow cytometry, after monocytes were incubated at set conditions for 30 minutes. (a) monocytes in the absence of serum or pHrodo E. coli; (b) monocytes incubated on ice with pHrodo E. coli without serum; (c) monocytes incubated on ice with pHrodo E. coli with 10% AB serum; (d) monocytes incubated at 37^{o}C with pHrodo E. coli without serum; (e) monocytes incubated at 37^{o}C with pHrodo E. coli with 10% AB serum.

3.3.1.2 60 minutes

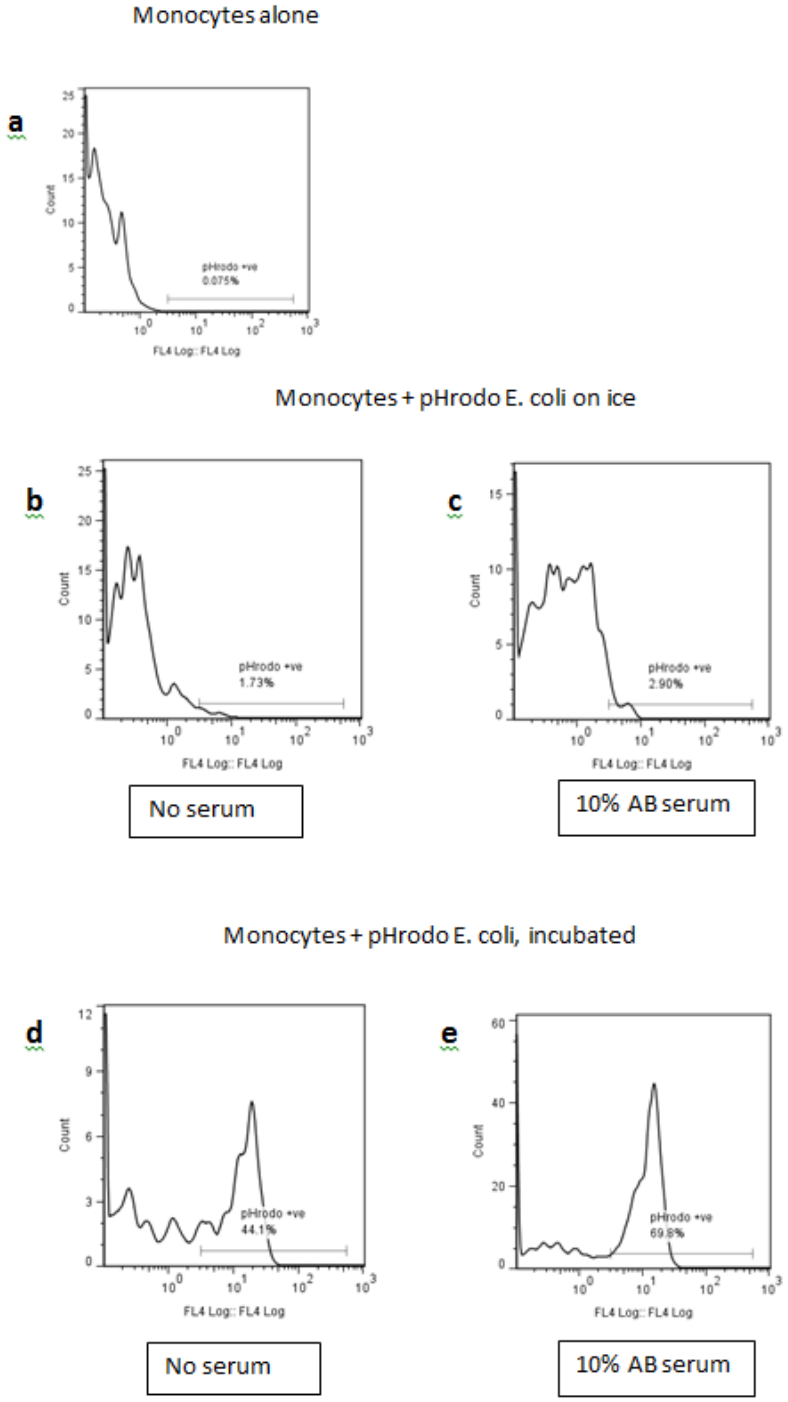


Figure 3-13 Flow Cytometry histograms of adult control monocytes incubated with pHrodo-stained E. coli for 60 minutes

These histograms show levels of pHrodo positivity detected by flow cytometry, after monocytes were incubated at set conditions for 60 minutes. (a) monocytes in the absence of serum or pHrodo E. coli; (b) monocytes incubated on ice with pHrodo E. coli without serum; (c) monocytes incubated on ice with pHrodo E. coli with 10% AB serum; (d) monocytes incubated at 37^{o}C with pHrodo E. coli without serum; (e) monocytes incubated at 37^{o}C with pHrodo E. coli with 10% AB serum.

3.3.1.3 90 Minutes

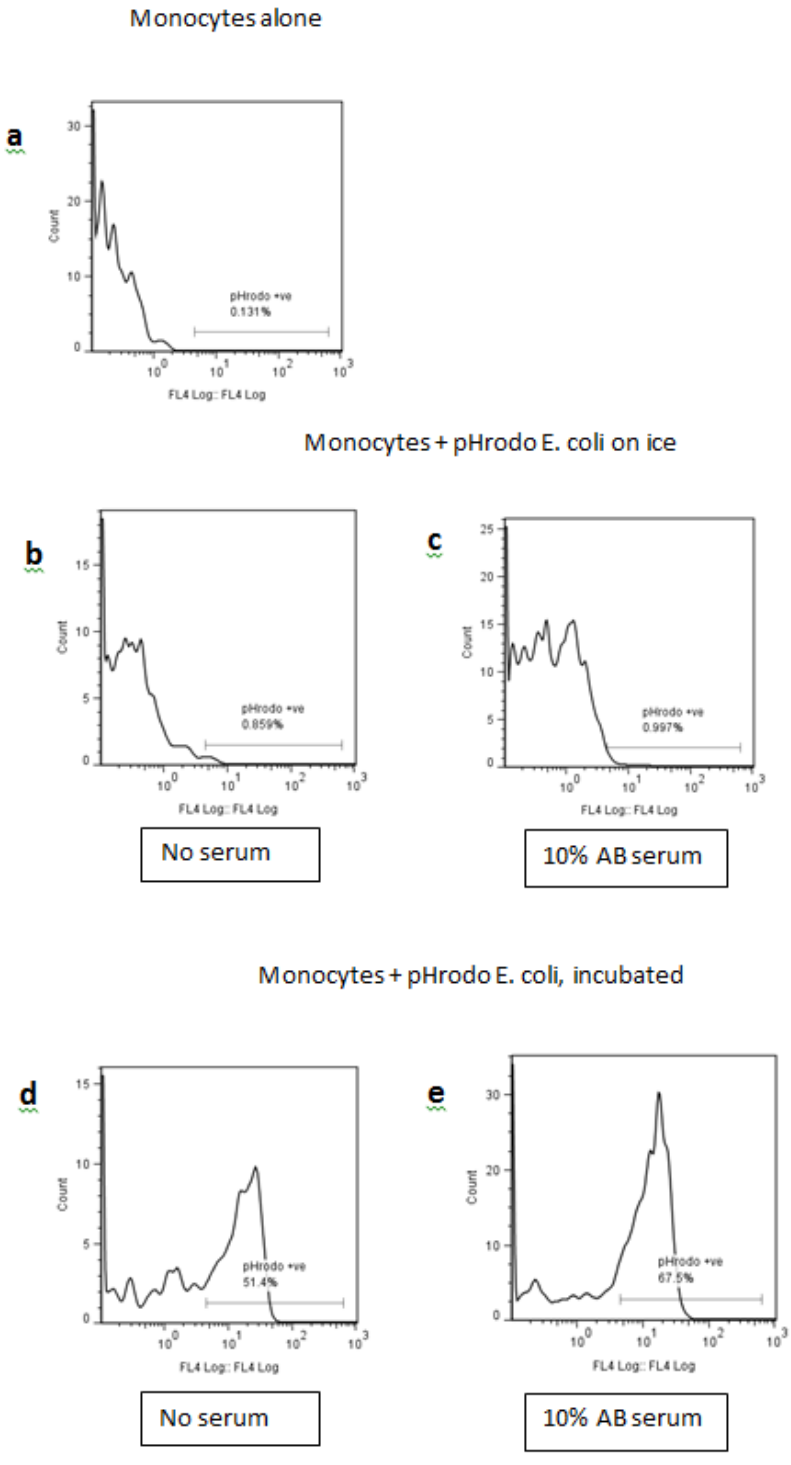


Figure 3-14 Flow Cytometry histograms of adult control monocytes incubated with pHrodo-stained E. coli for 90 minutes

These histograms show levels of pHrodo positivity detected by flow cytometry, after monocytes were incubated at set conditions for 90 minutes. (a) monocytes in the absence of serum or pHrodo E. coli; (b) monocytes incubated on ice with pHrodo E. coli without serum; (c) monocytes incubated on ice with pHrodo E. coli with 10% AB serum; (d) monocytes incubated at 37^{o}C with pHrodo E. coli without serum; (e) monocytes incubated at 37^{o}C with pHrodo E. coli with 10% AB serum.

The data from these histograms (Figure 3-12, Figure 3-13 and Figure 3-14) have been tabulated, and the differences calculated (Table 3-3). The results indicate that 30 minutes is a sufficient time-point to allow for phagocytosis of E. coli to occur. There is a slightly increased proportion of phagocytosis occurring at 60 minutes compared with 30 minutes, but there is no discernable increase between 60 minutes and 90 minutes. Certainly there is no increase in phagocytosis at 90 minutes compared with 60 minutes. The increase between 30 and 60 minutes is also quite small, suggesting that the majority of unrestricted phagocytosis occurs within the first 30 minutes in these experimental conditions. For these reasons, 30 minutes was selected as the time-point for experiments investigating monocyte phagocytosis of pHrodo E. coli.

a
No Serum

	% Phagocytosis		
	Ice	Incubated	Difference
30 mins	1.5	42.1	40.6
60 mins	1.7	44.1	42.4
90 mins	0.9	51.4	50.5

b
10% AB Serum

	% Phagocytosis		
	Ice	Incubated	Difference
30 mins	1.7	63.1	61.4
60 mins	2.9	69.8	66.9
90 mins	1.0	67.5	66.5

Table 3-3 Method validation and optimisation - percentage of phagocytosis across three time points

Tables presenting phagocytosis data with samples (n=3) placed on ice, incubated, and the difference between the two in no serum (a) and 10% AB serum (b) environments.

The importance of environment was also tested in these preliminary experiments. The results in Table 3-4 indicate that an environment of serum was more conducive to the occurrence of phagocytosis than in its absence.

	No serum	10% AB serum
30 mins	40.6	61.4
60 mins	42.4	66.9
90 mins	50.5	66.5

Table 3-4 Phagocytosis in a no serum environment compared with 10% AB serum

3.3.2 Monocyte phagocytosis of pHrodo E. coli in JSLE and HC serum and plasma

Figure 3-15 presents the results of comparing the performance of phagocytosis by adult HC monocytes in serum versus plasma. The four different sample conditions were tested in a repeated number of experiments (n=5). Phagocytosis of pHrodo E. coli in adult HC monocytes in HC serum (39.00 [29.20-52.40]) was comparable with those in HC plasma (43.30 [36.85-47.40]), with no significant difference (p=0.841). In JSLE environments, again, the results found showed similar phagocytic performance serum (23.20 [19.00-37.95]), as in plasma (27.10 [16.55-38.15]), with no statistically significant difference (p=1.000).

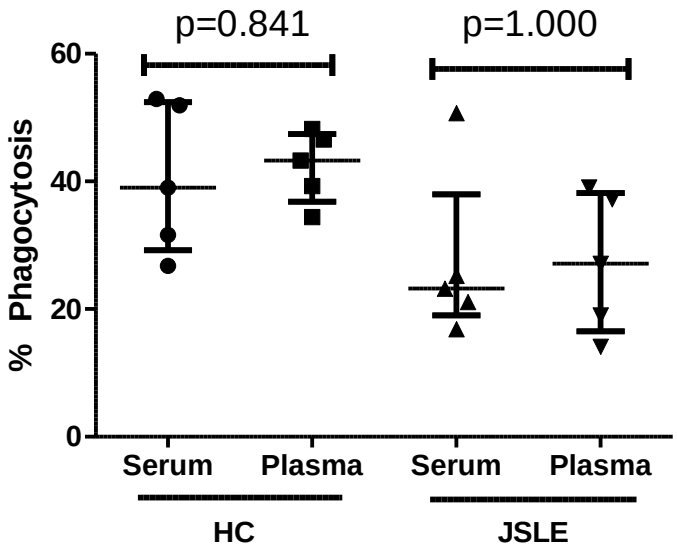


Figure 3-15 Monocyte phagocytosis of pHrodo E. coli, comparing performance in serum versus plasma

The vertical scatters above compare the % of monocytes that have phagcytosed pHrodo-stained E. coli within the 30 minute incubation period, in the HC group (n=5) and the JSLE group (n=5). Data are shown as median (interquartile range), and p-values were determined by the Mann-Whitney U test.

With the absence of a discernable difference between an environment of serum or plasma, experiments were thereafter continued in plasma. We compared the performance of adult HC monocytes in HC plasma (n=6) with JSLE plasma (n=5). Figure 3-16 is a vertical scatter illustrating the results found which suggest occurrence trend towards increased phagocytosis in HC plasma (44.95 [32.73-48.88]) compared with JSLE plasma (32.60 [22.85-38.15]). However, this difference did not reach statistical significance (p=0.126).

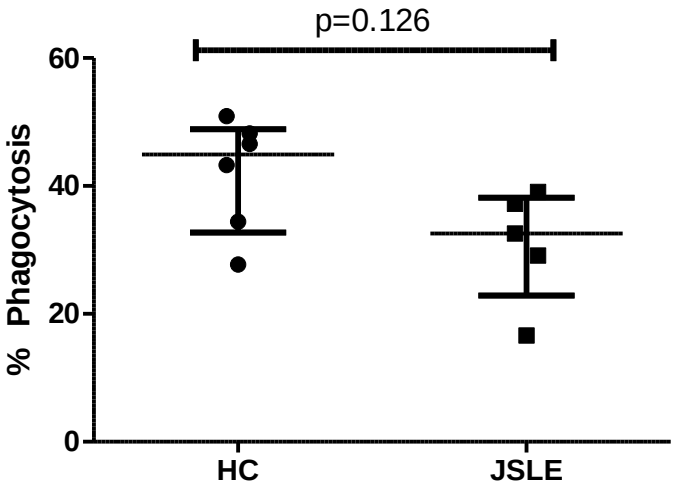


Figure 3-16 Monocyte phagocytosis of pHrodo E. coli comparing performance in plasma from HC with plasma from JSLE patients

The vertical scatters show the % monocytes that phagocytosed pHrodo-stained E. coli within the 30 minute incubation period, if exposed to HC (n=6) or JSLE (n=5) plasma. Data are shown as median (interquartile range), and p-values were determined by the Mann-Whitney U test.

3.4 Monocyte Stimulation

The results outlined here refer to experiments conducted to measure sMER concentration in supernatant, and to measure adult healthy control monocyte mRNA expression of CD36, MER and CR3 expression following exposure to LPS, IFNα, TNFα and apoptotic neutrophils (see

Objectives).

3.4.1 sMER ELISA

This sMER ELISA was performed with supernatant diluted in reagent diluent, to a working concentration of 1 in 5, as the amount of sMER that might be present was unknown. The standard curve is presented in Figure 3-17, and results calculated from this are displayed in Table 3-5.

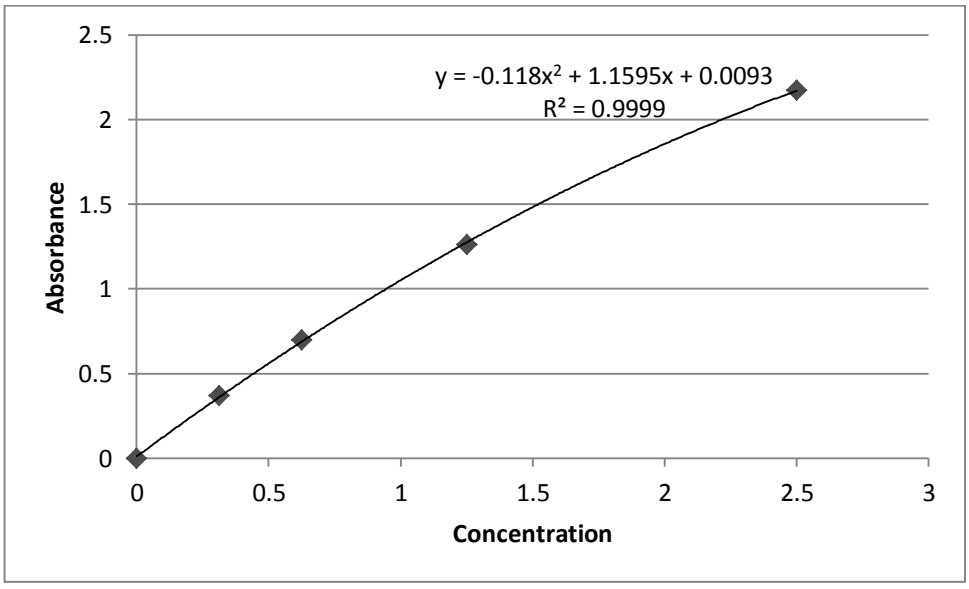


Figure 3-17 Monocyte Stimulation sMER ELISA Standard Curve

The standard curve graph charts the measured absorbance against the known concentrations of standard.

The results in Table 3-5 show that there were no measurable levels of sMER present in the samples.

	2 hour time-point supernatant sMER concentration (ng/mL)	6 hour time-point supernatant sMER concentration (ng/mL)
Monocytes alone	0.00	0.00
LPS 50 ng/mL	0.00	0.00
IFNα 100 pg/mL	0.00	0.00
IFNα 500 pg/mL	0.00	0.00
IFNα 1000 pg/mL	0.00	0.00
IFNα 2000 pg/mL	-	0.00
TNFα 100 pg/mL	-	0.00
TNFα 500 pg/mL	-	0.00
TNFα 1000 pg/mL	-	0.00
Apoptotic neutrophils (AN) 1:1	0.00	0.00
Apoptotic neutrophils (AN) 2:1	0.00	0.00
Apoptotic neutrophils (AN) 4:1	0.00	0.00

Table 3-5 sMER concentrations of supernatants following exposure of monocytes to test environments

Two separate experiments were undertaken with two separate adult HC donors' monocytes. The concentrations tested are presented in the table; apoptotic neutrophils (AN) ratios refer to the concentration of AN:monocytes. IFNα, interferon alpha; LPS, lipopolysaccharide; TNFα, tumour necrosis factor alpha.

The apoptotic profiles of neutrophils were as follows:

Net apoptosis at 2 hour time-point – 25% of neutrophils

Net apoptosis at 6 hour time-point – 63% of neutrophils

As a result, the experiment was repeated with supernatants diluted to a concentration of 1 in 2: the highest concentration possible with the volume of supernatant available. Once again, sMER was not present at detectable levels.

3.4.2 mRNA expression of phagocytic markers on JSLE and control monocytes following exposure to different environments

3.4.2.1 MER Expression

The results following qPCR for MER expression are shown in Figure 3-18. In response to the environments tested, expression of MER appeared to remain mostly stable at the two time-points. At the 2 hour time-point, the trend was for a down-regulation of expression observed in all except for monocytes exposed to IFNα at the concentration 1000 pg/mL. However, the down-regulation was generally small, except with LPS, IFNα (500 pg/mL) and apoptotic neutrophils (AN) 4:1. This down-regulation was again observed with LPS and AN 4:1 at the 6 hour time-point, albeit with only a small level of down-regulation. TNFα exposure appeared to up-regulate MER expression by monocytes across the three tested concentrations. This suggests that the presence TNFα may increase the expression of the MER tyrosine kinase receptor and potentially increase the capacity for apoptotic cell clearance. Other factors appear to down-regulate MER expression and may, therefore, negatively impact upon apoptotic cell clearance.

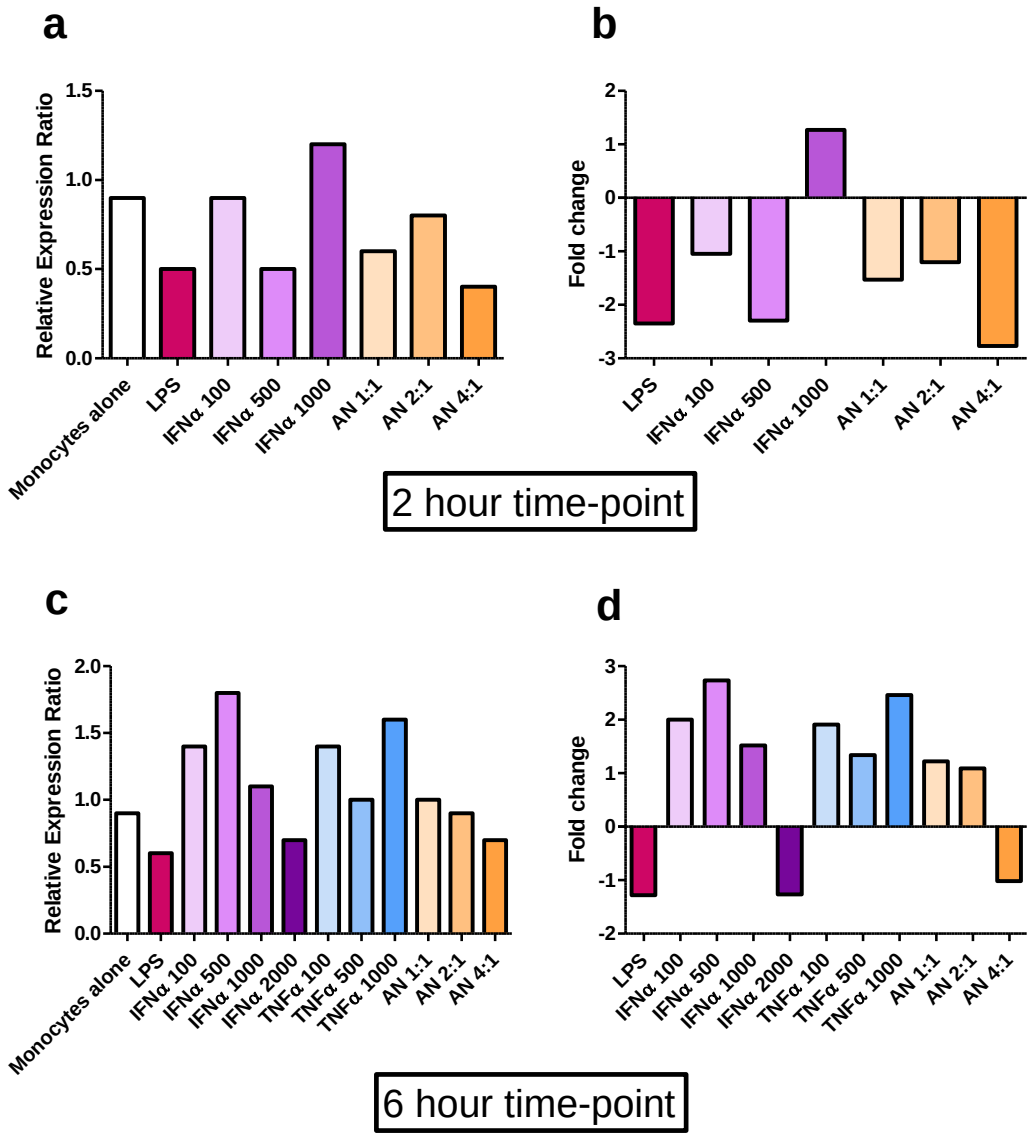


Figure 3-18 mRNA expression of MER in adult control monocytes in response to different environments

Bar charts present relative expression ratios and fold change data for the test conditions. (a) relative expression ratios of the gene expression of monocytes at the 2 hour time-point; (b) fold changes of the gene expression of monocytes at the 2 hour time-point; (c) relative expression ratios of the gene expression of monocytes at the 6 hour time-point; (d) fold changes of the gene expression of monocytes at the 6 hour time-point. Apoptotic neutrophils (AN) ratios refer to the concentration of AN:monocytes. IFNα, interferon alpha; LPS, lipopolysaccharide; TNFα, tumour necrosis factor alpha.

3.4.2.2 CD36 Expression

The results following qPCR for CD36 expression are shown in Figure 3-19. CD36 expression also appeared variable in the different test conditions. There was a negligible change in the majority of conditions, though some up-regulation observed in IFNα (500 pg/mL) at the two hour time point. The tendency was towards down-regulation of CD36 at the 6 hour time point, however, with IFNα seemingly triggering a dose-respondent trend of increasing down-regulation. This may indicate that chronic exposure to IFNα can act to reduce CD36 expression and thereby impair the clearance of apoptotic cells.

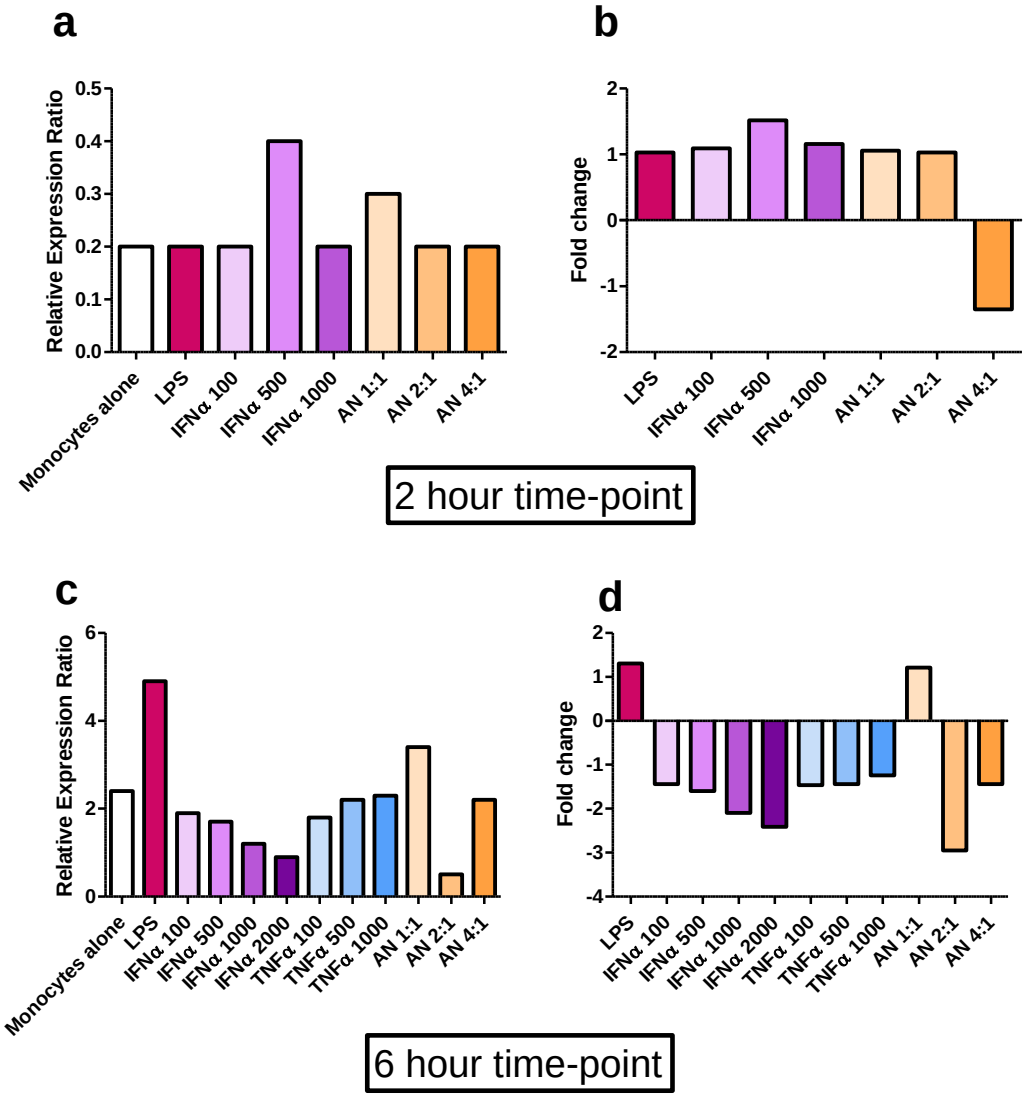


Figure 3-19 mRNA expression of CD36 in adult control monocytes in response to different environments

Bar charts present relative expression ratios and fold change data for the test conditions. (a) relative expression ratios of the gene expression of monocytes at the 2 hour time-point; (b) fold changes of the gene expression of monocytes at the 2 hour time-point; (c) relative expression ratios of the gene expression of monocytes at the 6 hour time-point; (d) fold changes of the gene expression of monocytes at the 6 hour time-point. Apoptotic neutrophils (AN) ratios refer to the concentration of AN:monocytes. IFNα, interferon alpha; LPS, lipopolysaccharide; TNFα, tumour necrosis factor alpha.

3.4.2.3 CR3 Expression

The results following qPCR for CR3 expression are shown in
Figure 3-20. CR3 expression by monocytes in response to the test conditions was generally consistent across the test conditions but varied between the two time points. At the 2 hour time-point, monocytes tended to up-regulate CR3 expression. This was particularly evidentwith exposure to apoptotic neutrophils (1:1), although at higher concentrations this up-regulation decreased. At the 6 hour time-point, expression of CR3 tended to be down-regulated, and with monocytes exposed to IFNα, fold change decreased, as observed with CD36, in a dose-dependent manner. These trends, as with those of CD36, may suggest that chronic exposure to IFNα can reduce CR3 expression and may impair phagocytic function as a result.

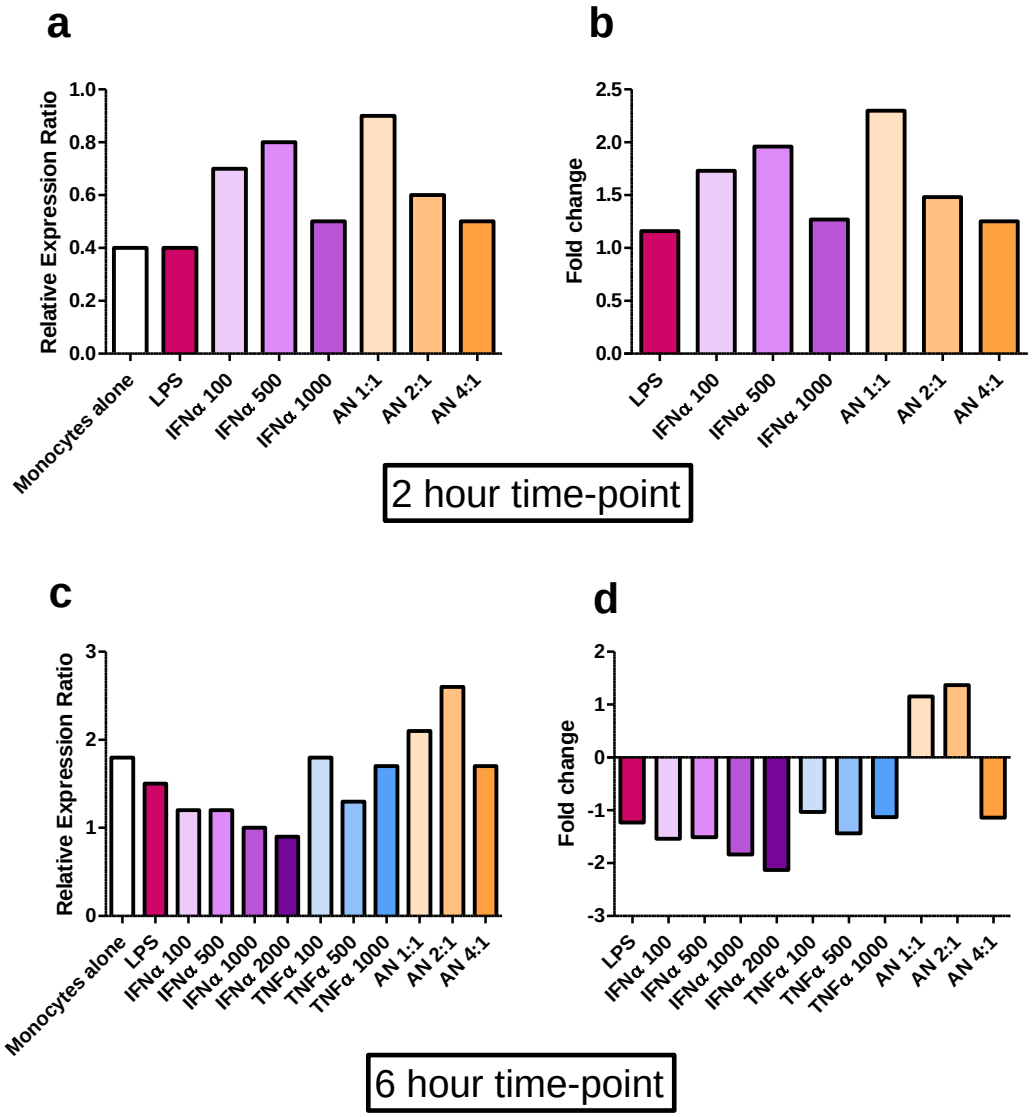


Figure 3-20 mRNA expression of CR3 in adult control monocytes in response to different environments

Bar charts present relative expression ratios and fold change data for the test conditions. (a) relative expression ratios of the gene expression of monocytes at the 2 hour time-point; (b) fold changes of the gene expression of monocytes at the 2 hour time-point; (c) relative expression ratios of the gene expression of monocytes at the 6 hour time-point; (d) fold changes of the gene expression of monocytes at the 6 hour time-point. Apoptotic neutrophils (AN) ratios refer to the concentration of AN:monocytes. IFNα, interferon alpha; LPS, lipopolysaccharide; TNFα, tumour necrosis factor alpha.

3.5 Summary of Results

- Increased mRNA levels of CD36 were found in JSLE monocytes ($p=0.025$) but not in macrophages. There was no statistical difference between expression levels of MER or CR3 in JSLE monocytes or macrophages compared with HC.
- The measured concentrations of sMER in plasma samples were found to be significantly greater in JSLE patients compared with HC ($p<0.001$) and JIA patients ($p=0.014$).
- There was a trend towards decreased phagocytosis of E. coli, when adult HC monocytes were incubated in JSLE serum and plasma compared with controls, but this difference was not statistically significant.
- No MER cleavage was detected following the exposure of adult HC monocytes to a number of test conditions; LPS, IFNα, TNFα and apoptotic neutrophils. The real-time qPCR results for CD36, MER and CR3 expression in response to these conditions were equivocal.

4 Discussion

4.1 Interpretation of research findings

4.1.1 Real-time qPCR of monocytes and macrophages

Overall, data gathered from real-time qPCR shows differential levels of gene expression of the phagocytic receptors. In monocytes, CD36 expression was increased in JSLE compared with HC (section 3.1.2), and there was no difference with MER and CR3. In macrophages, there was no difference found between JSLE and HC according to expression levels of CD36, MER or CR3 (section 3.1.3).

The results may indicate that CD36 may be upregulated in JSLE monocytes. These findings contrast with that reported for CD36 expression in adult-onset SLE: two previous studies (118, 119) found no significant differences between control and adult-onset SLE monocyte expression of CD36. The sample sizes across all groups is small, and differences may have been detected with greater numbers, though equally, greater numbers would be necessary for there to be certainty as to differences in monocyte expression of CD36.

It is unclear what the implications of the differential gene expressions of these receptors might be for JSLE pathogenesis and/or its pathology. With the characterised inherent phagocyte defects in SLE, one might expect the expression of receptors associated with apoptotic cell clearance to be decreased in JSLE than in HC. From analysing the results found in this study, one reason for the apparent upregulation of CD36 may be that the expressions found are an adaptation to the burden of apoptosis found in JSLE. The upregulation may be an unrelated characteristic of the JSLE phagocyte. Alternatively, the expression levels of these receptors may be unrelated to the actual function of the

phagocyte and the supposed dysfunction is not a result of the gene expression of these receptors. The gene expressions may also not be reflective of the actual protein levels expressed on the cell surface. The main reasons for such discrepancies as these may include the rate of protein turnover, poorly understood post-transcriptional mechanisms of protein synthesis, such that protein expression may not relate to mRNA expression, and also the potential for error and noise in laboratory experiments. Another disadvantage of qPCR data is that it reflects the cumulative total of mRNA from cells but provides no information as to the cellular profile of expression, i.e. the number of cells expression CD36/MER/CR3. This information could be obtained by flow cytometry analysis of monocytes and macrophages to investigate these receptors on a cell-by-cell basis.

4.1.2 sMER ELISA

The concentrations of sMER were measured in JSLE patients and this was of particular interest in light of published data suggesting increased levels in SLE patients compared with HC (109). The ELISA kit and methods used were first validated, which was important since a number of ELISA assays for cytokines tested within our laboratory were found to have been affected by matrix interference (147). The sMER levels were then measured, comparing JSLE patients with JIA patients, utilised as an inflammatory control, and HC. The JIA controls were particularly important to ensure that any differences found were specific to JSLE and not reflective of the inflammation common to both diseases.

The patient samples were age and sex-matched so that the study populations were demographically similar. A third of JSLE patients were defined as having active disease at the time of sample collection. The “other” category of medications also gives an indication of severity, with 20% of patients treated with cyclophosphamide or IVIG, drug treatments reserved for refractory forms of disease.

sMER concentrations were found to be significantly greater in JSLE patients than in JIA or HC (section 3.2.2.2). This echoed results previously described in adult-onset SLE that reported greater levels in SLE compared with controls (109). The differences observed between the JSLE and JIA concentrations suggest that this amount of sMER may be unique to JSLE pathology and not reflected by the general inflammation common to both diseases. These greater concentrations of sMER measured are of particular interest because of the potential implications they may have for JSLE.

To relate this to the qPCR data reported, presence of sMER may reflect losses incurred by phagocytes in JSLE and therefore reduce the amount of MER on the cell surface. More interesting still is the notion that sMER actually goes on to act as a competitive antagonist for ligands (105) (MER binds to Gas6 or protein S which in turn bridge to the apoptotic cell), and so with more sMER found in the plasma, this sMER may reduce the capacity of the phagocytes to internalise apoptotic cells using its MER receptors. Thus the impact of MER losses may occur in two ways. To confirm the sMER identified by ELISA, immunoblotting could be undertaken to determine molecular weight.

Concentrations of sMER were also found to be greater in JIA patients than in HC. Further investigation would be required to ascertain the reason for this difference. In the case of JIA, the presence of sMER may be the result of the associated inflammatory process. Inflammation is a disease feature common to both JSLE and JIA and may, at least in part, explain increased sMER concentrations.

Age showed strongly-inverse correlation with sMER concentration in JSLE patients ($r=-0.714$, $p=0.003$) (Figure 3-7). This correlation was not reproducible in either the JIA

(r=0.345, p=0.328) or HC (r=0.048, p=0.864) sample populations. In the same respect as JSLE often accompanies a more severe disease form of lupus than SLE (79), JSLE is observed to affect patients with greater severity in those with the disease from an early age of onset. Other factors involved may include different therapeutic regimen, so patients were grouped according to concurrent treatment to attempt to discern the potential role of this. This suggests that this is a feature unique to JSLE patients, although since JIA and HC sMER concentrations were within fairly small ranges (especially compared with those of JSLE), it is naturally difficult to use these data to correlate with sMER. Another disadvantage is that there were only three JSLE patients included under the age of 10, so it is difficult to predict how a greater sample size with more patients in this age range might have altered the results. The obvious difficulty however, is that JSLE patients under the age of 10 are a subgroup within a juvenile-form of an already rare disease.

A positive correlation of BILAG disease activity score and sMER was observed (r=0.472), although the correlation was not statistically significant (p=0.075). The suggestion is that sMER concentration could be a useful biomarker, reflective of JSLE disease activity, and associated with nephritis and presence of anti-dsDNA (109). A number of haematological biomarkers used to monitor JSLE disease were also correlated against sMER concentration; ESR, CRP, C3 and C4; but there was no evidence of correlation with these markers (Figure 3-8).

The potential relationship between sMER concentration and patient gender was also investigated. sMER concentration was found to be statistically significantly greater in males (Figure 3-9). This appears to add weight to the suggestion of correlation with age, with JSLE/SLE usually common in females, however in the young this gender difference is less noteworthy. This may be adding to the suggestion that sMER concentration is greater in

younger age of JSLE. The males tended to have greater BILAG disease activity scores (section 3.2.2.2), linking these discussed factors with disease severity and activity at the time of sample collection. The suggestion may be that sMER is increased at times of disease flare or increased activity. This would make it a useful biomarker in monitoring JSLE disease pathology.

JSLE patients were divided according to drug treatments being utilised at the time of sample collection. Potentially, these therapeutics may impact upon the sMER concentration found in plasma. The only drug treatment associated with differing levels of sMER in patients was hydroxychloroquine, with levels much greater in those not treated with the drug (Figure 3-10). Hydroxychloroquine has not been associated with affecting apoptotic cell clearance so this may simply represent a chance finding. The statistical power is weak, however, with only 3 patients not treated with the drug at the time of sample collection.

Observations of corticosteroids in apoptotic cell clearance have presented a number of different effects and associations. Recently, they have been shown to augment the clearance of apoptotic cells and this was linked to the MER receptor (104). However, there is also no indication from the literature that corticosteroids affect the rate of MER cleavage. The results from this set of experiments suggest that there is no effect of corticosteroids on the level of sMER present in plasma (Figure 3-11) and there is no reason to suspect a role in MER loss from the cell surface.

Dividing patients according to medications is difficult, since the effects of certain drugs may be long-lasting but in order to group patients, only the drugs they were prescribed at the time of sample collection were considered. However, patients could have been receiving

any other drugs in the preceding months. Another difficulty is that the sample size was only 15 patients and attempting to further characterise these patients reduces the statistical power.

4.1.3 Monocyte Phagocytosis of pHrodo E. coli

With the methods for assessing phagocytosis of pHrodo-stained E. coli first validated and optimised, a series of experiments were conducted on homologous adult HC monocytes to investigate the role of environment on phagocytosis. Monocytes were tested in JSLE and HC serum and plasma samples, with no differences in phagocytic performance in serum or plasma. The results indicated that phagocytosis performance is greater in control plasma than in JSLE plasma. The difference was, however, not statistically significant, with some tests showing greater phagocytosis in JSLE environments. Any differences in phagocytosis may be explained by differences found in the serum/plasma of JSLE and HC patients. Two of the main ways by which phagocytosis (see 1.3) of E. coli may have occurred are through the Fcγ receptors and complement receptors. JSLE patients may, at different times, have low levels of complement present and this may explain a defect in phagocytosis occurring via this mechanism.

Taken together, phagocytosis was generally greater in JSLE serum and plasma compared with control. This negative effect of serum has been reported previously by research into adult-onset SLE, in studies testing apoptotic cell clearance. Ren et al (38) and Bijl et al (58) both show marked reductions in phagocytosis by monocyte-derived macrophages in SLE serum, though one study reported no differences (59) between phagocytosis in HC or SLE serum. The implication is that a contribution is made to the impairment of phagocytes by the environment, seeming to suggest the involvement of extracellular factors in the hypothesised failing of apoptotic cell clearance.

In this study, only (adult) HC monocytes were tested, but reports are conflicting as to the potential of intrinsic cellular defects affecting phagocytosis. Herrmann et al (55) and Ren et al (38) reported decreased phagocytosis by SLE monocyte-derived macrophages, and Tas et al (57) found no differences in binding capability but identified defective internalisation. However, Bijl etl al (58) found no differences in phagocytic performance when comparing HC and SLE monocyte-derived macrophages in HC serum.

4.1.4 Monocyte Stimulation

Having exposed adult HC monocytes to a number of settings associated with lupus, an sMER ELISA was conducted to assess for evidence of MER cleavage. There was no sMER recovered from any of the test conditions. This was unexpected of the LPS-exposed monocytes, which was used at a concentration (50 ng/mL) that had been reported as sufficient to cause the cleavage of MER by Sather and colleagues (105). LPS was included as a positive control and the failure to detect sMER through ELISA reflects that this assay did not work to this end. It was difficult to apply similar conditions, however, as the authors used a murine macrophage cell line (J774), the cell count was reported as confluency, and MER cleavage was measured by immunoblotting. Exposure of cells to IFNα, TNFα and apoptotic neutrophils did not result in MER cleavage from the surface of the monocytes. This suggests that it is one or more other factors that are responsible for triggering MER cleavage. It is not necessarily the case that there was no MER present in supernatant, but it may have been at a concentration lower than was detectable by spectrophotometry. The number of monocytes may have been too small, or the incubation times may have been too short to allow for cleavage. JSLE is a chronic disease and the monocytes and macrophages of JSLE are therefore exposed to cytokines such as those tested for a longer period than 6 hours. Repeating the experiment with a greater number of cells, for a

number of time-points, may result in measurable levels. Flow cytometry could be used to assess the MER expression profiles of cells before and after stimulation with test conditions.

The mRNA from these monocytes was also tested through real-time qPCR to investigate for any effects that these conditions had caused in the genetic expression of the three receptors of interest in this project; CD36, MER and CR3. The changes in gene expression were fairly small, with all recorded fold changes between -3 and +3. In monocytes exposed for 2 hours, MER expression was generally downregulated, but at the 6 hour time-point the majority of expression was upregulated. This was in contrast to the general trends of CD36 and CR3, where at 2 hours gene expression was upregulated, but after 6 hours of exposure these markers were downregulated. CD36 and CR3 also shared a common reaction to increasing concentrations of IFNα, with decreasing levels of gene expression in response. At the 6 hour time-point, IFNα-exposed monocytes appeared to upregulate MER expression but this was not observed at the 2000 pg/mL concentration making this association unclear. This was, however, only observed at the 6 hour time point and not with monocytes exposed for 2 hours. These two time points were tested in turn and with different blood donors so may reflect an element of individuality in the response to different conditions.

How the JSLE/SLE environment may affect phagocyte gene expression or MER cleavage has not been investigated to date and this represents the first attempt to characterise any potential relationship between them.

4.2 Study limitations

All elements of the research conducted were approached rigorously to ensure the underlying methodology was sound. There are, however, a number of limitations of the

research which should be considered alongside the results found and conclusions reached. Time represented a major constraint, with needing to first develop and learn laboratory skills from scratch, and then the process of validating and optimising experiments; only after all these steps were completed could experiments be undertaken with results that would contribute to this thesis.

The real-time qPCR data for gene expressions of receptors in monocytes and macrophages are based on fairly small sample sizes. This is especially the case with JSLE macrophages (n=3). This lessens the extent to which the data can be interpreted, as a greater sample size may serve to alter the findings. However, the use of both macrophages and their precursors monocytes increases the power as, in general, trends were consistent in both cell types.

The cell culture technique for the differentiation of monocytes into macrophages *in vitro* also presented difficulties. Frequently this process was affected by contamination in spite of aseptic procedures with all work conducted within the sterile field of a ventilated hood. This continued to cause problems throughout the year and made progression difficult.

The RNA extraction process yields a greater amount of RNA with better quality, often relative to greater numbers of cells. After cell separation there may be relatively low numbers of $CD14^+$ cells, dependent in the main on the amount of whole blood available at the start. Whilst this could be accounted for when procuring adult HC blood, JSLE and control plasma samples may only be 5 mLs of blood. Further to this, JSLE patients are often found to have low levels of white cells, and in particular their monocytes have been observed to have a reduced capacity to adhere plastic (57). The result of these factors may be RNA that falls outside of the desired range of quantity and quality; these were

sometimes tested and then used providing the reference gene appeared to be stably expressed and there were no obvious abnormalities in subsequent PCR experiments.

The phagocytosis assay proved to be quite a difficult experiment to validate and optimise. In the end, adult HC monocytes were tested in JSLE and control serum and plasma. Ideally, macrophages would also have been tested under these conditions, then phagocytes from JSLE and paediatric controls also. The ultimate endpoint would have been the development of a phagocytosis assay in which apoptotic cell clearance was investigated as opposed to bacterial uptake.

The monocyte stimulation experiments used relatively small cell numbers (around 4×10^5 cells per test condition) so the RNA extracted was of poor quality and quantity. These were used and tested, however, and the reference gene expression was stable and expressions of receptors appeared unaffected, and the use of two separate cell sets with two different time points adds weight to the results. With different donors and performed separately, the nature of the neutrophils in which apoptosis was induced differed between the two experiments, but there could never be exactly the same response to apoptotic induction by cells. This may, in part, explain the discrepancies in the receptor expression levels observed between monocytes exposed for 2 hours and those exposed for 6 hours.

JSLE is a uniquely variable disease, with severity different from one patient to the next, and diagnosis is made upon satisfaction of 4 or more of 11 criteria. The result is a group with common pathological processes but with widely varying afflictions and manifestations. There is also a large range of therapeutic options available to clinicians, much of which is aimed at immunosuppression. These factors can present challenges to research in that

results can be affected in a number of different ways. JSLE is also a rare disease and therefore hospital attendances and blood samples are relatively infrequent.

4.3 Study strengths

Much of the pathogenesis of JSLE, and indeed adult-onset SLE, is not yet understood. As such, it is imperative that research is undertaken to attempt to characterise the associated disease processes. This is especially important in JSLE, which is often more severe than adult and may actually differ from SLE in currently unknown ways. This research project has involved rigorous scientific methodology to attempt to add to the knowledgebase of JSLE pathophysiology, at one of the considered earliest steps in the process. The functional phagocytosis assay showed results which appear consistent with similar reported studies in adult-onset SLE, therefore strengthening the hypothesis of the dysfunction of phagocyte function in lupus as a whole. sMER concentrations were also found to be greater in JSLE, as had been reported in adult-onset SLE, and the results found in relation to those of JIA suggest that this presence of sMER at these concentrations appears unique to the JSLE autoimmune process. Attempts to ascertain the effect of lupus-associated cytokines/other environmental factors gave rise to inconclusive results but this is a novel, previously untried set of experiments. All of the reported results are unique to JSLE and some are unique to both JSLE and adult-onset SLE, and can be precursors for additional research.

4.4 Potential research directions

There are many avenues available for future research projects to take with much yet to investigate. Results described in this study could also be enhanced by greater sample sizes, giving results greater statistical power. At the gene level, expression of other phagocytic receptors could be measured to look to capture differences between JSLE and control

monocytes, for example TIM4 and MARCO, two receptors associated with apoptotic cell clearance.

A natural progression from gene expression measurement would be to investigate protein levels through Western Blots. Assessment of the same receptors would indicate if the levels found at gene level were reflective of cell receptor expression, any discrepancies between these would be of interest and would naturally lead to investigation as to why these differed.

The results of the measurement of sMER levels in JSLE could be built upon by the continuation of investigation as to the potential triggers of MER cleavage, through further phagocyte stimulation assays. Experiments could be repeated in monocytes over a longer time-course, and macrophages could be exposed to the same environments. Cells could also be exposed to other cytokines associated with SLE, such as IL-6 (72, 148), IL-17 (149) and IL-21 (150). Cleavage has been observed to occur in response to LPS and PMA exposure in the literature (105). The identity of the molecule containing antigens found through the sMER ELISA experiments could be confirmed by immunoblotting to confirm molecular weight. The profile of monocytes and macrophages, with respect to MER expression, could be investigated through measurement by flow cytometry. Another potential direction of research is to investigate the effect of greater concentrations of sMER.

One method by which this could be investigated would be by further extension of the phagocytosis assay. The key would be successful validation and optimisation of a method of measuring phagocytosis of apoptotic cells by monocytes and macrophages. To these, plasma samples of known sMER concentration (confirmed by ELISA) could be added to assess the potential effect that the presence of sMER may exert on phagocytic function.

This could be done further to adding known quantities of exogenous MER and/or MER antibodies. The former would act as a control for the presumed action of sMER, and the latter would act as a blocking control.

There are also a number of interesting directions the phagocytosis assay could take, not in only relation to MER. Continuation of the phagocytosis assay described here could be used to investigate the ability of JSLE and control monocytes and macrophages to phagocytose either in JSLE or control serum or plasma. This would give indication as to whether any phagocytic impairment was reflective of inherent cellular defect or the proinflammatory, autoimmune environment. These could be conducted testing bacterial uptake and apoptotic cell uptake, comparing phagocytic function in both cases. Of particular relevance and interest to the research group would be the uptake of dying neutrophils which have been suggested as presenting a great apoptotic burden to the body (39).

5 Conclusion

Phagocytosis is an essential functional process designed to clear foreign bodies, debris, and cells undergoing apoptosis. Its underlying mechanisms are complicated and vary depending on circumstance. In the autoimmune disease JSLE, phagocytosis is believed to be impaired. This impairment, in relation to the clearance of dying cells, is thought to be a key factor involved with the pathogenesis of the disease.

In this study, JSLE monocytes were found to up-regulate gene expression of the phagocytic markers CD36 compared with HC, though no differences in MER or CR3 expression; there were no differences between JSLE and HC in macrophage expression of CD36, MER or CR3. Increased CD36 expression may suggest that JSLE monocytes may be adapted in light of a greater apoptotic cell burden comprised of cells such as neutrophils which have been shown undergo an accelerated cell death process in JSLE.

Control monocytes and macrophages were negatively affected in their capacity to phagocytose bacteria when exposed to JSLE plasma, suggesting that any phagocytic defect could be the result of extracellular factors. A cleaved form of the receptor MER, sMER, was found at greater concentrations in JSLE patients than in either inflammatory controls (JIA) or HC, as has been observed in adult-onset SLE. The increased quantities of sMER are probably causing a number of effects, such as reducing the cell's MER expression, but also antagonising the process of apoptotic cell clearance by competing for ligands.

Factors including IFNα, TNFα and apoptotic neutrophils failed to provide unequivocal evidence as to any potential role they may have in altered monocyte and macrophage phenotype, with no clear resulting changes in CD36, MER and CR3 gene expression levels.

Research is ongoing with the aim to discover more about the role the phagocytes may be playing in JSLE pathogenesis, with the ambition that through better understanding of disease processes may better therapeutics be tailored.

6 References

1. Mosser DM, Edwards JP. Exploring the full spectrum of macrophage activation. Nature reviews Immunology. 2008;8(12):958-69. Epub 2008/11/26.
2. Dale DC, Boxer L, Liles WC. The phagocytes: neutrophils and monocytes. Blood. 2008;112(4):935-45. Epub 2008/08/08.
3. Swirski FK, Nahrendorf M, Etzrodt M, Wildgruber M, Cortez-Retamozo V, Panizzi P, et al. Identification of Splenic Reservoir Monocytes and Their Deployment to Inflammatory Sites. Science. 2009;325(5940):612-6.
4. Crowe SM, Ziegler-Heitbrock L. Editorial: Monocyte subpopulations and lentiviral infection. Journal of leukocyte biology. 2010;87(4):541-3.
5. Geissmann F, Manz MG, Jung S, Sieweke MH, Merad M, Ley K. Development of monocytes, macrophages, and dendritic cells. Science. 2010;327(5966):656-61. Epub 2010/02/06.
6. Steinman RM. The dendritic cell system and its role in immunogenicity. Annual review of immunology. 1991;9:271-96. Epub 1991/01/01.
7. Serbina NV, Jia T, Hohl TM, Pamer EG. Monocyte-mediated defense against microbial pathogens. Annual review of immunology. 2008;26:421-52. Epub 2008/02/29.
8. Chow A, Brown BD, Merad M. Studying the mononuclear phagocyte system in the molecular age. Nature reviews Immunology. 2011;11(11):788-98. Epub 2011/10/26.
9. Passlick B, Flieger D, Ziegler-Heitbrock HW. Identification and characterization of a novel monocyte subpopulation in human peripheral blood. Blood. 1989;74(7):2527-34. Epub 1989/11/15.
10. Wright S, Ramos R, Tobias P, Ulevitch R, Mathison J. CD14, a receptor for complexes of lipopolysaccharide (LPS) and LPS binding protein. Science. 1990;249(4975):1431-3.
11. Clarkson SB, Ory PA. CD16. Developmentally regulated IgG Fc receptors on cultured human monocytes. The Journal of experimental medicine. 1988;167(2):408-20.
12. Fingerle G, Pforte A, Passlick B, Blumenstein M, Strobel M, Ziegler- Heitbrock H. The novel subset of CD14+/CD16+ blood monocytes is expanded in sepsis patients. Blood. 1993;82(10):3170-6.
13. Belge K-U, Dayyani F, Horelt A, Siedlar M, Frankenberger M, Frankenberger B, et al. The Proinflammatory CD14+CD16+DR++ Monocytes Are a Major Source of TNF. The Journal of Immunology. 2002;168(7):3536-42.
14. Skrzeczynska-Moncznik J, Bzowska M, Loseke S, Grage-Griebenow E, Zembala M, Pryjma J. Peripheral blood CD14high CD16+ monocytes are main producers of IL-10. Scandinavian journal of immunology. 2008;67(2):152-9. Epub 2008/01/19.
15. Murray PJ, Wynn TA. Protective and pathogenic functions of macrophage subsets. Nature reviews Immunology. 2011;11(11):723-37. Epub 2011/10/15.
16. Aderem A, Underhill DM. Mechanisms of phagocytosis in macrophages. Annual review of immunology. 1999;17:593-623. Epub 1999/06/08.
17. Galli SJ, Borregaard N, Wynn TA. Phenotypic and functional plasticity of cells of innate immunity: macrophages, mast cells and neutrophils. Nature immunology. 2011;12(11):1035-44. Epub 2011/10/21.
18. Kerr JF, Wyllie AH, Currie AR. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. British journal of cancer. 1972;26(4):239-57. Epub 1972/08/01.
19. Mevorach D, Trahtemberg U, Krispin A, Attalah M, Zazoun J, Tabib A, et al. What do we mean when we write “senescence,”“apoptosis,”“necrosis,” or “clearance of dying cells”? Annals of the New York Academy of Sciences. 2010;1209(1):1-9.

20. Taylor RC, Cullen SP, Martin SJ. Apoptosis: controlled demolition at the cellular level. Nature reviews Molecular cell biology. 2008;9(3):231-41. Epub 2007/12/13.
21. Verbrugge I, Johnstone RW, Smyth MJ. SnapShot: Extrinsic Apoptosis Pathways. Cell. 2010;143(7):1192-.e2.
22. Riedl SJ, Salvesen GS. The apoptosome: signalling platform of cell death. Nature reviews Molecular cell biology. 2007;8(5):405-13.
23. Trapani JA, Smyth MJ. Functional significance of the perforin/granzyme cell death pathway. Nature reviews Immunology. 2002;2(10):735-47. Epub 2002/10/03.
24. Majno G, Joris I. Apoptosis, oncosis, and necrosis. An overview of cell death. The American journal of pathology. 1995;146(1):3-15. Epub 1995/01/01.
25. Ravichandran KS. Find-me and eat-me signals in apoptotic cell clearance: progress and conundrums. The Journal of experimental medicine. 2010;207(9):1807-17. Epub 2010/09/02.
26. Ravichandran KS, Lorenz U. Engulfment of apoptotic cells: signals for a good meal. Nature reviews Immunology. 2007;7(12):964-74. Epub 2007/11/27.
27. Fadok VA, de Cathelineau A, Daleke DL, Henson PM, Bratton DL. Loss of phospholipid asymmetry and surface exposure of phosphatidylserine is required for phagocytosis of apoptotic cells by macrophages and fibroblasts. The Journal of biological chemistry. 2001;276(2):1071-7. Epub 2000/09/15.
28. Casciola-Rosen L, Rosen A, Petri M, Schlissel M. Surface blebs on apoptotic cells are sites of enhanced procoagulant activity: implications for coagulation events and antigenic spread in systemic lupus erythematosus. Proceedings of the National Academy of Sciences of the United States of America. 1996;93(4):1624-9. Epub 1996/02/20.
29. Wu Y, Tibrewal N, Birge RB. Phosphatidylserine recognition by phagocytes: a view to a kill. Trends in cell biology. 2006;16(4):189-97. Epub 2006/03/15.
30. Henson PM, Bratton DL, Fadok VA. The phosphatidylserine receptor: a crucial molecular switch? Nature reviews Molecular cell biology. 2001;2(8):627-33. Epub 2001/08/03.
31. Kagan VE, Gleiss B, Tyurina YY, Tyurin VA, Elenstrom-Magnusson C, Liu SX, et al. A role for oxidative stress in apoptosis: oxidation and externalization of phosphatidylserine is required for macrophage clearance of cells undergoing Fas-mediated apoptosis. J Immunol. 2002;169(1):487-99. Epub 2002/06/22.
32. Hoffmann PR, deCathelineau AM, Ogden CA, Leverrier Y, Bratton DL, Daleke DL, et al. Phosphatidylserine (PS) induces PS receptor-mediated macropinocytosis and promotes clearance of apoptotic cells. The Journal of cell biology. 2001;155(4):649-59. Epub 2001/11/14.
33. Jacobson MD, Weil M, Raff MC. Programmed cell death in animal development. Cell. 1997;88(3):347-54. Epub 1997/02/07.
34. Fadeel B, Orrenius S. Apoptosis: a basic biological phenomenon with wide-ranging implications in human disease. Journal of internal medicine. 2005;258(6):479-517. Epub 2005/11/30.
35. Reed JC. Apoptosis-based therapies. Nature reviews Drug discovery. 2002;1(2):111-21. Epub 2002/07/18.
36. Munoz LE, van Bavel C, Franz S, Berden J, Herrmann M, van der Vlag J. Apoptosis in the pathogenesis of systemic lupus erythematosus. Lupus. 2008;17(5):371-5. Epub 2008/05/21.
37. Emlen W, Niebur J, Kadera R. Accelerated in vitro apoptosis of lymphocytes from patients with systemic lupus erythematosus. J Immunol. 1994;152(7):3685-92. Epub 1994/04/01.
38. Ren Y, Tang J, Mok MY, Chan AW, Wu A, Lau CS. Increased apoptotic neutrophils and macrophages and impaired macrophage phagocytic clearance of apoptotic neutrophils

in systemic lupus erythematosus. Arthritis and rheumatism. 2003;48(10):2888-97. Epub 2003/10/15.
39. Midgley A, McLaren Z, Moots RJ, Edwards SW, Beresford MW. The role of neutrophil apoptosis in juvenile-onset systemic lupus erythematosus. Arthritis and rheumatism. 2009;60(8):2390-401. Epub 2009/08/01.
40. Bengtsson AA, Sturfelt G, Gullstrand B, Truedsson L. Induction of apoptosis in monocytes and lymphocytes by serum from patients with systemic lupus erythematosus - an additional mechanism to increased autoantigen load? Clinical and experimental immunology. 2004;135(3):535-43. Epub 2004/03/11.
41. Midgley A, Beresford MW. Cellular localization of nuclear antigen during neutrophil apoptosis: mechanism for autoantigen exposure? Lupus. 2011;20(6):641-6. Epub 2011/03/03.
42. Platt N, da Silva RP, Gordon S. Recognizing death: the phagocytosis of apoptotic cells. Trends in cell biology. 1998;8(9):365-72. Epub 1998/09/05.
43. Hall JE, Guyton AC. Guyton and Hall textbook of medical physiology. Philadelphia, Pa.: Saunders/Elsevier; 2011.
44. Gasque P. Complement: a unique innate immune sensor for danger signals. Molecular immunology. 2004;41(11):1089-98. Epub 2004/10/13.
45. Turk B, Turk V. Lysosomes as “Suicide Bags” in Cell Death: Myth or Reality? Journal of Biological Chemistry. 2009;284(33):21783-7.
46. Henson PM, Bratton DL, Fadok VA. Apoptotic cell removal. Current biology : CB. 2001;11(19):R795-805. Epub 2001/10/10.
47. Krysko DV, Vanden Berghe T, D'Herde K, Vandenabeele P. Apoptosis and necrosis: detection, discrimination and phagocytosis. Methods. 2008;44(3):205-21. Epub 2008/03/04.
48. Krysko DV, Denecker G, Festjens N, Gabriels S, Parthoens E, D'Herde K, et al. Macrophages use different internalization mechanisms to clear apoptotic and necrotic cells. Cell death and differentiation. 2006;13(12):2011-22.
49. Underhill DM, Ozinsky A. Phagocytosis of microbes: complexity in action. Annual review of immunology. 2002;20:825-52. Epub 2002/02/28.
50. Elliott MR, Ravichandran KS. Clearance of apoptotic cells: implications in health and disease. The Journal of cell biology. 2010;189(7):1059-70. Epub 2010/06/30.
51. Munoz LE, Lauber K, Schiller M, Manfredi AA, Herrmann M. The role of defective clearance of apoptotic cells in systemic autoimmunity. Nature reviews Rheumatology. 2010;6(5):280-9. Epub 2010/05/01.
52. Gaipl US, Franz S, Voll RE, Sheriff A, Kalden JR, Herrmann M. Defects in the disposal of dying cells lead to autoimmunity. Current rheumatology reports. 2004;6(6):401-7. Epub 2004/11/06.
53. Henson PM, Tuder RM. Apoptosis in the lung: induction, clearance and detection. American journal of physiology Lung cellular and molecular physiology. 2008;294(4):L601-11. Epub 2008/01/08.
54. Brandt L, Hedberg H. Impaired phagocytosis by peripheral blood granulocytes in systemic lupus erythematosus. Scandinavian journal of haematology. 1969;6(5):348-53. Epub 1969/01/01.
55. Herrmann M, Voll RE, Zoller OM, Hagenhofer M, Ponner BB, Kalden JR. Impaired phagocytosis of apoptotic cell material by monocyte-derived macrophages from patients with systemic lupus erythematosus. Arthritis and rheumatism. 1998;41(7):1241-50. Epub 1998/07/15.
56. Brandt L, Hedberg H. Impaired Phagocytosis by Peripheral Blood Granulocytes in Systemic Lupus Erythematosus. Scandinavian journal of haematology. 1969;6(5):348-53.

57. Tas SW, Quartier P, Botto M, Fossati-Jimack L. Macrophages from patients with SLE and rheumatoid arthritis have defective adhesion in vitro, while only SLE macrophages have impaired uptake of apoptotic cells. Annals of the rheumatic diseases. 2006;65(2):216-21. Epub 2005/07/15.
58. Bijl M, Reefman E, Horst G, Limburg PC, Kallenberg CG. Reduced uptake of apoptotic cells by macrophages in systemic lupus erythematosus: correlates with decreased serum levels of complement. Annals of the rheumatic diseases. 2006;65(1):57-63. Epub 2005/05/28.
59. Grevink ME, Horst G, Limburg PC, Kallenberg CG, Bijl M. Levels of complement in sera from inactive SLE patients, although decreased, do not influence in vitro uptake of apoptotic cells. Journal of autoimmunity. 2005;24(4):329-36. Epub 2005/06/02.
60. Baumann I, Kolowos W, Voll RE, Manger B, Gaipl U, Neuhuber WL, et al. Impaired uptake of apoptotic cells into tingible body macrophages in germinal centers of patients with systemic lupus erythematosus. Arthritis and rheumatism. 2002;46(1):191-201. Epub 2002/01/31.
61. Crow MK. Developments in the clinical understanding of lupus. Arthritis research & therapy. 2009;11(5):245. Epub 2009/10/24.
62. Nightingale AL, Farmer RD, de Vries CS. Incidence of clinically diagnosed systemic lupus erythematosus 1992-1998 using the UK General Practice Research Database. Pharmacoepidemiology and drug safety. 2006;15(9):656-61. Epub 2006/01/04.
63. Kamphuis S, Silverman ED. Prevalence and burden of pediatric-onset systemic lupus erythematosus. Nature reviews Rheumatology. 2010;6(9):538-46. Epub 2010/08/05.
64. Buyon JP, Petri MA, Kim MY, Kalunian KC, Grossman J, Hahn BH, et al. The effect of combined estrogen and progesterone hormone replacement therapy on disease activity in systemic lupus erythematosus: a randomized trial. Annals of internal medicine. 2005;142(12 Pt 1):953-62. Epub 2005/06/22.
65. Pluchinotta FR, Schiavo B, Vittadello F, Martini G, Perilongo G, Zulian F. Distinctive clinical features of pediatric systemic lupus erythematosus in three different age classes. Lupus. 2007;16(8):550-5. Epub 2007/08/23.
66. Watson L, Gohar F, Beresford MW. Diagnosis and management of juvenile-onset SLE. Paediatrics and Child Health. 2011;21(12):539-45.
67. Sinha A, Lopez M, McDevitt H. Autoimmune diseases: the failure of self tolerance. Science. 1990;248(4961):1380-8.
68. Pham BN, Prin L, Gosset D, Hatron PY, Devulder B, Capron A, et al. T lymphocyte activation in systemic lupus erythematosus analysed by proliferative response to nucleoplasmic proteins on nitrocellulose immunoblots. Clinical and experimental immunology. 1989;77(2):168-74. Epub 1989/08/01.
69. Aringer M, Smolen JS. The role of tumor necrosis factor-alpha in systemic lupus erythematosus. Arthritis research & therapy. 2008;10(1):202. Epub 2008/01/30.
70. Kontoyiannis D, Kollias G. Accelerated autoimmunity and lupus nephritis in NZB mice with an engineered heterozygous deficiency in tumor necrosis factor. European journal of immunology. 2000;30(7):2038-47. Epub 2000/08/15.
71. Brennan DC, Yui MA, Wuthrich RP, Kelley VE. Tumor necrosis factor and IL-1 in New Zealand Black/White mice. Enhanced gene expression and acceleration of renal injury. J Immunol. 1989;143(11):3470-5. Epub 1989/12/01.
72. Davas EM, Tsirogianni A, Kappou I, Karamitsos D, Economidou I, Dantis PC. Serum IL-6, TNFalpha, p55 srTNFalpha, p75srTNFalpha, srIL-2alpha levels and disease activity in systemic lupus erythematosus. Clinical rheumatology. 1999;18(1):17-22. Epub 1999/03/24.
73. Svenungsson E, Fei GZ, Jensen-Urstad K, de Faire U, Hamsten A, Frostegard J. TNF-alpha: a link between hypertriglyceridaemia and inflammation in SLE patients with cardiovascular disease. Lupus. 2003;12(6):454-61. Epub 2003/07/23.

74. Fitzgerald-Bocarsly P, Dai J, Singh S. Plasmacytoid dendritic cells and type I IFN: 50 years of convergent history. Cytokine & growth factor reviews. 2008;19(1):3-19.
75. Niewold TB, Clark DN, Salloum R, Poole BD. Interferon alpha in systemic lupus erythematosus. Journal of biomedicine & biotechnology. 2010;2010:948364. Epub 2010/07/24.
76. Crow MK, Kirou KA. Interferon-alpha in systemic lupus erythematosus. Current opinion in rheumatology. 2004;16(5):541-7. Epub 2004/08/18.
77. Niewold TB, Hua J, Lehman TJ, Harley JB, Crow MK. High serum IFN-alpha activity is a heritable risk factor for systemic lupus erythematosus. Genes and immunity. 2007;8(6):492-502. Epub 2007/06/22.
78. Kirou KA, Lee C, George S, Louca K, Peterson MG, Crow MK. Activation of the interferon-alpha pathway identifies a subgroup of systemic lupus erythematosus patients with distinct serologic features and active disease. Arthritis and rheumatism. 2005;52(5):1491-503. Epub 2005/05/10.
79. Hoffman IE, Lauwerys BR, De Keyser F, Huizinga TW, Isenberg D, Cebecauer L, et al. Juvenile-onset systemic lupus erythematosus: different clinical and serological pattern than adult-onset systemic lupus erythematosus. Annals of the rheumatic diseases. 2009;68(3):412-5. Epub 2008/10/22.
80. Brunner HI, Gladman DD, Ibanez D, Urowitz MD, Silverman ED. Difference in disease features between childhood-onset and adult-onset systemic lupus erythematosus. Arthritis and rheumatism. 2008;58(2):556-62. Epub 2008/02/02.
81. Hochberg MC. Updating the American College of Rheumatology revised criteria for the classification of systemic lupus erythematosus. Arthritis and rheumatism. 1997;40(9):1725. Epub 1997/10/27.
82. Rahman A, Isenberg DA. Systemic lupus erythematosus. The New England journal of medicine. 2008;358(9):929-39. Epub 2008/02/29.
83. Marks SD, Tullus K. Modern therapeutic strategies for paediatric systemic lupus erythematosus and lupus nephritis. Acta Paediatr. 2010;99(7):967-74. Epub 2010/03/13.
84. Ruiz-Irastorza G, Ramos-Casals M, Brito-Zeron P, Khamashta MA. Clinical efficacy and side effects of antimalarials in systemic lupus erythematosus: a systematic review. Annals of the rheumatic diseases. 2010;69(1):20-8. Epub 2008/12/24.
85. Kyburz D, Brentano F, Gay S. Mode of action of hydroxychloroquine in RA[mdash]evidence of an inhibitory effect on toll-like receptor signaling. Nat Clin Pract Rheum. 2006;2(9):458-9.
86. Hedrich CM, Zappel H, Straub S, Laass MW, Wieczorek K, Hahn G, et al. Early onset systemic lupus erythematosus: differential diagnoses, clinical presentation, and treatment options. Clinical rheumatology. 2011;30(2):275-83. Epub 2010/10/05.
87. Wiglesworth AK, Ennis KM, Kockler DR. Belimumab: a BLyS-specific inhibitor for systemic lupus erythematosus. The Annals of pharmacotherapy. 2010;44(12):1955-61. Epub 2010/11/18.
88. van Vollenhoven RF, Petri MA, Cervera R, Roth DA, Ji BN, Kleoudis CS, et al. Belimumab in the treatment of systemic lupus erythematosus: high disease activity predictors of response. Annals of the rheumatic diseases. 2012;71(8):1343-9. Epub 2012/02/18.
89. García-Carrasco M, Jiménez-Hernández M, Escárcega RO, Mendoza-Pinto C, Galarza-Maldonado C, Sandoval-Cruz M, et al. Use of rituximab in patients with systemic lupus erythematosus: An update. Autoimmunity reviews. 2009;8(4):343-8.
90. Kausar F, Mustafa K, Sweis G, Sawaqed R, Alawneh K, Salloum R, et al. Ocrelizumab: a step forward in the evolution of B-cell therapy. Expert opinion on biological therapy. 2009;9(7):889-95. Epub 2009/05/26.

91. Dorner T, Kaufmann J, Wegener WA, Teoh N, Goldenberg DM, Burmester GR. Initial clinical trial of epratuzumab (humanized anti-CD22 antibody) for immunotherapy of systemic lupus erythematosus. Arthritis research & therapy. 2006;8(3):R74. Epub 2006/04/25.
92. Devitt A, Marshall LJ. The innate immune system and the clearance of apoptotic cells. Journal of leukocyte biology. 2011;90(3):447-57. Epub 2011/05/13.
93. Lai C, Lemke G. An extended family of protein-tyrosine kinase genes differentially expressed in the vertebrate nervous system. Neuron. 1991;6(5):691-704. Epub 1991/05/01.
94. O'Bryan JP, Frye RA, Cogswell PC, Neubauer A, Kitch B, Prokop C, et al. axl, a transforming gene isolated from primary human myeloid leukemia cells, encodes a novel receptor tyrosine kinase. Molecular and cellular biology. 1991;11(10):5016-31. Epub 1991/10/01.
95. Huang M, Rigby AC, Morelli X, Grant MA, Huang G, Furie B, et al. Structural basis of membrane binding by Gla domains of vitamin K-dependent proteins. Nature structural biology. 2003;10(9):751-6. Epub 2003/08/19.
96. Nagata K, Ohashi K, Nakano T, Arita H, Zong C, Hanafusa H, et al. Identification of the product of growth arrest-specific gene 6 as a common ligand for Axl, Sky, and Mer receptor tyrosine kinases. The Journal of biological chemistry. 1996;271(47):30022-7. Epub 1996/11/22.
97. Lemke G, Rothlin CV. Immunobiology of the TAM receptors. Nature reviews Immunology. 2008;8(5):327-36. Epub 2008/04/19.
98. Scott RS, McMahon EJ, Pop SM, Reap EA, Caricchio R, Cohen PL, et al. Phagocytosis and clearance of apoptotic cells is mediated by MER. Nature. 2001;411(6834):207-11. Epub 2001/05/11.
99. Todt JC, Hu B, Curtis JL. The receptor tyrosine kinase MerTK activates phospholipase C gamma2 during recognition of apoptotic thymocytes by murine macrophages. Journal of leukocyte biology. 2004;75(4):705-13. Epub 2004/01/06.
100. Shao W-H, Eisenberg RA, Cohen PL. The Mer Receptor Tyrosine Kinase Is Required for the Loss of B Cell Tolerance in the Chronic Graft-versus-Host Disease Model of Systemic Lupus Erythematosus. The Journal of Immunology. 2008;180(11):7728-35.
101. Rahman ZS, Shao WH, Khan TN, Zhen Y, Cohen PL. Impaired apoptotic cell clearance in the germinal center by Mer-deficient tingible body macrophages leads to enhanced antibody-forming cell and germinal center responses. J Immunol. 2010;185(10):5859-68. Epub 2010/10/19.
102. Shao W-H, Kuan AP, Wang C, Abraham V, Waldman MA, Vogelgesang A, et al. Disrupted Mer receptor tyrosine kinase expression leads to enhanced MZ B-cell responses. Journal of autoimmunity. 2010;35(4):368-74.
103. Galvan MD, Foreman DB, Zeng E, Tan JC, Bohlson SS. Complement Component C1q Regulates Macrophage Expression of Mer Tyrosine Kinase To Promote Clearance of Apoptotic Cells. The Journal of Immunology. 2012;188(8):3716-23.
104. McColl A, Bournazos S, Franz S, Perretti M, Morgan BP, Haslett C, et al. Glucocorticoids induce protein S-dependent phagocytosis of apoptotic neutrophils by human macrophages. J Immunol. 2009;183(3):2167-75. Epub 2009/07/15.
105. Sather S, Kenyon KD, Lefkowitz JB, Liang X, Varnum BC, Henson PM, et al. A soluble form of the Mer receptor tyrosine kinase inhibits macrophage clearance of apoptotic cells and platelet aggregation. Blood. 2007;109(3):1026-33. Epub 2006/10/19.
106. Park IC, Park MJ, Rhee CH, Lee JI, Choe TB, Jang JJ, et al. Protein kinase C activation by PMA rapidly induces apoptosis through caspase-3/CPP32 and serine protease(s) in a gastric cancer cell line. International journal of oncology. 2001;18(5):1077-83. Epub 2001/04/11.

107. Feng X, Deng T, Zhang Y, Su S, Wei C, Han D. Lipopolysaccharide inhibits macrophage phagocytosis of apoptotic neutrophils by regulating the production of tumour necrosis factor alpha and growth arrest-specific gene 6. Immunology. 2011;132(2):287-95. Epub 2010/11/03.
108. Thorp E, Vaisar T, Subramanian M, Mautner L, Blobel C, Tabas I. Shedding of the Mer Tyrosine Kinase Receptor Is Mediated by ADAM17 Protein through a Pathway Involving Reactive Oxygen Species, Protein Kinase Cδ, and p38 Mitogen-activated Protein Kinase (MAPK). Journal of Biological Chemistry. 2011;286(38):33335-44.
109. Wu J, Ekman C, Jonsen A, Sturfelt G, Bengtsson AA, Gottsater A, et al. Increased plasma levels of the soluble Mer tyrosine kinase receptor in systemic lupus erythematosus relate to disease activity and nephritis. Arthritis research & therapy. 2011;13(2):R62. Epub 2011/04/19.
110. Wu Y, Singh S, Georgescu MM, Birge RB. A role for Mer tyrosine kinase in alphavbeta5 integrin-mediated phagocytosis of apoptotic cells. Journal of cell science. 2005;118(Pt 3):539-53. Epub 2005/01/28.
111. Akakura S, Singh S, Spataro M, Akakura R, Kim J-I, Albert ML, et al. The opsonin MFG-E8 is a ligand for the αvβ5 integrin and triggers DOCK180-dependent Rac1 activation for the phagocytosis of apoptotic cells. Experimental Cell Research. 2004;292(2):403-16.
112. Savill J, Hogg N, Haslett C. Macrophage vitronectin receptor, CD36, and thrombospondin cooperate in recognition of neutrophils undergoing programmed cell death. Chest. 1991;99(3 Suppl):6S-7S. Epub 1991/03/01.
113. Sipes JM, Krutzsch HC, Lawler J, Roberts DD. Cooperation between thrombospondin-1 type 1 repeat peptides and alpha(v)beta(3) integrin ligands to promote melanoma cell spreading and focal adhesion kinase phosphorylation. The Journal of biological chemistry. 1999;274(32):22755-62. Epub 1999/07/31.
114. Hanayama R, Tanaka M, Miwa K, Shinohara A, Iwamatsu A, Nagata S. Identification of a factor that links apoptotic cells to phagocytes. Nature. 2002;417(6885):182-7.
115. Ren Y, Silverstein RL, Allen J, Savill J. CD36 gene transfer confers capacity for phagocytosis of cells undergoing apoptosis. The Journal of experimental medicine. 1995;181(5):1857-62. Epub 1995/05/01.
116. Voll RE, Herrmann M, Roth EA, Stach C, Kalden JR, Girkontaite I. Immunosuppressive effects of apoptotic cells. Nature. 1997;390(6658):350-1. Epub 1997/12/06.
117. Fadok VA, Warner ML, Bratton DL, Henson PM. CD36 is required for phagocytosis of apoptotic cells by human macrophages that use either a phosphatidylserine receptor or the vitronectin receptor (alpha v beta 3). J Immunol. 1998;161(11):6250-7. Epub 1998/12/02.
118. Cairns AP, Crockard AD, Bell AL. CD36-mediated apoptotic cell clearance in SLE. Lupus. 2001;10(9):656-7. Epub 2001/10/27.
119. Yassin LM, Rojas M, Ramirez LA, Garcia LF, Vasquez G. Monocyte activation by apoptotic cells removal in systemic lupus erythematosus patients. Cellular immunology. 2010;266(1):52-60. Epub 2010/09/25.
120. Ren Y, Stuart L, Lindberg FP, Rosenkranz AR, Chen Y, Mayadas TN, et al. Nonphlogistic clearance of late apoptotic neutrophils by macrophages: efficient phagocytosis independent of beta 2 integrins. J Immunol. 2001;166(7):4743-50. Epub 2001/03/20.
121. Toda S, Hanayama R, Nagata S. Two-step engulfment of apoptotic cells. Molecular and cellular biology. 2012;32(1):118-25. Epub 2011/11/01.
122. Mevorach D, Mascarenhas JO, Gershov D, Elkon KB. Complement-dependent clearance of apoptotic cells by human macrophages. The Journal of experimental medicine. 1998;188(12):2313-20. Epub 1998/12/22.

123. van Lookeren Campagne M, Wiesmann C, Brown EJ. Macrophage complement receptors and pathogen clearance. Cellular microbiology. 2007;9(9):2095-102. Epub 2007/06/26.
124. Pickering MC, Botto M, Taylor PR, Lachmann PJ, Walport MJ. Systemic lupus erythematosus, complement deficiency, and apoptosis. Advances in immunology. 2000;76:227-324. Epub 2000/11/18.
125. Truedsson L, Bengtsson AA, Sturfelt G. Complement deficiencies and systemic lupus erythematosus. Autoimmunity. 2007;40(8):560-6. Epub 2007/12/14.
126. Wu YL, Higgins GC, Rennebohm RM, Chung EK, Yang Y, Zhou B, et al. Three distinct profiles of serum complement C4 proteins in pediatric systemic lupus erythematosus (SLE) patients: tight associations of complement C4 and C3 protein levels in SLE but not in healthy subjects. Advances in experimental medicine and biology. 2006;586:227-47. Epub 2006/08/09.
127. Watson L, Leone V, Pilkington C, Tullus K, Rangaraj S, McDonagh JE, et al. Disease activity, severity, and damage in the UK juvenile-onset systemic lupus erythematosus cohort. Arthritis & Rheumatism. 2012;64(7):2356-65.
128. Petty RE, Southwood TR, Baum J, Bhettay E, Glass DN, Manners P, et al. Revision of the proposed classification criteria for juvenile idiopathic arthritis: Durban, 1997. The Journal of rheumatology. 1998;25(10):1991-4. Epub 1998/10/21.
129. Riedy MC, Timm EA, Jr., Stewart CC. Quantitative RT-PCR for measuring gene expression. BioTechniques. 1995;18(1):70-4, 6. Epub 1995/01/01.
130. VanGuilder HD, Vrana KE, Freeman WM. Twenty-five years of quantitative PCR for gene expression analysis. BioTechniques. 2008;44(5):619-26. Epub 2008/05/14.
131. Zipper H, Brunner H, Bernhagen J, Vitzthum F. Investigations on DNA intercalation and surface binding by SYBR Green I, its structure determination and methodological implications. Nucleic acids research. 2004;32(12):e103. Epub 2004/07/14.
132. SIGMA-ALDRICH. SYBR Green based qPCR technical animation. SIGMA-ALDRICH; 2012 [cited 2012 8 August]; Available from: http://www.sigmaaldrich.com/sigma-aldrich/areas-of-interest/life-science/molecular-biology/pcr/quantitative-pcr/sybr-green-based-qpcr/syber-green-animation.html.
133. Radonic A, Thulke S, Mackay IM, Landt O, Siegert W, Nitsche A. Guideline to reference gene selection for quantitative real-time PCR. Biochemical and biophysical research communications. 2004;313(4):856-62. Epub 2004/01/07.
134. Zhang X, Ding L, Sandford AJ. Selection of reference genes for gene expression studies in human neutrophils by real-time PCR. BMC molecular biology. 2005;6(1):4. Epub 2005/02/22.
135. Gussow D, Rein R, Ginjaar I, Hochstenbach F, Seemann G, Kottman A, et al. The human beta 2-microglobulin gene. Primary structure and definition of the transcriptional unit. The Journal of Immunology. 1987;139(9):3132-8.
136. Barber RD, Harmer DW, Coleman RA, Clark BJ. GAPDH as a housekeeping gene: analysis of GAPDH mRNA expression in a panel of 72 human tissues. Physiological Genomics. 2005;21(3):389-95.
137. Zhu G, Chang Y, Zuo J, Dong X, Zhang M, Hu G, et al. Fudenine, a C-terminal truncated rat homologue of mouse prominin, is blood glucose-regulated and can up-regulate the expression of GAPDH. Biochemical and biophysical research communications. 2001;281(4):951-6. Epub 2001/03/10.
138. Goidin D, Mamessier A, Staquet MJ, Schmitt D, Berthier-Vergnes O. Ribosomal 18S RNA prevails over glyceraldehyde-3-phosphate dehydrogenase and beta-actin genes as internal standard for quantitative comparison of mRNA levels in invasive and noninvasive human melanoma cell subpopulations. Analytical biochemistry. 2001;295(1):17-21. Epub 2001/07/31.

139. Ishii T, Wallace AM, Zhang X, Gosselink J, Abboud RT, English JC, et al. Stability of housekeeping genes in alveolar macrophages from COPD patients. The European respiratory journal : official journal of the European Society for Clinical Respiratory Physiology. 2006;27(2):300-6. Epub 2006/02/03.
140. Engvall E, Perlmann P. Enzyme-linked immunosorbent assay (ELISA). Quantitative assay of immunoglobulin G. Immunochemistry. 1971;8(9):871-4. Epub 1971/09/01.
141. Ternynck T, Avrameas S. Avidin-biotin system in enzyme immunoassays. Methods in enzymology. 1990;184:469-81. Epub 1990/01/01.
142. Paulie S, Perlmann H, Perlmann P. Enzyme-linked Immunosorbent Assay. eLS: John Wiley & Sons, Ltd; 2001.
143. Wilkerson MJ. Principles and applications of flow cytometry and cell sorting in companion animal medicine. The Veterinary clinics of North America Small animal practice. 2012;42(1):53-71. Epub 2012/01/31.
144. Rahman M. Introduction to Flow Cytometry. AbD Serotec; 2006 [cited 2006 8 August]; Available from: http://static.abdserotec.com/uploads/Flow-Cytometry.pdf.
145. Brown M, Wittwer C. Flow cytometry: principles and clinical applications in hematology. Clinical chemistry. 2000;46(8 Pt 2):1221-9. Epub 2000/08/06.
146. Miksa M, Komura H, Wu R, Shah KG, Wang P. A novel method to determine the engulfment of apoptotic cells by macrophages using pHrodo succinimidyl ester. Journal of immunological methods. 2009;342(1-2):71-7. Epub 2009/01/13.
147. Ong J, Harris D, Jeffers G, Watson L, Ballantine L, Beresford M. Matrix interference of IL-6, IL-17, IL-21 And TNF-alpha measurement in juvenile-onset systemic lupus erythematosus serum and plasma. Annals of the rheumatic diseases. 2012;71((Suppl3)):255.
148. Linker-Israeli M, Deans RJ, Wallace DJ, Prehn J, Ozeri-Chen T, Klinenberg JR. Elevated levels of endogenous IL-6 in systemic lupus erythematosus. A putative role in pathogenesis. J Immunol. 1991;147(1):117-23. Epub 1991/07/01.
149. Wong CK, Lit LC, Tam LS, Li EK, Wong PT, Lam CW. Hyperproduction of IL-23 and IL-17 in patients with systemic lupus erythematosus: implications for Th17-mediated inflammation in auto-immunity. Clin Immunol. 2008;127(3):385-93. Epub 2008/04/01.
150. Wong C, Wong P, Tam L, Li E, Chen D, Lam C. Elevated Production of B Cell Chemokine CXCL13 is Correlated with Systemic Lupus Erythematosus Disease Activity. Journal of clinical immunology. 2010;30(1):45-52.

Appendices

Appendix 1: Research Ethics Approval

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		

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

North West 3 Research Ethics Committee - Liverpool East
Bishop Goss Complex
Victoria Building
Rose Place
Liverpool
L3 3AN

Tel: 0151 330 2077
Fax: 0151 330 2075

03 November 2009

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

Study title:	**UK Juvenile Systemic Lupus Erythematosus Cohort Study & Repository: "Clinical characteristics and immunopathology of juvenile-onset systemic lupus erythematosus"**
REC reference:	**06/Q1502/77**
Amendment number:	**1.3**
Amendment date:	**16 October 2009**

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

Ethical opinion

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

Approved documents

The documents reviewed and approved at the meeting were:

Document	Version	Date
Letter to adult rheumatologists	v1.doc	18 January 2008
Participant Consent Form	1.3	16 October 2009
Participant Information Sheet	1.3	16 October 2009
Protocol	1.3	16 October 2009
Notice of Substantial Amendment (non-CTIMPs)		16 October 2009
Letter to patients diagnosed as child	1.1	16 October 2009

Membership of the Committee

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

R&D approval

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

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (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**

Yours sincerely

Ron Wall
Committee Co-ordinator

E-mail: Ronald.Wall@liverpoolpct.nhs.uk

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

Copy to: *Dot Lambert, R & D manager ,Alder Hey Children's NHS Foundation Trust*

Appendix 2: Information sheets for the UK Juvenile Lupus Cohort Study and Repository

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).

Age-appropriate information sheets are given to patients: the above reproduced information sheets are aimed at patients of 16 years or older.

Appendix 3: Patient's consent form for the UK Juvenile Lupus Cohort Study and Repository

Alder Hey Children's NHS
NHS Foundation Trust

Patient's Consent Form
(Liverpool only)

UK Juvenile SLE Cohort Study and Repository

Please INITIAL box

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

Name of patient	Date	Signature
Name of person taking consent (if different from researcher)	Date	Signature
Researcher	Date	Signature

1 copy for patient, 1 for researcher, 1 to be kept with hospital notes

UK JSLE Cohort Study & Repository - COREC Consent Forms Version 1.3 – 16th October 2009

Example consent form (used if patient is able to consent for him/herself)

Appendix 4: mRNA expression of JSLE and HC Monocytes and Macrophages

	CR3	CD36	MER
C136a	0.7206	0.3573	0.1718
C137a	0.6945	0.4048	0.4197
C138a	0.7251	0.7781	0.2040
C140a	1.1407	0.4795	0.4889
C142a	0.8059	0.5756	0.3052
C143a	1.8595	0.6693	0.2754
L17p	0.3163	0.5336	0.3751
L21g	0.8847	1.5133	0.7596
L30i	0.6765	1.2755	0.5482
L42l	0.5550	0.9611	0.1021
L52b	1.2077	0.7350	0.7343
L55c	0.6176	0.9650	0.3609

Monocytes mRNA expression

	CR3	CD36	MER
C135a	0.3122	0.4280	0.5857
C136a	0.3900	0.5294	0.3999
C137a	0.8340	0.5848	0.9561
C138a	0.1540	0.4122	1.4129
C142a	1.4024	1.1415	1.1330
C143a	2.2203	1.5129	2.0548
C152a	3.7391	0.5672	2.1093
C154a	2.2350	0.6542	1.2650
C155a	1.9328	0.8280	1.1397
L17p	0.2629	0.6355	0.7368
L40h	0.6225	1.0196	0.8474
L41k	0.4857	2.1756	0.8629

Macrophages mRNA expression

Relative expression ratios of monocytes and macrophages
C, Control; L, Lupus (JSLE)
Numbers indicate anonymised unique patient/control code
Lower case letter codes for the case attendance number

Appendix 5: sMER ELISA

	Blank	ng/ml	x 5
L2e	0.689	2.224	11.120
L3f	1.070	4.068	20.338
L4j	1.208	4.889	24.445
L17h	1.014	3.760	18.800
L21d	0.872	3.031	15.155
L30e	1.460	6.599	32.995
L33d	0.692	2.236	11.180
L35h	0.677	2.174	10.869
L40e	0.775	2.587	12.933
L43f	1.101	4.245	21.225
L45k	0.613	1.930	9.649
L46c	1.574	7.462	37.309
L49d	0.979	3.570	17.852
L50f	0.453	1.391	6.957
L51d	0.739	2.431	12.155
J3c	0.683	2.200	10.999
J15b	0.635	2.012	10.062
J24b	0.988	3.621	18.103
J25b	0.710	2.310	11.548
J26c	0.667	2.134	10.672
J27b	0.644	2.045	10.225
J40b	0.475	1.459	7.295
J41a	0.731	2.397	11.985
J43a	0.550	1.703	8.515
J44a	0.458	1.407	7.033
C54a	0.369	1.152	5.762
C59a	0.674	2.164	10.820
C60a	0.393	1.216	6.081
C61a	0.465	1.428	7.140
C66a	0.433	1.330	6.651
C67a	0.567	1.764	8.819
C71a	0.558	1.732	8.662
C73a	0.542	1.677	8.387
C74a	0.396	1.225	6.123
C76a	0.556	1.724	8.619
C85a	0.324	1.037	5.184
C87a	0.391	1.212	6.061
C90a	0.336	1.067	5.334
C91a	0.311	1.005	5.025
C100a	0.398	1.230	6.151

Absorbances measured by spectrophotometer from ELISA assay; concentrations calculated using the standard curve generated; corrected sMER concentrations, multiplied by 5x to correct for 1 in 5 dilution.
C, Control; J, JIA; L, Lupus (JSLE)
Numbers indicate anonymised unique patient/control code
Lower case letter codes for the case attendance number

Appendix 6: Monocyte Phagocytosis of pHrodo E. coli

	% Phagocytosis
C154a	34.4
C169a	48.2
C159a	46.6
C132a	43.3
C171a	50.9
C174a	27.7
L42h	16.6
L52i	37.2
L59a	39.1
L43k	29.1
L50k	32.6

Measured phagocytosis of pHrodo E. coli by adult HC monocytes in plasma samples (expressed as a percentage of all monocytes)
C, Control; L, Lupus (JSLE)
Numbers indicate anonymised unique patient/control code
Lower case letter codes for the case attendance number

Appendix 7: Monocyte Stimulation

	MER	CD36	CR3
Exp 1 Mo alone	0.9353	0.2104	0.3752
Exp 1 LPS	0.4552	0.2289	0.4372
Exp 1 IFNα 100	0.9436	0.2153	0.6584
Exp 1 IFNα 500	0.4695	0.3973	0.7562
Exp 1 IFNα 1000	1.1780	0.2400	0.4773
Exp 1 apoptotic neutrophils 1:1	0.6314	0.2584	0.8886
Exp 1 apoptotic neutrophils 2:1	0.7795	0.2404	0.5657
Exp 1 apoptotic neutrophils 4:1	0.3627	0.1816	0.4799
Exp 2 Mo alone	0.8955	2.4304	1.8493
Exp 2 LPS	0.6469	4.8915	1.5194
Exp 2 TNFα 100	1.3589	1.8905	1.2050
Exp 2 TNFα 500	1.0073	1.6736	1.2376
Exp 2 TNFα 1000	1.6153	1.2224	1.0158
Exp 2 IFNα 100	1.4106	0.9043	0.8640
Exp 2 IFNα 500	1.8420	1.8434	1.8392
Exp 2 IFNα 1000	1.0579	2.2089	1.3020
Exp 2 IFNα 2000	0.6698	2.3082	1.6703
Exp 2 apoptotic neutrophils 1:1	1.0307	3.4161	2.1381
Exp 2 apoptotic neutrophils 2:1	0.9306	0.5424	2.5552
Exp 2 apoptotic neutrophils 4:1	0.7351	2.2392	1.6565

Relative expression ratios of mRNA from monocytes exposed to different conditions

	MER	CD36	CR3
Exp 1 LPS	-2.3457	1.0281	1.1567
Exp 1 IFNα 100	-1.0497	1.0943	1.7291
Exp 1 IFNα 500	-2.2974	1.5157	1.9588
Exp 1 IFNα 1000	1.2746	1.1567	1.2658
Exp 1 apoptotic neutrophils 1:1	-1.5263	1.0570	2.2974
Exp 1 apoptotic neutrophils 2:1	-1.1975	1.0281	1.4845
Exp 1 apoptotic neutrophils 4:1	-2.7702	-1.3472	1.2483
Exp 2 LPS	-1.2834	1.3013	-1.2311
Exp 2 TNFα 100	1.9053	-1.4439	-1.5369
Exp 2 TNFα 500	1.3379	-1.6021	-1.5052
Exp 2 TNFα 1000	2.4623	-2.0994	-1.8404
Exp 2 IFNα 100	2.0000	-2.4116	-2.1287
Exp 2 IFNα 500	2.7321	-1.4641	-1.0281
Exp 2 IFNα 1000	1.5157	-1.4439	-1.4439
Exp 2 IFNα 2000	-1.2658	-1.2397	-1.1251
Exp 2 apoptotic neutrophils 1:1	1.2226	1.2142	1.1487
Exp 2 apoptotic neutrophils 2:1	1.09	-2.9485	1.3660
Exp 2 apoptotic neutrophils 4:1	-1.02	-1.4439	-1.1408

Fold changes of mRNA from monocytes exposed to different conditions

Exp (experiment) 1: 2 hour exposure; Exp 2: 6 hour exposure
Mo, monocytes; LPS, lipopolysaccharide; IFN, interferon alpha; AN, apoptotic neutrophils; TNF, tumour necrosis factor alpha