Clinical Validation of an assay for Type 1 interferon in systemic lupus erythematosus

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Abstract

Systemic Lupus Erythematosus (SLE) is a multi-organ autoimmune disease characterised by the breakdown of immune tolerance, and a defect in the clearance of apoptotic material. There is an unmet need for better biomarkers to diagnose and monitoring of SLE. Type I interferon (IFN-I) has a crucial role in pathogenesis of SLE and IFN-I varies between patients. IFN-I has previously been measured using a signature of IFN-I-inducible genes but these have not been applied in routine clinical practice due to lack of validated assays and clinical validation. Tetherin (CD317) is an interferon-inducible protein expressed on the cell surface, and therefore, amenable to measurement using flow cytometry. Measurement of tetherin in specific cell subsets appeared to be a useful biomarker in SLE in discovery studies.

In this study tetherin protein expression was assessed on whole blood and peripheral blood mononuclear cells using flow cytometry. It was determined that a whole blood assay reporting median tetherin MFI was convenient and applicable in a routine diagnostic laboratory. IFN-I inducible gene expression was measured by quantitative reverse transcription polymerase chain reaction (qRT-PCR) for comparison with tetherin. Healthy control (n=20), SLE-remission (n=66) and SLE-flare (n=65) groups were recruited from Leeds Lupus Clinic and tetherin levels were compared. Tetherin was increased in SLE patients with sensitivity (65.56%) and specificity (70%), with similar findings for gene expression. Tetherin expression on memory B cells, but not monocytes, predicted flare in patients in remission. However, repeat measurement of tetherin at follow up in flare patients (n=15) did not significantly reduce. The

potential use of tetherin and IFN-I inducible gene expression to stratify patients to appropriate biologics was explored.

These results demonstrate the potential value of tetherin as a biomarker in a routine clinical practice setting. Results have been used to design definitive validation studies.

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Abbreviations

- ACR American College of Rheumatology
- Ab Antigen-antibody
- ATP Adenosine Triphosphate
- ANA Antinuclear Antibodies
- APLs Antiphospholipid Antibodies
- BAFF B-cell Activating Factor
- BAFF/BlyS B-cell Activating Factor/B lymphocyte Stimulator
- BLK B-cell Lymphocyte Kinase
- BANK1 B-Cell Scaffold Protein with Ankyrin Repeats 1
- BCR B-cell Receptor
- BILAG British Isles Lupus Activity Group
- BLyS B-lymphocyte Stimulator
- BST-2 Bone marrow Stromal Antigen 2
- CSIM Cell Specific Interferon Marker
- CD Cluster of Differentiation
- C4 Complement component 4
- C1Q Complement component 1q
- cDNA complementary DNA
- CNS Central Nervous System
- CRP C-Reactive Protein
- CLIIF Crithidia luciliae Immunofluorescence
- CT Cytoplasmic Tail
- DC Dendritic cell
- DNA Deoxyribonucleic Acid

- dsDNA double-stranded DNA
- E-ILE Early Incomplete Lupus Erythematosus
- EDTA Ethylenediaminetetraacetic acid
- Env Envelope glycoprotein
- ELISA Enzyme Linked Immunosorbent Assay
- FBS Foetal Bovine Serum
- FITC Fluorescein Isothiocyanate
- FMO Florescent Minus One
- FSC Forward Side Scatter
- GE Gene Expression
- GEO Gene Expression Omnibus
- GC Germinal Centre
- GWAS Genome-Wide Association Scans
- GWAS Genome-Wide Association Studies
- GPI Glycosyl Phosphatidyl Inositol
- HIV-1 Human Immunodeficiency Virus type 1
- HLA Human Leukocyte Antigen
- HPRT1 Hypoxanthine-guanine phosphoribosyltransferase
- ICs Immune Complexes
- ICC Immune Complex bound Complement components
- ID Immunodiffusion
- IIF Immunofluorescence
- IFC Integrated Fluidics Circuit
- IFN Interferon
- IFN-α Interferon alpha

- IFNAR1 Interferon α Receptor 1
- ILE Incomplete Lupus Erythematosus
- IQR Interquartile Range
- ISGs Interferon Stimulated Genes
- IFNSGS Interferon-stimulated Gene Signature
- IRF7 Interferon Regulatory Factor 7
- Ig Immunoglobulin
- ILT7 Immunoglobulin-Like Transcript 7
- IL Interleukin
- IL-6 Interleukin 6
- IRF5 Interferon Regulatory Factor 5
- IRAK1 Interleukin 1 Receptor-Associated Kinase 1
- ISCs Immunoglobulin Secreting Cells
- ITAM Immunoreceptor Tyrosine-based Activation Motif
- KSHV Kaposi's Sarcoma-associated Herpesvirus
- LUMINA Lupus in Minorities: Nature versus Nurture
- MFI Median fluorescence intensity
- MARCH Membrane-Associated RING-CH
- MFIs Median Fluorescence Intensities
- MHP Mitochondrial Hyperpolarisation
- MMF Mycophenolate Mofetil
- mDC Myeloid Dendritic Cell
- NK Natural killer
- NFkB Nuclear Factor kappa-light-chain-enhancer of activated B cells
- PPIA Peptidylprolyl isomerase A

- PBMC Peripheral Blood Mononuclear Cell
- PBS Phosphate Buffered Saline
- PCR Polymerase Chain Reaction
- pDCs Plasmacytoid Dendritic Cells
- PDCP1 Programmed Cell Death Protein 1
- PE Phycoerythrin
- qRT-PCR quantitative reverse transcription Polymerase Chain Reaction
- ROC Receiver Operator Curve
- RBCs Red Blood Cells
- RT Reverse Transcriptase
- RNPs Ribonucleoprotein Particles
- RNP Ribonuclear Proteins
- RNA Ribonucleic Acid
- RTX Rituximab
- TCR T-cell Receptor
- TCGA The Cancer Genome Atlas
- Tr1 T regulatory cells
- TM Transmembrane
- TNF-α Tumour Necrosis Factor Alpha
- TNFAIP3 Tumour Necrosis Factor Alpha-Induced Protein 3
- TRAF3 TNF Receptor-Associated Factor 3
- TRAP Tartrate-resistant Acid Phosphatase
- TREX1 Three Prime Repair Exonuclease 1
- TLR Toll-Like Receptor

- TREX1 Three prime Repair Exonuclease 1
- SSC Side Scatter
- SIVs Simian Immunodeficiency Viruses
- SLE Systemic Lupus Erythematosus
- SOP Standard Operating Procedures
- STAT4 Signal Transducer and Activator of Transcription 4
- SLEDAI SLE Disease Activity Index
- UV Ultraviolet
- VH Variable Heavy
- Vpu Viral Protein U
- ^{o}C degree Celsius
- Mins Minutes
- mL Millilitre
- µL Microliter
- G Gravity
- S Second

1.0 Introduction

Overview

Systemic lupus erythematosus (SLE) is a prototypic autoimmune disease with heterogeneous clinical manifestations and immunopathogenesis (Tsokos, 2011, Sieber et al., 2014). It is a chronic inflammatory disease that is characterised by the dysfunction of T lymphocytes, dendritic cells, B cells and by the production of antinuclear autoantibodies (Perl, 2010). SLE is also associated with abnormally raised cytokine levels, including interleukin 6 (IL-6), B-cell activating factor (BAFF), and most notable, type I interferon (IFN). These atypical levels are thought to have fundamental roles in the maintenance and progression of the disease (Grondal et al., 2000, Munroe et al., 2014). The exact aetiology of SLE is unclear, however, initiation of the disease appears to depend on a combination of genetic susceptibility, immunological and environmental factors (Figure 1) that predispose and/or contribute to pathogenic autoimmunity (Grammer and Lipsky, 2003).

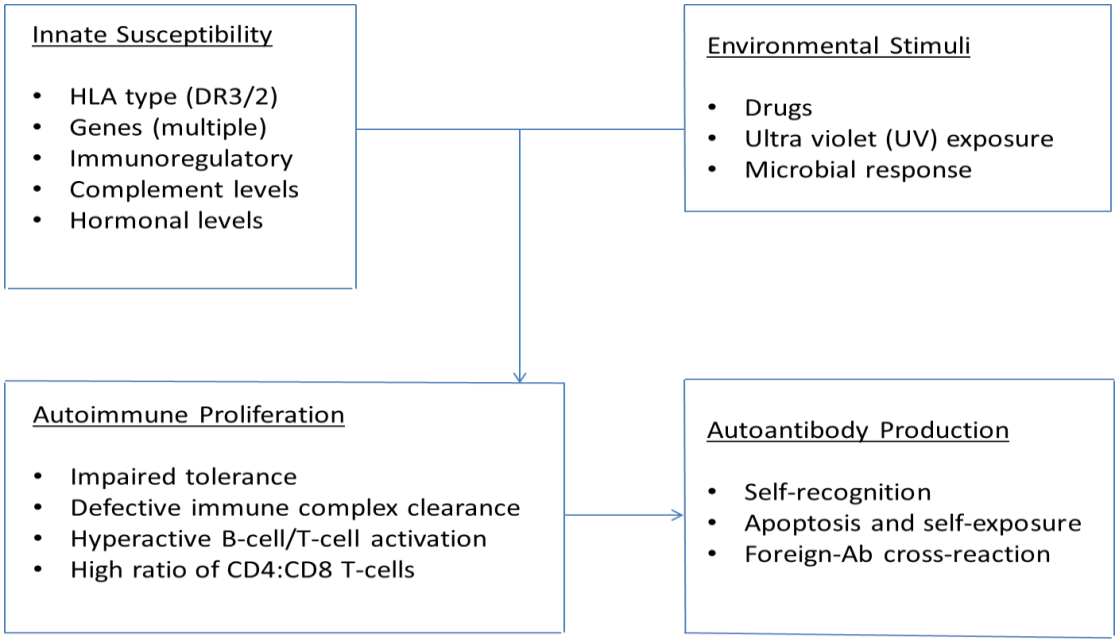
More than 90% of cases of SLE are diagnosed in women of childbearing age. The incidence rates in Europe vary; however, they are usually between 2 to 4.7 per 100,000 population per year and prevalence range from 20 to 150 cases per 100,000 in the overall population. The prevalence of SLE in the UK is approximately 25-30 per 100 000 adults (Haque et al., 2008). The concordance of SLE in monozygotic twins is approximately 25–50% and in dizygotic twins is around 2-5% (Harley et al., 2009). Aforementioned suggests that genetic factors play an influential role in predisposition to the disease. However, most cases of SLE are sporadic, indicating that multiple environmental or unknown factors may influence occurrence (Mok and Lau, 2003).

The disease's clinical manifestations range from mild symptoms such as skin rash, fever, joint pain, fatigue and arthralgia to severe forms of the disease affecting the kidneys, heart and central nervous system (CNS) (Rahman and Isenberg, 2008). However, the gastrointestinal tract is the most commonly affected system in SLE patients, with its manifestations similar to that of viral infections. The disease show highest prevalence and can be most aggressive in the African-American and African-Caribbean populations, however, SLE also affects Asian and Caucasian populations (Danchenko et al., 2006).

Genetic susceptibility includes genes that are involved in multiple functions such as; activation, proliferation, differentiation, survival of immunoglobulin secreting cells (ISCs). Additionally, the pathogenesis of SLE includes genes involved in the presentation and clearance of apoptotic materials and autoantigens by antigen presenting cells and other phagocytes (Mok and Lau, 2003). It has been shown that the progressive loss of tolerance to nuclear antigens over time, impaired clearance of immune complexes (ICs) and apoptotic materials, and the production of autoantibodies results in clinical disease (Elkon and Stone, 2011). Also there has been a strong correlation between elevated IFNs and SLE disease activity (Banchereau and Pascual, 2006, Landolt-Marticorena et al., 2009). Type I IFN has been shown to be one of the most important factors in the pathogenesis of SLE. This is because genes in the IFN pathway and regulation of innate immune responses have been identified in SLE susceptibility such as interferon regulatory factor 5 (*IRF5)* and signal transducer and activator of transcription 4 (*STAT4*). The heterogeneity of clinical features and the disease's unpredictable course can be characterised by flares and remissions (Obermoser and Pascual, 2010).

Over the past decade the type I IFN cytokine family has been suggested to play a central role in SLE pathogenesis (Obermoser and Pascual, 2010), by promoting feedback loops that progressively disrupt peripheral immune tolerance, which drives disease activity. The identification of novel molecules involved in the pathogenesis of SLE will improve understanding of the complexity of the disease, and may also help to identify novel targets for biological intervention (Obermoser and Pascual, 2010). Tetherin, an alpha interferon-inducible cellular factor, is a possible key molecule that could play a role in predicting flare or disease activity in SLE patients, and is therefore the target molecule in this study.

Figure 1: Factors that trigger the development of SLE



Genetic-susceptibility factors, environmental factors, antigen-antibody (Ab) responses, B-cell and T-cell interactions, and immune clearance processes interact to generate and perpetuate autoimmunity. HLA = human leukocyte antigen; UV = ultraviolet light.

http://emedicine.medscape.com/article/332244-overview#a4.

1.1 The role of interferon (IFN) in pathogenesis of SLE

IFNs are signalling proteins (De Andrea et al., 2002) and they were first recognised for their ability to impede viral replication (Vilcek, 2006). However, the antiviral potency of individual IFN varies considerably, and they modulate functions of the immune system. There are three types of IFNs, and they are categorised based upon the amino acid sequence and recognition of specific receptors (Theofilopoulos et al., 2005). Type I IFN comprises; IFN-α, IFN-β, IFN-ε, IFN-κ and IFN-ω (van Boxel-Dezaire et al., 2006, Noppert et al., 2007). Whereas, Type II IFN consists of a single member, IFN-γ and Type III IFN (IFN-λ1-4) (Obermoser and Pascual, 2010).

IFN-α is produced in virally infected leukocytes while IFN-β is from virally infected fibroblasts and keratinocytes. IFN-γ is induced by the stimulation of sensitised lymphocytes with antigen or non-sensitised lymphocytes with mitogens (Imanishi, 1994). IFNs do not only have antiviral activity but also various kinds of biological activities including cell growth inhibition, immunosuppressive effects, natural killer (NK) cells, killer (K) cells and neutrophil functions, enhancement of macrophage activity and cell differentiation-inducing activity (Imanishi, 1994). IFNs are also involved in the pathogenesis of various diseases, including, such as SLE, rheumatoid arthritis, insulin-dependent diabetes mellitus, severe pancreatitis, nephritis, multiple sclerosis, and atherosclerosis. IFNs are clinically used in therapy against viral infections such as hepatitis B and C (Parkin and Cohen, 2001), and for malignancies, such as skin cancers, renal cell carcinoma, and chronic myelogenous leukaemia (Imanishi, 1994).

IFN activity was first discovered in the serum of patients suffering from several autoimmune diseases in 1979 (Hooks et al., 1979, de Weerd et al., 2007), a finding which was later confirmed mainly in SLE patients (Preble et al., 1982, Kirou et al., 2005). The role of type I IFN-α and IFN-β in SLE was observed from the induction of autoimmunity during IFN-α/β infusion (Ronnblom et al., 1991) and the increased levels presence of IFN-α/β in SLE patients' blood. This confirmed that IFNs levels directly correlated with SLE disease activity (Blanco et al., 2001).

1.2 Mechanisms associated with the pathogenesis of SLE.

Multiple immune abnormalities contribute to the pathogenesis of SLE. These include abnormal clearance of apoptotic cells, immune complexes (ICs) and low thresholds of activation of B and T cells which leads to loss of self-tolerance and autoantibody production (Elkon and Stone, 2011). It has been shown that these autoantibodies are directed against nucleic acids (DNA) and associated nuclear proteins as well as ribonuclear proteins (RNP) such as Ro, La, and Sm (Tan, 1989).

Both environmental and genetic risk factors are critical in the development of SLE. The disease is nine folds higher in females than in males; indicating that hormones make a major contribution in the development of SLE. Human leukocyte antigen (HLA) associations HLA-B8 and HLA-DR3 and complement deficiencies, especially of early complement components C1q, C2, and C4 are associated with an increased risk of SLE (Truedsson et al., 2007). It has been suggested that more than 90% of individuals who have a homozygous deficiency of C1q develop SLE with more severe disease manifestations

(Pickering and Walport, 2000). C1q deficiency inhibits the clearance of apoptotic cells and can also upregulate cytokine production by other immune cells (Fraser et al., 2009).

Kirou *et al* (2005) reported that low complement levels are associated with the activation of the type I IFN pathway, which could explain the increased disease activity (Kirou et al., 2005). Studies performed by Lood *et al* and Santer *et al* showed that C1q plays a direct role in the regulation of IFN-α stimulation by SLE ICs (Lood et al., 2009, Santer et al., 2010). There has been evidence of three monogenic deficiencies of C1q, three prime repair exonuclease 1 (TREX1), and tartrate-resistant acid phosphatase (TRAP) have been identified to show in clinical phenotypes consistent with lupus (Elkon and Stone, 2011). *TREX1* mutations were found in up to 2.7% of SLE patients (Lee-Kirsch et al., 2007, Namjou et al., 2011). In an analysis of over 8,000 multi-ancestral lupus patients two discoveries were made (Namjou et al., 2011), 1) a *TREX1* risk allele was linked with neurologic manifestations, especially seizure in patients of European descent. 2), a strong association between a *TREX1* single-nucleotide polymorphism and anti-nRNP antibodies was seen (Namjou et al., 2011).

There are more than 100 genetic variants (only eight consistently replicated) that confer the increased risk of SLE susceptibility. However, only a small effect has been identified by genome-wide association studies (GWAS) (Harley et al., 2009). The three monogenic syndromes and multiple genetic variants identified by GWAS are involved in the type I IFN pathway, which further highlight the significance of this pathway in SLE (Deng and Tsao, 2010, Sestak et al., 2011).

1.2.2 Diagnosis of SLE

There are numerous diagnostic tests available to aid the diagnosis of SLE; however, no laboratory assay has 100 percent (100%) accuracy and precision, and clinical tests are often compromised by subjectivity. SLE patients are primarily women who present with chronic nonspecific symptoms such as weight loss, fever and fatigue (Banchereau and Pascual, 2006). The American College of Rheumatology (ACR) criteria for the diagnosis of SLE includes detection of several autoantibodies (Table 1). Confirming the diagnosis of SLE requires the fulfilment of four out of the eleven clinical and immunological criteria to be present at some time-point. One of those includes the presence of anti-nuclear antibodies (ANAs); ANAs are detected in >95% of SLE patients (Banchereau and Pascual, 2006). Antiphospholipid antibodies (APLs) are detected in approximately 60% of patients with SLE and Anti-Sm protein is found in approximately 30% of people with lupus. A positive test supports a lupus clinical diagnosis; however, relying on these antibody results alone could lead to a false positive or false negative diagnosis.

Classification and Clinical Features of SLE by ACR Criteria

Criteria	**Definition**
Malar Rash	Fixed erythema, flat or raised, over the malar eminences, tending to spare the nasolabial folds
Discoid Rash	Erythematous raised patches with adherent keratotic scaling and follicular plugging; atrophic scarring occurs in older lesions
Photosensitivity	Skin rash as a result of unusual reaction to sunlight, by patient history or physician observation
Oral Ulcers	Oral or nasopharyngeal ulceration, usually painless, observed by a physician
Arthritis	Non-erosive arthritis involving two or more peripheral joints, characterised by tenderness, swelling or effusion
Serositis	a) Pleuritis: convincing history of pleuritic pain or rub heard by a physician or evidence of pleural effusion or b) Pericarditis: documented by ECG or rub or evidence of pericardial effusion
Renal Disorder	a) Persistent proteinuria >0.5 g per day or >3+ if quantitation is not performed or b) Cellular casts: may be red cell, haemoglobin, granular tubular, or mixed.
Neurological Disorder	a) Seizures: in the absence of off ending drugs or known metabolic derangements (e.g., uraemia, acidosis, or electrolyte imbalance) or b) Psychosis: in the absence of off ending drugs or known metabolic derangements (e.g., uraemia, acidosis, or electrolyte imbalance)
Haematological Disorder	a) Haemolytic anaemia with reticulocytosis, or b) Leucopoenia: <4000/mm^3, or c) Lymphopenia: <1500/mm^3, or

Criteria	Definition
	d) Thrombocytopenia: <100,00mm^3, in the absence of off ending drugs
Immunological Disorder	a) Anti-DNA: antibody to native DNA in abnormal titre, or b) Anti-Sm: presence of antibody to Sm nuclear antigen, or c) Positive finding of antiphospholipid antibodies based on: (1) an abnormal serum concentration of IgG or IgM anticardiolipin antibodies, (2) a positive test result for lupus anticoagulant using a standard method, or (3) a false positive serologic test for syphilis known to be positive for at least 6 months and confirmed by Treponema pallidum immobilisation or fluorescent treponemal antibody absorption test.
Antinuclear Antibody	An abnormal titre of antinuclear antibody by immunofluorescence or an equivalent assay at any point in time and in the absence of drugs known to be associated with 'drug-induced lupus' syndrome.

Table 1: The American College of Rheumatology revised classification criteria for SLE.

The classification of SLE is based on the presence of at least 4 of 11 criteria according to the American College of Rheumatology revised in 1997 (Hochberg, 1997). The criteria present high sensitivity (>85%) and specificity (>95%) and include both clinical and serological parameters.

Due to the positive contribution of serology in SLE, the criteria were revised in 2012 by the Systemic Lupus International Collaborating Clinics (SLICC). As it is now at least one clinical and one laboratory criteria in the total 4 are required

for the diagnosis of SLE (Table 2) (Petri et al., 2012). However, the criteria were validated for SLE patients with longstanding disease history.

Clinical Criteria	**Immunologic Criteria**
Acute Cutaneous Lupus	ANA
Chronic Cutaneous Lupus	Anti-DNA
Oral or Nasal Ulcers	Anti-Smith (anti-Sm)
Non-scaring Alopecia	Antiphospholipid Ab
Arthritis	Low Complement (C3, C4, CH50)
Serositis	Direct Coombs' Test
Renal	
Neurologic	
Haemolytic Anaemia	
Leukopenia	
Thrombocytopenia (<100,000/mm^3)	

Table 2: The SLICC classification criteria for SLE

There are several laboratory techniques used to detect antinuclear antibodies (ANA) such as: Immunodiffusion (ID), which detects high affinity antibodies, immunofluorescence (IIF), which detects moderate and high affinity antibodies, and finally, enzyme linked immunosorbent assay (ELISA) which can identify low and high affinity antibodies. The most common techniques used in the UK are dsDNA ELISA, Crithidia luciliae IIF (CLIIF), or Farr immunoprecipitation assays (Egner, 2000).

Farr assays have been shown to be specific, but also detect high affinity IgM anti-dsDNA. IgG specific ELISA or CLIIF methods may produce similar results to Farr assays. It is believed that local validation of each assay is essential to ensure adequate diagnostic performance (Egner, 2000). There are limitations of these tests; for example, purified antigens maybe contaminated, or may not contain the full complement of native proteins. Recombinant antigens might lack certain epitopes, or contain contaminating bacterial antigens. All assays require careful validation to determine whether they perform adequately for detecting human autoantibodies (Egner, 2000). There is a need to develop specific assays for diagnosis of SLE, and there are on-going experiments for the development of biomarkers and more accurate assays. The use of serology tests to diagnose SLE is that they are not specific. For instance, ANA is present in individuals that do not have SLE. Currently there is the use to anti-dsDNA titres, erythrocyte sedimentation rate (ESR), complement levels and immunoglobulin titres to predict flare which are not reliable and specific. Hence why the discovering of a reliable and specific biomarker to predict flare will be beneficial to the patients and it will save cost for the National Health Service (NHS).

1.2.3 Treatment of SLE

Treatment of SLE depends on disease severity and disease manifestations (Hahn, 2005). Hydroxychloroquine has a central role for long-term treatment in all SLE patients. The Lupus in Minorities: Nature versus Nurture (LUMINA) study (2007 with 608 patients) and other trials have offered evidence of a decrease in flares and prolonged life in patients given hydroxychloroquine,

making it the basis of SLE management (Alarcon et al., 2007). Anti-malarials such as hydroxychloroquine, cyclophosphamide, methotrexate and mycophenolate mofetil (MMF) are widely used for the treatment of mild manifestations of SLE. For SLE patients without major organ manifestations, glucocorticoids and antimalarial agents are usually given (Bertsias et al., 2008). Glucocorticoids are the backbone of therapy in the acute phase. Furthermore, immunosuppressive or immunomodulatory drugs alone or in combination can be used for the control or reduction of disease activity in the long-term.

Biologic therapies have recently been added to the SLE therapeutic armamentarium such as belimumab and rituximab. These drugs act by inhibiting the number of circulating B cells function. B-cell depletion can be achieved by targeting the cell surface marker CD20 (rituximab (RTX)). RTX is a common treatment option, particularly in the case of more severe disease. There is clinical improvement in patients treated with the B cell-depleting CD20 antibody compared to those taking hydroxychloroquine (Vital et al., 2012, Md Yusof et al., 2017).

The field of biological therapies has encountered many setbacks regarding SLE treatment. However, belimumab, monoclonal antibody against B-lymphocyte stimulator (BLyS) stands out, with two phase 3 trials (BLISS-52 and BLISS-76) that met the primary outcome, the SLE responder index (Lateef and Petri, 2010). Belimumab is licensed for add-on therapy in adults with active, autoantibody-positive SLE with a high degree of disease activity. In the United Kingdom (UK) belimumab is commissioned only if: the SLE Disease Activity

Index (SLEDAI) score is ≥10 at baseline, anti-double stranded deoxyribonucleic acid (dsDNA) antibodies titers are raised and complement levels (C3 and C4) are low. This is because these criteria predicted a higher response rate in clinical trials. SLEDAI and British Isles Lupus Activity Group (BILAG) are validated scales of disease activity.

A phase 1 dose-escalation study (Yao et al., 2009) evaluated the effects of a single dose of anti-IFN monoclonal antibody therapy in SLE. Anifrolumab is an antagonist human monoclonal antibody (IgG1 κ) that targets interferon α receptor 1 (IFNAR1) and prevents signalling by all type I IFNs. Anifrolumab has been established to treat autoimmune diseases (Peng et al., 2015) and has been assessed in a phase IIb, randomized, double-blind, placebo-controlled study of adult patients with moderate to severe SLE (Merrill, 2016). It has proven to substantially reduced disease activity compared with placebo across multiple clinical endpoints. It is currently in pivotal phase III studies.

Despite the therapeutic advances, side effects affect many patients. Potential side effects are gastrointestinal disturbance, increased risk of infection, liver toxicity, decreased fertility and an increased risk of cancer as well as long-term glucocorticoid toxicity. Many patients fail to respond to existing treatment. Cardiovascular toxicity is markedly increased. It is thought that long-term outcomes would be improved if patients could maintain in a state of low disease activity (i.e. without flare). For this reason, predictors of flare would be valuable to (i) give pre-emptive therapy if a flare is imminent to prevent the flare; and (ii) reduce potentially toxic therapy in patients whose risk of flare is low.

1.3 The role of B cells in SLE

B-cells play a key role in the pathogenesis of SLE by secreting autoantibodies. The presence of autoantibodies in SLE patients is a defining characteristic of the disease, with many of these making a major contribution to disease pathogenesis, for example anti-DNA antibodies. B-cells also act as antigen presenting cells, activating T-cells. Finally, they secrete both pro-inflammatory and anti-inflammatory cytokines (Nashi et al., 2010, Sieber et al., 2014); such as IL-4, IL-6, IL-10, IFN-γ, Transforming Growth Factor-β and Lymphotoxin-α (Anolik, 2007).

1.4 Lymphocytes as antigen-presenting cells in SLE

Several studies have demonstrated that B-cells are active participants in humoral immune responses that lead to differentiation of ISCs (Grammer and Lipsky, 2003). Furthermore, differentiation of ISCs has been shown to be affected by stimulation from B-cell activating factor (BAFF/BlyS/TNFSF13B). BAFF is coded for in an SLE susceptibility locus (13q32-34) (Blomberg et al., 2001, Honda et al., 2005) and has been shown to be higher in the serum of patients with active SLE (Jahnsen et al., 2000, Le Bon et al., 2001).

Studies have revealed that B-cells play a role in auto-regulating humoral immune responses, and data suggested that B cells from active SLE patients have an intrinsic tendency to overreact to immunologic stimulation during antigenic challenge (Grammer and Lipsky, 2003). This sets the stage for novel hypotheses regarding therapeutic approaches to interfere with the development and progression of SLE (Grammer and Lipsky, 2003). The B-cell abnormalities observed in SLE patients may either reflect the impact of multiple genetic

factors that affect intrinsic B cell function and/or they may be secondary to other primary immunologic abnormalities (Shlomchik et al., 2001). Evidence has suggested there is alteration of a tolerance checkpoint in SLE patients (Yurasov et al., 2005). It has been shown that altered B-cell tolerance checkpoints in SLE comes from B cells expressing the VH4-34 gene, that encodes autoantibodies of different specificities (Banchereau and Pascual, 2006). Data has shown that in healthy individuals, VH4-34+ B cells are excluded during the early stages of the germinal centre (GC) reaction, therefore representing a second tolerance checkpoint in the life of a B cell. Whereas, in SLE patients, VH4-34+ B cells progress through this checkpoint, participate in GC reactions, and also expanded within the post-GC IgG memory and plasma cell compartments (Cappione et al., 2005).

1.5 Autoantibodies

Anti-DNA antibodies are extensively studied in lupus. It has been shown that in SLE patients 50-70% of these antibodies are present at some stage in the disease (Nashi et al., 2010) and their presence supports diagnosis of the disease. A number of studies have shown that titres of anti-DNA antibodies tend to rise during flares of SLE disease activity particularly in lupus nephritis. Despite these findings, it is important to remember not all anti-DNA antibodies are pathogenic. Indeed, some anti-DNA antibodies have no pathogenic effect regardless of their DNA-binding affinities being equivalent to those of pathogenic antibodies (Nashi et al., 2010). It has been suggested that certain isotypes and antigen binding properties are associated with pathogenicity. IgG anti-double-stranded DNA (anti-dsDNA) antibodies for instance, are more

clinically significant and are associated with increased disease activity and tissue damage compared with IgM antibodies (Isenberg et al., 1997), which have been shown to be protective (Witte, 2008). It was stated that anti-dsDNA antibodies have been shown to be more pathogenic than anti-single-stranded DNA antibodies. Anti-DNA antibodies from SLE patients with renal lupus exhibit a high affinity for DNA (Williams et al., 1999).

Anti-nucleosome antibodies may be more important than anti-DNA antibodies in the aetiology of SLE. Nucleosomes, which consist of DNA wrapped around a core of histone proteins, are more important antigenic targets in lupus than naked DNA (Nashi et al., 2010). The levels of circulating nucleosomes have been shown to be increased in the plasma of lupus patients (Williams et al., 2001). Furthermore, the levels of anti-nucleosome antibodies correlate strongly with lupus disease activity (Min et al., 2002), particularly with renal flare (Simon et al., 2004). The study by Ng *et al* (2006) showed that patients with higher titres of anti-nucleosome antibodies have a shorter time to flare after a serologically active but clinically quiescent period (Ng et al., 2006). These studies propose that titres of anti-nucleosome antibodies could be a better tool and have greater clinical significance than titres of anti-DNA antibodies in predicting flare (Nashi et al., 2010).

1.6 The role of T-cells in SLE

Studies in patients with SLE have established that autoantigen-reactive T-cells can be isolated from peripheral blood and these cells can support autoantibody production *ex vivo* (Hoffman, 2004). This suggests that they have a central role in the pathogenesis of the disease. Previous work has identified and characterised signalling abnormalities in T-cells from SLE patients that may be fundamental to the disease (Hoffman, 2004). T-cells contribute to the initiation and development of autoimmunity in SLE and seem to be involved in the development of related organ damage (Crispin et al., 2010). The cytokine expression pattern is characterised by decreased expression of interleukin-2 (IL-2) and increased production of IL-17 and related cytokines (Crispin et al., 2010). Biochemical description of SLE T-cells has revealed distinct early and late signalling abnormalities, and has enabled the identification of novel molecular targets that can be corrected with small molecules, and biomarkers that may predict disease activity and organ damage (Crispin et al., 2010).

Abnormal T-lymphocyte activation and cell death signalling underlie the pathology of SLE (Kyttaris et al., 2005). Mitochondria, which control death signal processing, are dysfunctional in lupus T-cells (Perl et al., 2009). This is believed to manifest as a persistent elevation of the mitochondrial trans-membrane potential or mitochondrial hyperpolarisation (MHP) (Gergely et al., 2002). Whereas, adenosine triphosphate (ATP) depletion, which predisposes the cell to death by necrosis. The increased release of necrotic materials from T-cells may drive disease pathogenesis by activating macrophages and dendritic cells (DCs) and enhancing their capacity to produce nitric oxide and IFN-α in SLE (Perl et al., 2004).

Preliminary observations by Banchereau and Pascual (2006) suggest that IFN-α/β stimulated DCs might favour the generation of these pathogenic cells. IFN-α was shown to strongly enhance IL-10 induced differentiation of functional $CD4^+$ T regulatory cells (Tr1) ($IL\text{-}10^+$, IFN-g^+, IL-22/lo). Blanco *et al* (2005) showed that SLE patients with disease flares display greater proportions of perforin and/or granzyme B-positive lymphocytes with a differentiated effector phenotype (CCR72 and $CD45RA^+$) (Blanco et al., 2001, Blanco et al., 2005). The administration of IFN-α/β to metastatic melanoma patients has also showed an increase of circulating fatally differentiated effectors (Di Pucchio et al., 2006).

1.7 Apoptosis and SLE

Apoptosis an active programmed cell death may be induced by a variety of soluble and surface signals (Mok and Lau, 2003). Apoptosis is a process by which nuclear material may become a source of autoantigens which results in systemic autoimmunity, through defective clearance of dead and dying cells (Mok and Lau, 2003). For instance, in healthy individuals, apoptotic cells are promptly removed by macrophages in a process that is anti-inflammatory. However, it has been shown that in SLE patients, there is evidence of defective clearance of apoptotic cells (Munoz et al., 2009) leading to secondary necrosis. IFN promotes loss of self-tolerance, production of autoantibodies against nuclear material, and IC formation (Martin and Elkon, 2005). The process of IC formation between SLE autoantibodies and antigen derived from dead and dying cells can be replicated *in vitro* using apoptotic or necrotic debris. Evidence has shown that the ability of SLE sera to induce IFN-α production appears to be strongly correlated with the presence of antibodies against small nuclear

ribonucleoprotein particles (RNPs) such as Sm or U1RNP. Furthermore, the presence of antibodies specific for Sm, U1RNP, Ro, and dsDNA in serum has been linked with higher expression of interferon stimulated genes (ISGs) in SLE patients, supporting the clinical significance of these observations (Kirou et al., 2005).

1.8 Impaired clearance of immune complexes (antigen-antibody) in SLE

Impaired clearance is the inability to completely eliminate apoptotic cell material which has been shown to contribute in the pathogenesis of SLE (Mok and Lau, 2003). Deficiencies of early components of complement, such as C1q, C2 or C4 are rare, but they have been shown to be the strongest genetic susceptibility to SLE in humans, with a penetrance rate from 30% (C4 deficiency) to over 90% (C1q deficiency) (Walport, 2001, Manderson et al., 2004, Harley et al., 2006). A study carried out by Lood *et al* (2009) reported that circulating C1q inhibits formation of immune-complexes thus preventing activation of type 1 IFN-α production by pDC, suggesting a novel link between complement deficiency and the activation of the type I IFN pathway in SLE (Lood et al., 2009). Evidence has also shown that patients with SLE have relative deficiencies of the C3b/C4b receptor (CR1, CD35) on erythrocytes (E). This receptor is involved in the binding, transport and endocytosis of circulating immune complex bound complement components (ICC). Besides the influence of autoantibodies in SLE, the abnormalities in IC elimination are fundamental to the pathogenesis of SLE (Kavai, 2008).

1.9 SLE has a complex genetic basis

SLE involves a combination of both environmental and genetic factors. Genetic components include a high sibling risk ratio (Fairhurst et al., 2008), high heritability (greater than 66%), and higher concordance rates between monozygotic twins (20 to 40%) compared to dizygotic twins (2 to 5%) (Deapen et al., 1992, Alarcon-Segovia et al., 2005). A large number of genetic risk factors are associated with increased susceptibility to SLE. The genetically determined increased risk status has been referred to as a “threshold liability” (Wandstrat and Wakeland, 2001), which is expected to be highly polygenic in nature and widely variable between individuals. Environmental factors also affect SLE susceptibility and likely interact with this “threshold liability” (Niewold et al., 2010). Genetic contribution has been shown to be important in the development of the disease even though the concordance rate for SLE is only 25% among monozygotic twins (Rahman and Isenberg, 2008). More than 25 genetic risk loci have been identified in genome-wide association scans (GWAS). Interestingly, despite this remarkable progress, it is estimated that less than 10% of the total genomic susceptibility to SLE has been characterised (Moser et al., 2009). The genetic risk for SLE is likely derived from variation in many (perhaps as many as 100) genes, each of modest effect size with odds ratios between 1.15 and 2.0 (Harley et al., 2009).

1.10 Frequently observed alleles in SLE

Studies have shown that HLA-DRB1, interferon regulatory factor 5 (IRF5) and signal transducer and activator of transcription 4 (STAT4) are the three most often observed alleles contributing each for a little more than 1% of the variance

in genome-wide association studies (GWAS) (Gateva et al., 2009). Together they contribute to the alterations in the innate and adaptive immune systems. IRF5 is the gene outside the HLA locus that is most strongly and consistently associated with SLE (Niewold et al., 2010). IRF5 is a transcription factor expressed in plasmacytoid dendritic cells (pDCs) and B-cells. It is involved in the transcription of type I interferon (IRF5 activates IFNα production) and pro-inflammatory cytokines triggered by toll-like receptor 7 (TLR7), TLR8 and TLR9 signalling (Armstrong et al., 2009). IRF5 acts as a downstream of TLR-MyD88 signalling pathway in the induction of pro-inflammatory cytokines (Honda et al., 2005, Takaoka et al., 2005). IRF5 polymorphisms have been observed in the pathogenesis of SLE and distinctive IRF-5 isoforms have been confirmed across different ethnic backgrounds (Lee and Song, 2009, Graham et al., 2007). STAT4 plays a vital role in type I and type II IFN signalling pathways (Obermoser and Pascual, 2010). It has been shown to involved in proliferation, differentiation, and apoptosis in SLE (Niewold et al., 2010). Evidence has shown a solid relationship between STAT4 and SLE in GWAS and candidate-gene studies (Deng and Tsao, 2010). As mentioned above, genetic susceptibility (risk alleles) plays a major role in the pathogenesis of SLE; STAT4 and IRF5 have been established as addition risk factors for SLE.

Other studies have also shown that genes associated with SLE are involved in the following pathways as highlighted below (Gateva et al., 2009, Obermoser and Pascual, 2010):

1. Antigen presentation to the T-cell receptor of $CD4^+$ T-cells by HLA-DR1 HLA-DR2 and HLADR3.

2. The pathways involved in upstream and downstream of type I IFN: (a) components of TLR signalling pathways (interleukin 1 receptor-associated kinase 1 (IRAK1), IRF5, IRF7, IRF8, and tumour necrosis factor alpha-induced protein 3 (TNFAIP3)), (b) IFN signalling (STAT4), (c) intracellular DNA degradation (TREX1).
3. Signalling molecules activated after engagement of the T-cell receptor (TCR; such as TNFSF4/OX40L, Programmed cell death protein 1 (PDCD1), PTPN22, STAT4).
4. Signalling molecules activated after engagement of the B-cell receptor (BCR; such as B-cell scaffold protein with Ankyrin repeats 1 (BANK1), B-cell lymphocyte kinase (BLK), LYN, PTPN22) (Rieck et al., 2007, Arechiga et al., 2009).
5. Molecules involved in the clearance of apoptotic debris and immune complexes, such as FCGR2A/CD32 and FCGR3A/CD16 (Coxon et al., 1996). Additionally, C4A, C4B, C2 and C1Q.

1.11 Dendritic Cells (pDCs) in the pathogenesis of SLE

Dendritic cells (DCs) are antigen presenting cells that initiate adaptive immune responses. They are also capable of interacting with and influencing the responses of cells in the innate immune system. They are the initiators and regulators of immune responses (Steinman et al., 2003). It is believed that persistent activation of DCs could contribute to the pathogenesis of SLE (Banchereau and Pascual, 2006).

In humans and other mammals, pDCs are specialised immune cells that selectively express TLR7 and TLR9, which are key endosomal sensors of

microbial and self-ribonucleic acid (RNA) or DNA, respectively (Liu, 2005, Gilliet et al., 2008). It was evidenced that the activation of TLR7 or TLR9 by nucleic acids in pDCs triggers signal transduction, resulting in rapid and vigorous secretion of type I IFN, inflammatory cytokines, and chemokines (Honda et al., 2005, Gilliet et al., 2008). The TLR-induced IFN response is regulated by several immunoreceptor tyrosine-based activation motif (ITAM) bearing signalling receptors on pDCs (Blasius et al., 2006, Cao et al., 2009, Gilliet et al., 2008).

The above mechanisms play a fundamental role in the pathogenesis of SLE, although the definitive cause of SLE remains unclear.

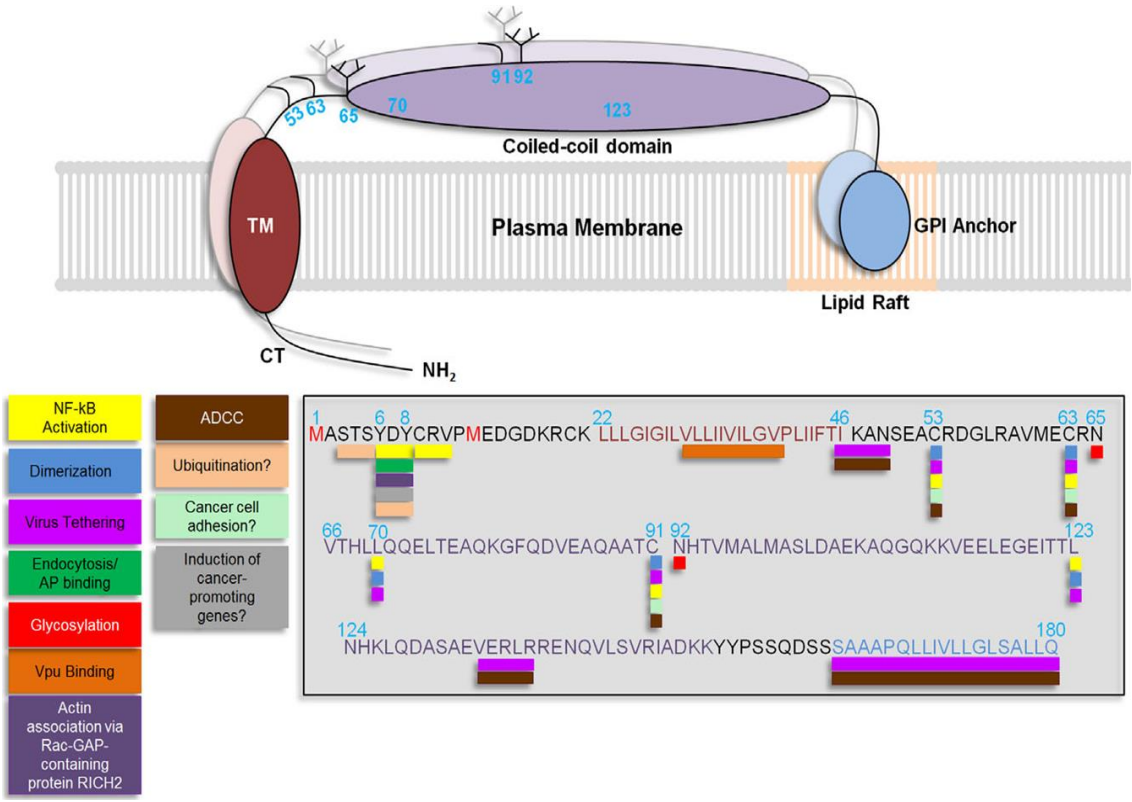
Tetherin

This study focuses on measuring the level of tetherin using flow cytometry, compared to IFN-inducible gene expression measured by quantitative reverse transcription polymerase chain reaction (qRTPCR).

Tetherin/CD317/HM1.24, also known as bone marrow stromal antigen 2 (BST-2), is a lipid raft associated protein that in humans is encoded by the *BST2* gene (Sauter, 2014, Mahauad-Fernandez et al., 2015). BST-2 was initially identified as a membrane protein in terminally differentiated human B-cells of patients with multiple myeloma (Goto et al., 1994, Ohtomo et al., 1999). It was later rediscovered as a potent antiviral restriction factor with the ability to tether enveloped viruses to the cell membrane of infected cells via its GPI anchor (Neil et al., 2008). It also potently inhibits virus replication in cultured cells and *in vivo* (Mahauad-Fernandez et al., 2014b, Mahauad-Fernandez et al., 2015). BST-2 is an alpha interferon-inducible cellular molecule that impairs and/ or inhibits the

release of Human immunodeficiency virus type 1(HIV-1) and other enveloped viruses (Neil et al., 2008, Van Damme et al., 2008) (Figure 3). It functions as a negative-feedback regulator of IFN production by pDCs (Bego et al 2012). BST-2 has been stated to consists of an N-terminal transmembrane region, a central coiled coil motif, and a putative C-terminal glycosylphosphatidylinositol (GPI) anchor motif (Kupzig et al., 2003, Andrew et al., 2011, Sauter, 2014) (Figure 2). It is a 30-36 kDa type II transmembrane protein that consists of 180 amino acids (Ishikawa et al., 1995). The BST-2 ectodomain encodes three cysteine residues (Goto et al., 1994, Andrew et al., 2009, Perez-Caballero et al., 2009)), which are believed to autonomously contribute to the formation of covalent cysteine-linked dimers (Andrew et al., 2009, Perez-Caballero et al., 2009). BST-2 inhibits virus release by physically tethering viral particles to the cell surface via its TM motif and GPI anchor. This protein is constitutively expressed in mature B cells, plasma cells and pDCs, and in many other cells; but only as a response to IFN stimulation. This study will investigate the role of tetherin in the pathogenesis of SLE.

Figure 2: BST-2 structure

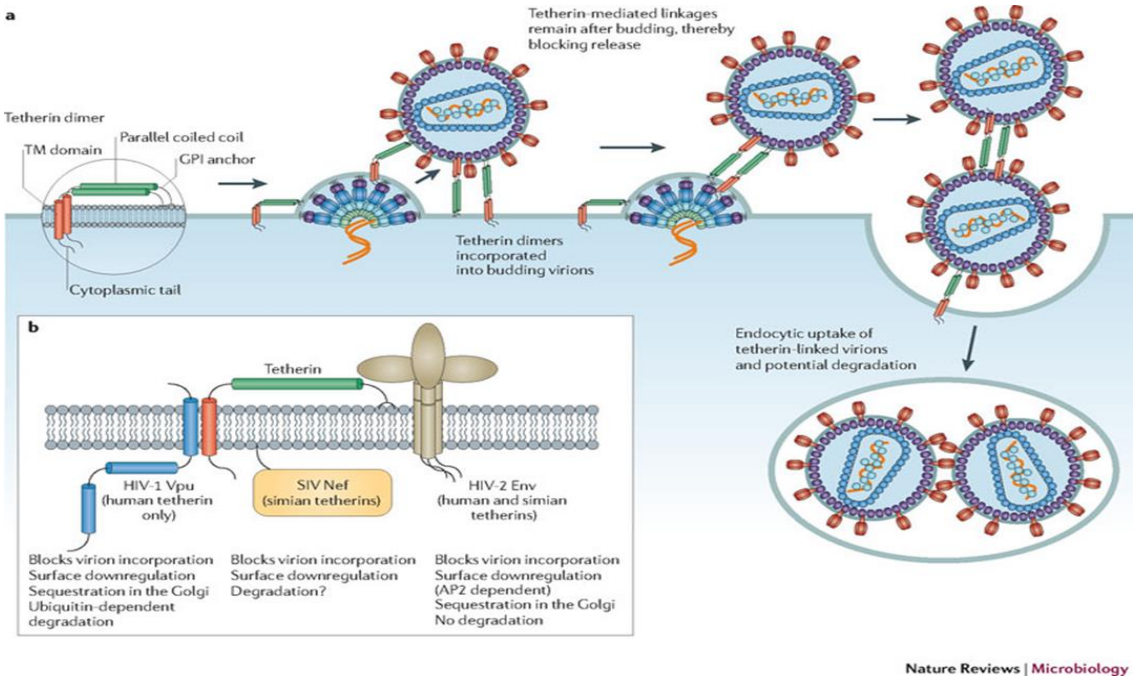


BST-2 is a type II transmembrane protein with a N-terminal cytoplasmic tail (CT) , a transmembrane domain (TM), a coiled-coil domain and a glycosylphosphatidylinositol (GPI) anchor embedded in lipid rafts in the cell membrane (Mahauad-Fernandez and Okeoma, 2016). The amino acid sequence of BST-2 depicted in the grey box is colour coded with their respective domains. Numbers on top of amino acids correspond to amino acid location. Underneath the amino acid sequences are colour boxes corresponding to different functions and characteristics of BST-2 as shown on the left corner of the Fig2. BST-2 contains two translational start sites at methionine 1 and 13 (red) generating a long and short isoform, respectively (Mahauad-Fernandez and Okeoma, 2016). The short isoform cannot induce NF-kB activation since it lacks the YXY motif. BST-2 forms homo-dimers and tetramers through three conserved cytosine residues at positions 53, 63 and 91. Leucine residues at positions 70 and 123 are important for maintaining the structure of BST-2 and for virus tethering, which also requires the C-terminal GPI anchor. Taken from Mahauad-Fernandez and Okeoma, 2016. The role of BST-2/Tetherin in host protection and disease manifestation, ***Immunity, Inflammation and Disease***; 4(1): 4-23.

1.12 The Tetherin Protein and Mechanism of Virion Retention

BST-2 was rediscovered in 2008 as the host agent responsible for preventing and/or inhibiting the release of HIV-1 with mutated viral protein U (Vpu) from host cells (Neil et al., 2008, Van Damme et al., 2008); and it was later renamed tetherin (Neil et al., 2008). HIV-1 counteracts the antiviral function of BST2 by expressing VPU (Neil et al., 2008, Van Damme et al., 2008). However, in the absence of VPU, virus particles are prevented from budding off the cellular membrane in cells that express BST-2, resulting in virions being tethered to the plasma membrane (Figure 3) (Neil et al., 2008). By tethering enveloped viruses, BST-2 prevents virus release which in return stimulates and amplifies innate immune responses through the induction of cytokine/ chemokine expression (Galao et al., 2012, Mahauad-Fernandez et al., 2014b). This process is believed to largely involve BST-2 cytoplasmic tail (Mahauad-Fernandez and Okeoma, 2016). It is not clear whether BST-2 acts as the actual tether or whether BST2-dependent tethering occurs in all BST-2 expressing cell types (Miyagi et al., 2009). BST-2 was shown to be induced by type I, type II and type III IFNs (Blasius et al., 2006), suggesting that BST-2 is part of the innate antiviral response triggered in infected cells.

Figure 3: The mechanism of tetherin as an antiviral molecule



A, In macrophages and in interferon-stimulated CD4⁺ T-cells, the antiviral membrane protein tetherin (also known as M1.24, BST2 or CD317) becomes incorporated into the nascent virion. However, the parallel tetherin dimers do not inhibit viral assembly and membrane scission; they are thought to form physical crosslinks between the cell and the virion by virtue of their dual-membrane anchors (Martin-Serrano and Neil, 2011). This leads to virion accumulation on the cell surface and subsequent internalisation to late endosomes. **B**, Primate immunodeficiency viruses encode countermeasures that interact with tetherin (Vpu for HIV-1, Nef for most simian immunodeficiency viruses (SIVs) and envelope glycoprotein (Env) in the case of HIV-2). These interactions can be species specific and result in inhibition of the antiviral activity of tetherin, often accompanied by cell surface removal of the protein, its intracellular sequestration and, ultimately, its degradation (Martin-Serrano and Neil, 2011). Image was from http://www.nature.com/nrmicro/journal/v9/n7/fig_tab/nrmicro2596_F4.html.

BST-2 is underrepresented in plasma membranes from cells expressing VPU (Douglas et al., 2009) and also the K5 protein of Kaposi's sarcoma-associated herpesvirus (KSHV) (Bartee et al., 2006). K5 is a viral homologue of the cellular

transmembrane ubiquitin ligases, called membrane-associated RING-CH (MARCH) proteins (Bartee et al., 2004), which mediate the ubiquitination of the cytoplasmic portion of the transmembrane proteins (Nathan and Lehner, 2009). Each member of this family targets a subset of cellular membrane proteins with both unique and shared specificities (Bartee et al., 2006, Wang et al., 2008). The downregulation of BST2 by K5 suggests that K5 also counteracts innate antiviral responses (Mansouri et al., 2009). Studies have shown that most transmembrane proteins targeted by viral or cellular MARCH proteins are type I transmembrane proteins of the immunoglobulin superfamily; whereas, BST2 is a type II transmembrane protein (Kupzig et al., 2003).

pDC activation and IFN production are associated in autoimmune diseases therefore a mechanism controlling pDC IFN production is essential (Cao et al., 2009). Activation of TLR7 and / or TLR9 by nucleic acids in pDCs triggers signal transduction, resulting in rapid and abundant secretion of type I IFN, chemokines and inflammatory cytokines (Honda et al., 2005, Gilliet et al., 2008). A study carried out by Cao *et al* (2009) identified BST2 as a physiological ligand for a human pDC-specific receptor immunoglobulin-like transcript 7 (ILT7) (Cao et al., 2009). They also discovered that pDCs play an important role in antiviral innate immune responses by secreting large quantities of IFN-α/β. BST2-ILT7 interaction, serves as an important negative feedback mechanism to prevent prolonged IFN production after viral infection (Cao et al., 2009). Despite BST-2 antiviral functions, it is believed to be involved in SLE disease manifestation, a function associated to the ability of BST-2 to encourage cell to cell interaction (Mahauad-Fernandez and Okeoma, 2016).

Looking at the above mechanisms that contribute to the development of SLE, there is clearly a need for an accurate assay and the discovery of new biomarkers for routine clinical practice. There has been extensive investigation on the understanding of SLE pathogenesis and only a few biomarkers have been validated and widely accepted (Liu et al., 2005). However, there is a lack of reliable, specific biomarkers for SLE which can precisely assess disease activity, identify patients at risk for flares and organ damage and aid in the management of patients. Severe flares of disease may occur unpredictably, and markers of response to therapy are needed to guide the effective use of immunosuppressant drugs and glucocorticoids. Despite the fundamental role played by IFN in pathogenesis, there is currently no assay for IFN used in routine clinical practice. Additionally, accurate tests for IFN activity are needed to stratify patients for IFN-blocking biologics, and achieve highest response rates.

1.13 Biomarkers for SLE

There has been extensive effort and devotion to approach the several challenges of SLE including the development of diagnostic tests and biomarkers to inform the clinical management of SLE patients (Liu et al., 2013). The investigations for SLE (lupus) biomarkers to diagnose, stratify, monitor, and predict an individual's response to therapy has been remarkable. However, there are still numerous unmet needs in SLE research and patient care (Liu et al., 2013). These are largely due to the lack of reliable lupus biomarkers for diagnosis, stratification, monitoring, and prediction of response to treatment (Liu et al., 2013). A biomarker is defined as ''a measurement, including but not

limited to a genetic, biochemical, molecular, biological, or imaging event whose alterations correlate with the pathogenesis and/or manifestations of a disease and can be evaluated quantitatively and/or qualitatively in laboratories'' (Illei et al., 2004a, Illei et al., 2004b).

SLE biomarker reports have been published during the past years; however, the majority of these studies were carried out on small numbers of patients and they have been limited to cross-sectional observations.

The development of biomarkers has improved diagnosis and monitoring of the disease, however, there is no specific biomarker. This is the reason why, researchers have evolved toward the discovery and validation of lupus biomarker 'panels' for diagnosis and disease activity monitoring (Liu et al., 2013).

This project will focus on investigating a biomarker and developing an accurate assay for SLE diagnosis and/or prognosis. The purpose of the biomarker is to assist in making a precise diagnosis and/or monitoring disease activity, which may help in predicting the onset of SLE in susceptible individuals and/or development of flares in patients with established SLE. It will also aid in assessing the effectiveness of therapeutic interventions. If the outcomes of the findings are successful and reproducible this may allow the proactive institution of therapeutic and preventive strategies so that the therapeutic efficacy can be improved with minimised side effects.

1.14 Discovery

A previous study by Dr Vital's group in Leeds (El-Sherbiny et al., 2016) speculated that changes in numbers of strongly IFN-stimulated gene signature (IFNGS) positive populations, such as B-cells, which are characteristic of autoimmunity, could lead to falsely positive or negative IFNGS analysed using whole blood. They performed a gene expression analysis of unsorted peripheral blood mononuclear cells (PBMCs) and sorted cell subsets, with B-cells that were physically sorted and analysed separately by gene expression. This demonstrated a positive IFNGS, which was not apparent if the whole blood unsorted sample was analysed. The greatest contribution to overall IFNGS status was made by monocytes, which have both highest expressions of interferon-inducible genes as well as being a large proportion of PBMCs.

Cell sorting is not feasible as a diagnostic test so they therefore sought a cell surface protein marker that represents IFN activity to allow analysis of individual populations conveniently using flow cytometry. Tetherin was found to be expressed on all blood cell types and whose level corresponds to subset-specific gene expression IFNGS (El-Sherbiny et al., 2016).

In cross sectional studies, Tetherin was measured on several circulating cell subsets and compared to disease activity as well as plasmablast numbers (another biomarker of B cell activity and disease activity in research studies). Memory B cell tetherin was shown to correlate better with these parameters than tetherin on monocytes or interferon-stimulated gene expression (El-Sherbiny et al., 2015). This is consistent with the prominence of B-cells in models of SLE pathogenesis.

Our group developed and clinically validated a 2-score system (IFN-Score-A and -B) using Factor Analysis of 30 ISGs measured by TaqMan selected from 3-IFN annotated modules. These scores were evaluated using in-vitro IFN stimulation as well as in sorted cells and they were then clinically validated in a cohort of 328 autoimmune disease patients and healthy controls. ISGs varied in response to IFN-subtypes and both scores varied between cell subsets. However, IFN-Score-A differentiated Systemic Lupus Erythematosus (SLE) from both Rheumatoid Arthritis (RA) and Healthy Controls (HC), while IFN-Score-B differentiated SLE and RA from HC (El-Sherbiny et al., 2015). In SLE, both scores were associated with cutaneous and haematological but not musculoskeletal disease activity. Due to these outcomes, Score A would be compere against tetherin expression level on the cell subsets.

1.15 Hypothesis

The overall hypothesis of this study is: *flow cytometric assessment of tetherin will provide a convenient assay for Type I interferon activity that has clinical utility*. I will investigate this under the following sub-hypotheses:

Hypothesis 1: Cell surface tetherin can be measured accurately using a whole blood assay in a routine diagnostic laboratory.

Hypothesis 2: Tetherin will differentiate SLE from Healthy control

Hypothesis 3: SLE patients in remission with higher level of tetherin on memory B cells have significantly higher risk of flare compare to patients in remission with lower or normal level of tetherin.

Hypothesis 4: SLE patients who are flaring will show a reduction in tetherin level following treatment.

Hypothesis 5: Tetherin may have value as part of a biomarker panel to select patients for targeted therapy.

1.16 Aims

To validate a flow-cytometry based assay for IFN activity as a clinically useful biomarker in SLE.

1.17 Objectives

In order to test my hypothesis I will:

1. Optimise a whole blood assay for tetherin in a routine clinical laboratory and compared to isolated peripheral blood mononuclear cells (PBMCs) assay results.

2. Collect a cross-sectional sample of SLE patients (and healthy controls) through Leeds SLE clinic via the standard pathology blood service and measure tetherin by flow cytometry. I will also collect diagnosis and treating physician opinion of flare or remission as well BILAG disease activity score and extract RNA and perform an existing interferon-stimulated gene expression score as an alternative interferon biomarker for each analysis.

3. Collect flare rate in follow up for patients in remission at the time of initial sampling

4. Evaluate tetherin as an ‘‘IFN high’’ biomarker (for interferon-blocking therapy) alongside the criteria for an alternative biologic therapy (belimumab).

2.0 Materials and methods

2.1 Ethical Considerations

As the project involved acquiring human blood samples, it was necessary to obtain ethical approval before the project could commence. Ethical approval was provided by Leeds East National Research Ethics committee, approval reference number (REC 10/H1306/88).

In order to minimise distress to the patients, blood samples for the study were collected during venepuncture carried out as part of routine clinical care. For collection of blood from both the patients and healthy controls, samples were collected in ethylenediaminetetraacetic acid (EDTA) tubes. Samples collected were used for cell surface phenotyping (whole blood lysis) and PBMC isolation and were used within 24 hours of collection. For whole blood lysis and staining 5 mL of EDTA blood samples were sent to the laboratory for each patient and control. Samples were stored at 4^{o}C. All data related to the study were stored on password protected computer systems.

2.2 Patients and Control Selection

Patients with an established diagnosis of SLE were identified from the Leeds Teaching Hospitals NHS Trust connective tissue clinic in Leeds, and categorised as in remission or flare according to BILAG 2004 and by physicians treating. Informed written consent was obtained from patients and suitable healthy volunteers.

Samples obtained from patients were divided in two groups as follows:

1. For SLE patients in remission, can Cell-Specific Interferon Marker (CSIM) predict flare in subsequent 12 months (n=80 patients).
2. For new onset SLE or undifferentiated connective tissue disease (CTD), can CSIM predict progression to further organ involvement (n=40 patients).
3. For SLE patients with current flare who start treatment, is CSIM responsive (n=40 patients).

2.3 Preliminary Investigation whole blood assay: titrations, template and compensations

2.3.1 Equipment and consumables

FACSCanto II Flow Cytometer (Diva 7 software) (Becton Dickinson), FACS flow tubes (Becton Dickinson) 50 mL Falcon tubes (Becton Dickinson), haemocytometer, light microscope, laboratory booking in forms, laboratory coat, gloves disposable (Regional Supplies Dept), goggles coverall (BDH). Pastettes (Regional Supplies Dept), Eppendorf pipette 0.5-10 µL, Eppendorf pipette 200-1000 µL. Pipette tips polypropylene 5-100 µL yellow, pack of 1000 (Regional Supplies Dept). Pipette tips polypropylene 200-1000 µL blue, pack of 100 (Regional Supplies Dept). Monoclonal antibodies and isotypes, (Miltenyi Biotec and Biolegend).

2.3.2 Antibody optimisation

The optimal antibody concentration was determined experimentally for each antibody by using a series of dilutions of antibodies. Blood samples from two healthy volunteers were used. Eight antibodies were used for this project (Table 3) and for each antibody six FACS flow tubes (Becton Dickinson) were labelled with the antibody (Miltenyi Biotec and Biolegend) and the volume to be added into the tubes for the titration method. The antibody volume (0 µL, 1 µL, 2.5 µL, 5 µL, 7.5 µL and 10 µL) was carefully pipetted into the correct test tube. The samples were processed as described in 2.3.3.

2.3.3 Preparation of lymphocytes for whole blood lysis staining

50 mL Falcon tubes (Becton Dickinson) were labelled with the patients' or healthy volunteers' initials. The blood samples were mixed well and 4 mL of healthy sample (EDTA whole blood) was added into the appropriate Falcon tube and 46 mL of the freshly prepared working red blood cells (RBCs) lysing buffer was added (working RBCs lysing buffer was prepared as manufacturer recommendation catalogue number 349202 (Becton Dickinson) diluted 1:10 with distilled water)). The tubes were vortexed and incubated for 10 min at room temperature and were subsequently centrifuged at 500 g for 10 min. The supernatant was decanted. The cells were suspended and washed by adding 50 mL of Phosphate-buffered saline / 1% foetal bovine serum (%PBS / FBS) solution (FACS wash buffer) into the Falcon tube; and the tubes were centrifuged at 500 g for 10 min. The washing procedure was repeated. After centrifugation 50 mL of 1% PBS / FBS wash buffer was added into the Falcon tubes and the samples were mixed gently by inversion and 10 µL of the sample

was pipetted into the haemocytometer and the cells were counted using haemocytometer and light microscope and the cells number recorded. After cells were counted for each sample, the tubes were centrifuged at 500 g for 10 min.

After centrifugation, supernatant was decanted. Cells were resuspended in blocking buffer (composed of IgG from human serum (appendix 1)) at a cell density of 20 million/mL and left for 5 min at room temperature. 50 µL of the cells was added into the series of dilutions of antibodies, 0 µL, 1 µL, 2.5 µL, 5 µL, 7.5 µL and 10 µL (Table 3). The antibodies were pipetted to the bottom of the FACS flow tubes and the pipette tip was changed for each antibody. The tubes were mixed gently by vortexed for 3 s and tubes were incubated for 30 min at 4°C; samples were protected from direct light. The cells were subsequently washed by adding 3 mL of 1% PBS / FBS into each tube, then vortexed and centrifuged at 500 g for 5 min. Supernatants were decanted and the washing procedure repeated. The cells were then resuspended in 400 µL cell fix buffer (PBS + 0.5% formaldehyde). The tubes solution were mixed and left in ice for 10 min, to prevent the formation of aggregates. The samples were acquired on FACSCanto II Flow Cytometer (Diva 7 software). The samples were acquired at threshold of 50,000 events per sample tube (Figure 4). The voltages of the forward scatter and side scatter were set using the unstained (0 µL) tube.

Antibodies used for cellular staining list were added into each tube as listed

Antigen	Isotype	Fluorochrome	Manufacturer	Clone
CD27	Mouse IgG1κ	Viobright FITC	Miltenyi Biotec (cat #130-104-845)	M-T271
CD19	Mouse IgG1	VioBlue	Miltenyi Biotec (cat # 130-098-598)	LT19
CD38	Recombinant human IgG	PE-vio770	Miltenyi Biotec (cat # 130-108-838)	REA572
CD8	Mouse IgG2a	PerCP-Cy5.5	Miltenyi Biotec (cat # 130-094-972)	BW135/80
CD56	Recombinant human IgG	APC	Miltenyi Biotec (cat # 130-100-698)	REA196
CD3	Mouse IgG2a	VioGreen	Miltenyi Biotec (cat # 130-096-910)	BW264/56
CD4	Mouse IgG2a	APC-Vio770	Miltenyi Biotec (cat # 130-096-652)	VIT4
CD317	Mouse IgG1κ	PE	Biolegend (cat # 348406)	RS38E

Table 3: Antibodies and volumes used for titration procedure

2.3.4 Data analysis for titration

During acquisition, the cell populations (lymphocytes, monocytes and neutrophils) were identified by their light scatter using forward side scatter (FSC) and side scatter (SSC), and voltages were adjusted to determine the cell populations (Figure 4). Voltages were set using unstained cells. On the

lymphocytes population a gate was drawn (P1) which helped with determining the population on the second dot plot SSC against CD19 VioBlue (Figure 4). The positive and negative population, were defined by quadrant gating in the dot plots and interval gates on histogram plots (Figure 4). The procedure was repeated for the remaining antibodies (Table 3). Statistical analysis was acquired of the data corresponding percentage (%), mean fluorescence intensity and median fluorescence intensity as shown in (Figure 4).

Figure 4 shows titration analysis between unstained and stained

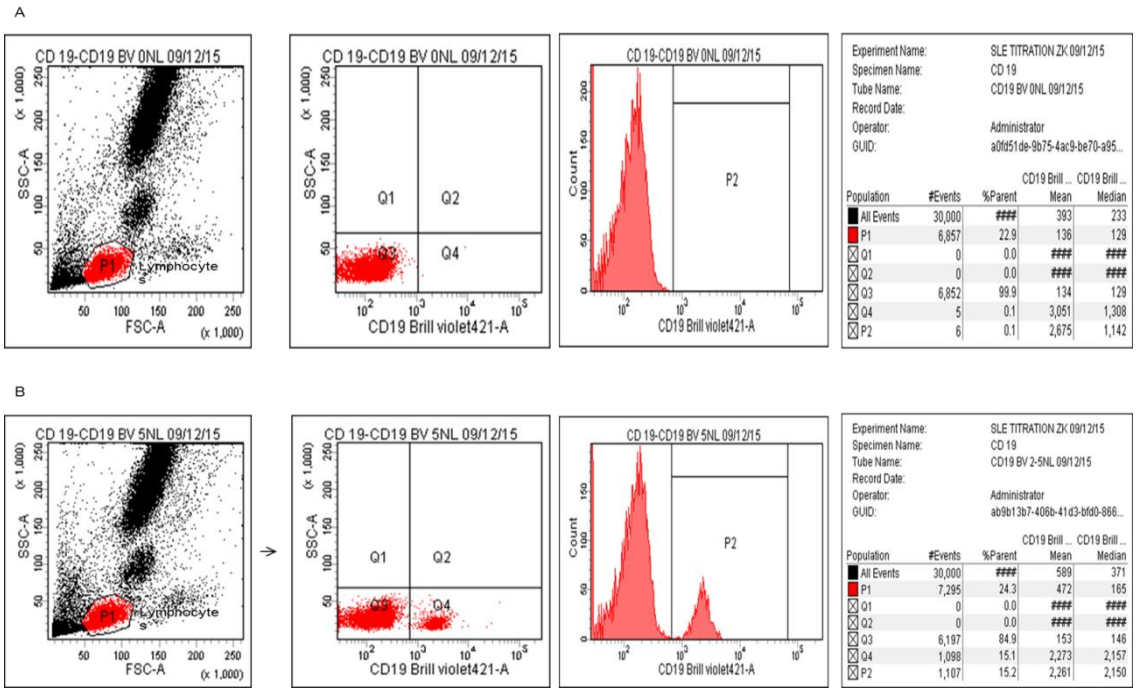


Fig 4: A snap shot of the titration analysis procedure. A, shows the unstained tube. B shows the stained tube (5 µL) of CD19. The voltages of the forward scatter and side scatter to determine the cells population. The other antibodies population were determined in a similar manner. The % parent, mean and median was determined by P2 = the positive population stained by antibody plot on the histogram, as showed above. The statistical analysis generated the mean and median values.

Figure 5 : Monoclonal antibodies titration as part of optimisation process.

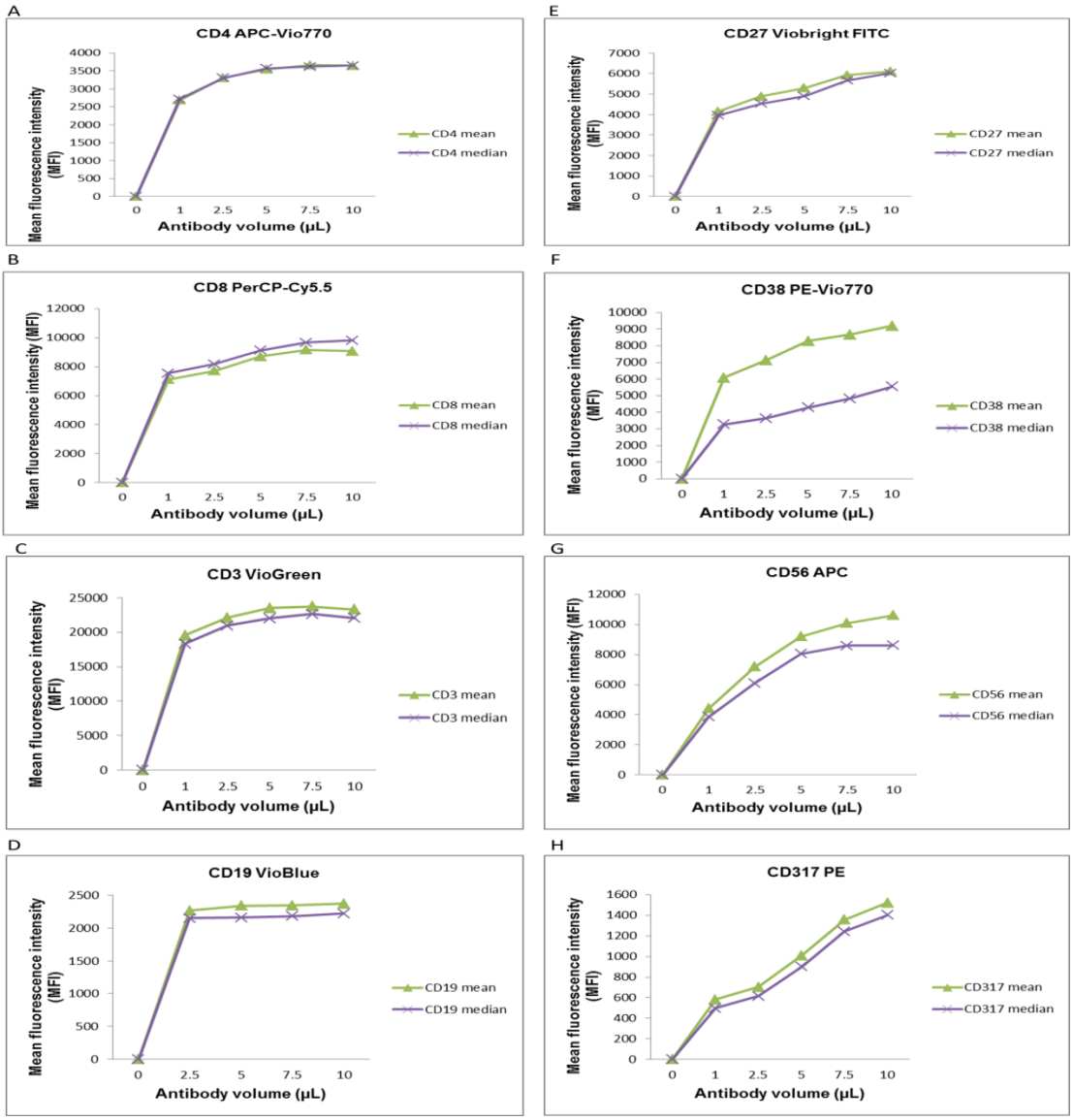


Fig 5: Titrations**:** Image A-H shows the series of titration of the antibodies. 10 µL was recommended by the manufactures; however, after titration and data were plotted we decided to use 5 µL for each antibody. This volume will deduce accurate and reliable results at a reduced cost.

Figure E, F, G and H shows an increased above 5 µL, however, 5 µL was used in the study. This was because the negative population bleed (spread) into the positive population. We found that 5 µL / 50 µL staining buffer was effectively saturating the cells for staining and maintain MFI, noting that manufacturer

recommended dose was equivalent to my finding (10 µL per 100 µL staining buffer for 10 million cells maximum) (Table 4 and Figure 5) a template was created (Figure 6) to assess the phonotype of B-cells, T-cells and Natural Killer (NK) cells. Compensation was carried out to ensure there is no bleeding in other channels (Figure 7and Figure 8).

2.3.5 Preparing compensation control samples

Compensation control samples were prepared, processed and acquired as described previously in 2.3.3. However, with the compensation control samples, eleven FACS Flow tubes were labelled; eight tubes for the single stain, one unstained, one Isotype (ISO) and one test. The Test tube had all eight antibodies and single stain tubes had one antibody into each tube (Table 4). The antibodies were pipetted into the bottom of the FACS Flow tubes. The test tube had premade cocktail of antibodies of 40 µL. An ISO (Miltenyi Biotec) cocktail of corresponding antibodies (40 µL) was added into the labelled ISO tube.

Antibodies were added into each tube as listed below into the labelled test tube

Monoclonal antibody	Monoclonal	Fluorochrome	Manufacturer	Clone	Volume per test (µL)
Human CD27	Mouse IgG1κ	Viobright FITC	Miltenyi Biotec (cat #130-104-845)	M-T271	5
Human CD19	Mouse IgG1	VioBlue	Miltenyi Biotec (cat # 130-098-598)	LT19	5
Human CD38	Recombinant human IgG	PE-vio770	Miltenyi Biotec (cat # 130-108-838)	REA572	5
Human CD8	Mouse IgG2a	PerCP-Cy5.5	Miltenyi Biotec (cat # 130-094-972)	BW135/ 80	5
Human CD56	Recombinant human IgG	APC	Miltenyi Biotec (cat # 130-100-698)	REA196	5
Human CD3	Mouse IgG2a	VioGreen	Miltenyi Biotec (cat # 130-096-910)	BW264/ 56	5
Human CD4	Mouse IgG2a	APC-Vio770	Miltenyi Biotec (cat # 130-096-652)	VIT4	5
Human CD317	Mouse IgG1κ	PE	Biolegend (cat # 348406)	RS38E	5

Table 4: Monoclonal antibodies and volumes used to process or stain the labelled test tubes

Acquisition of sample- Samples were acquired and recorded on the SLE template panel.

Panel	Stopping GATE Population	Number to acquire
Singles stained	Lymphocytes	50,000 events
Unstained	Lymphocytes	50,000 events
Iso	Lymphocytes	50,000 events
Test panel	$CD19^{+}$	30,000 events

Table 5: Results were recorded on this format and then exported as an experiment or FCS files for backup.

Figure 6- Acquisition template, shows a snap shot of the labelled test tube after compensation had been carried out.

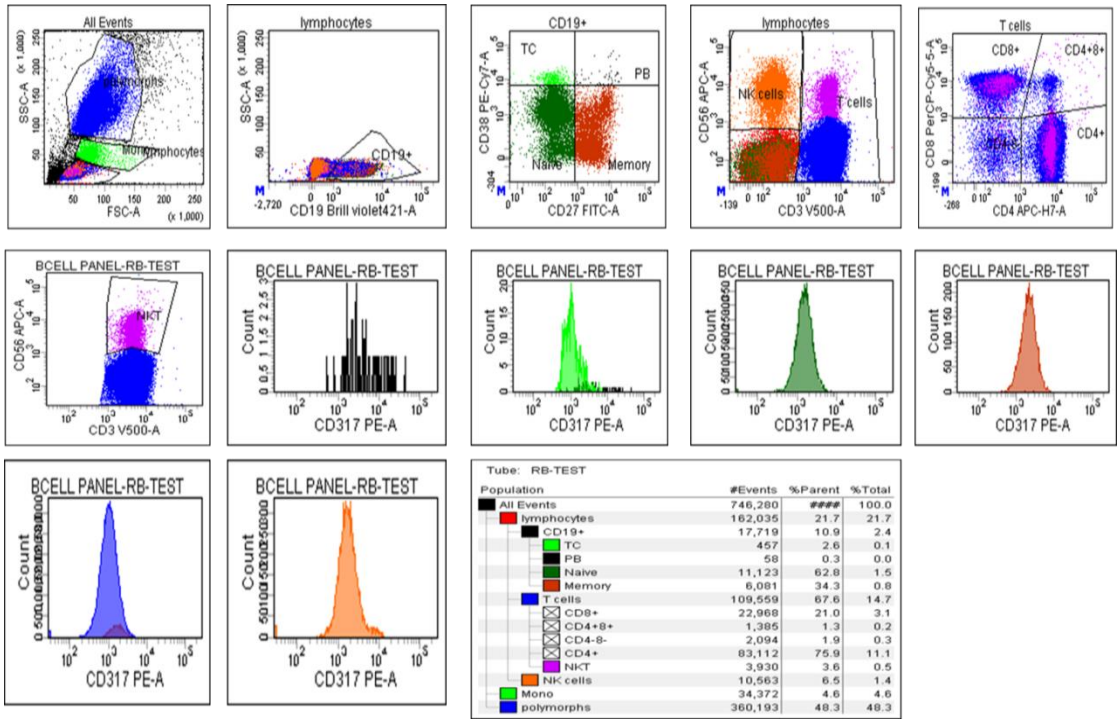


Fig 6: A screen shot of the SLE panel template of whole blood staining. The dot plot shows the identification of naïve and memory B-cells, plasmablasts, CD3+ T-cells, NK-cells and monocytes. The histogram represents the intensity level of tetherin protein present in each of these cell types.

2.3.6 Compensation analysis

Compensation was carried out to correct for the signal spillover from a given fluorochrome into the neighbouring channels (Figure 7 and Figure 8). To correct spillover, spectral overlap values were adjusted for all fluorophores and in all detectors, via single-colour controls. Compensation was performed either by decreasing or increasing the values in equation. As indication of good compensation, the mean/median fluorescence intensities (MFIs) of the positive and negative populations of the compensation control were aligned in the neighbouring channels (Figure 8). Compensation was correctly set when the median of the negative population is equal to the median of the positive population in the spillover channel.

The procedure was repeated after each fluorochrome that was compensated. Compensated fluorochromes were saved on the cytometer settings and on to the tubes, to ensure each fluorochrome was compensated against each other (Figure 8). Fluorescent minus one (FMO) were run once to ensure appropriate gating positioning.

Figure 7 and Figure 8 shows a snap shot of how compensation was performed.

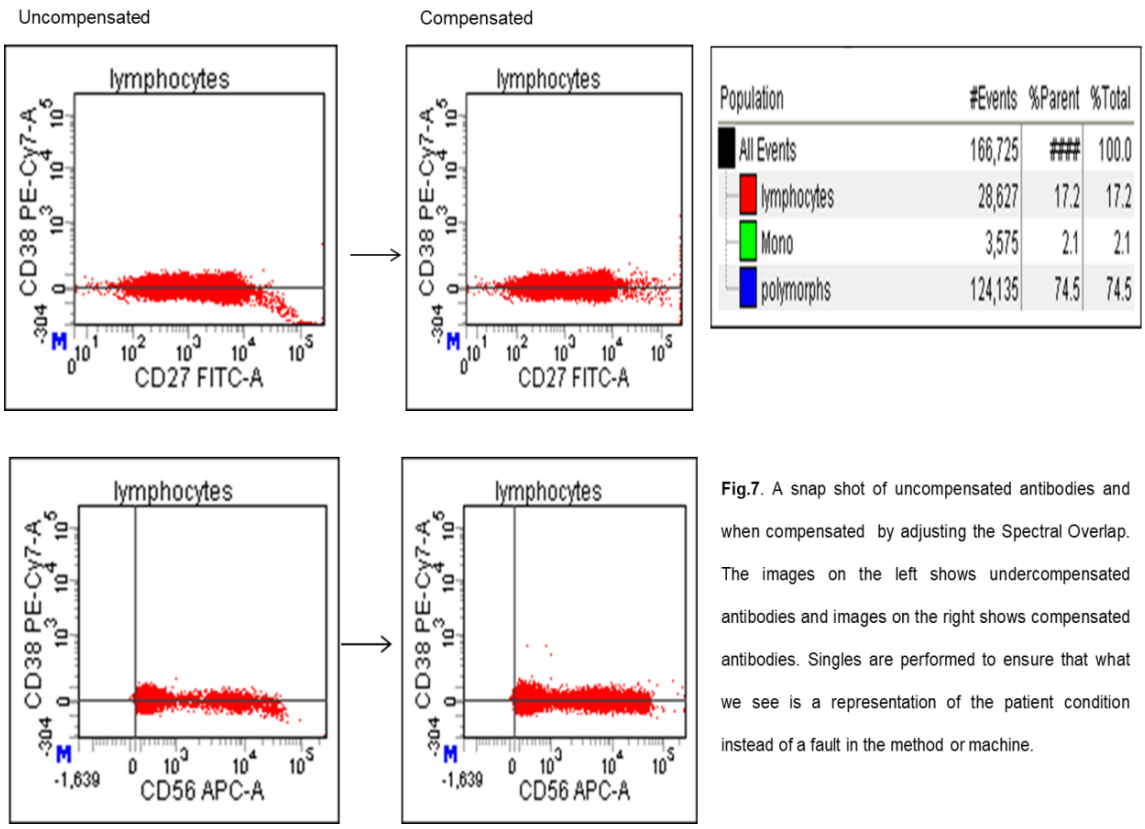


Fig.7. A snap shot of uncompensated antibodies and when compensated by adjusting the Spectral Overlap. The images on the left shows undercompensated antibodies and images on the right shows compensated antibodies. Singles are performed to ensure that what we see is a representation of the patient condition instead of a fault in the method or machine.

Figure 8 shows how each antibody is compensated against the other antibodies.

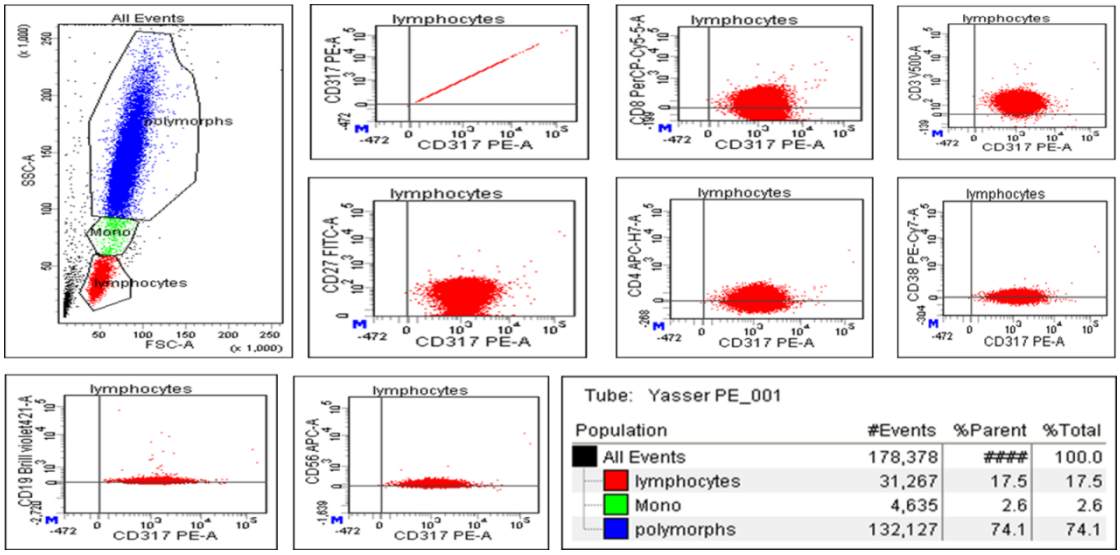


Fig.8. A snap shot of a manually compensated CD317 PE against all eight antibodies. The SSC and FSC voltages were set with unstained sample. Antibodies were compensated by clicking on the cytometer window compensation and adjusted the Spectral Overlap, either by decreasing or increasing the values as some antibodies were either under or over compensated. This process was repeated for each antibody.

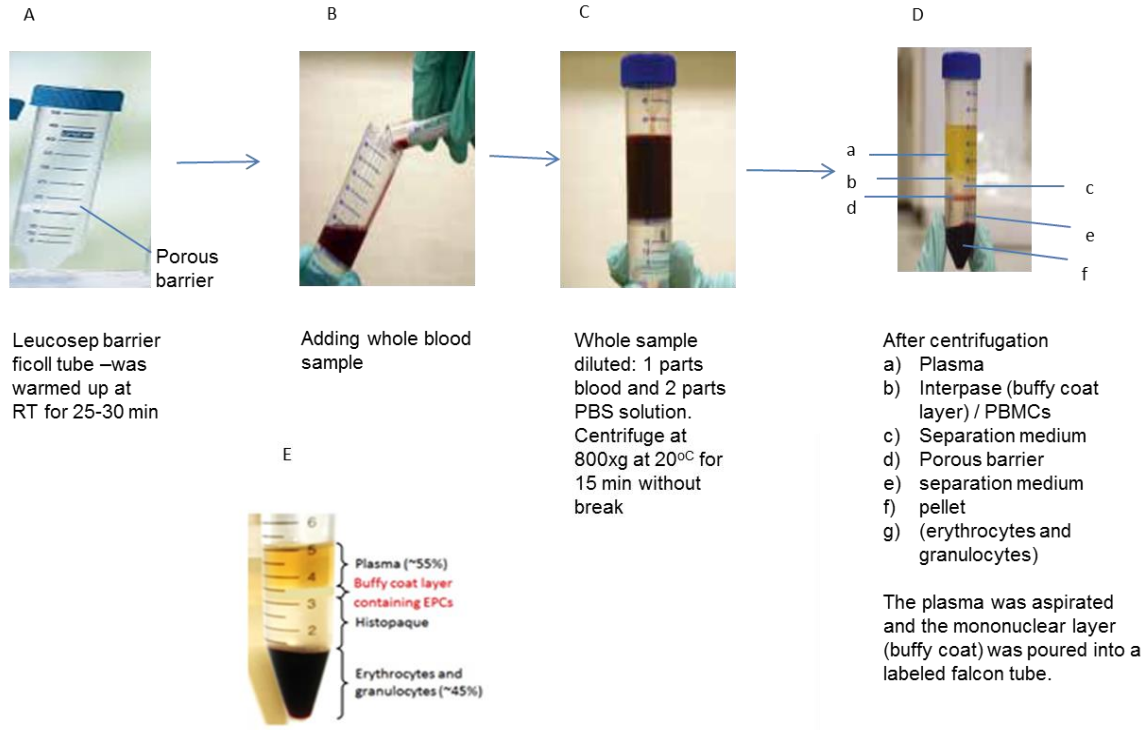
2.4 Main study methods

2.4.1 Preparing patients' whole blood samples for flow cytometry

Patients' samples were prepared and acquired as described previously in 2.3.3 However, quality control single labelled cells (single staining) was performed every two months or when a new vial was opened. Unstained, Isotype control stained, and Test staining were performed (processed) for every sample processed. Figure 6 shows a representation of a test sample.

2.4.2 Isolation of peripheral blood mononuclear cells (PBMCs) from whole blood using Leucosep Barrier Ficoll Tube

Figure 9 PBMCs separation



These images demonstrate the steps in obtaining PBMCs from blood samples. Images A-D were taken from http://www.greinerbioone.com/UserFiles/File/Catalogue%202007/Kapitel_9_E.pdf. Taken 17/07/17

Image E was taken from https://www.google.co.uk/search?q=Leucosep&source=lnms&tbm=isch&sa=X&ved=0ahUKEwiMmlHT45DVAhUqC8AKHfoSCNIQ_AUIBigB&biw=1280&bih=929&dpr=1#imgdii=YGvAa8Hf-hmaxM:&imgrc=FNhnssQj8OJGqM:&spf=1500310586248. Taken 17/07/17

Fig 9: Snapshot of PBMCs separation procedures

The required numbers of Leucosep tubes were left at room temperature (25-30°C) prior to use. Prior to the isolation of PBMCs from whole blood specimens, the Leucosep Barrier Ficoll tubes and the 50 mL Falcon tubes were labelled with patients' identifiers on the tubes and on the lids, obtained from the 20 mL of EDTA whole blood. The whole blood samples were thoroughly mixed before

adding into the appropriate Leucosep Barrier Ficoll tube and were diluted at a ratio of 1 part blood to 2 parts PBS solution. Leucosep Barrier Ficoll tubes were centrifuged at 800 g at 20^{o}C for 15 min in a bucket and rotor centrifuge (Eppendorf). After centrifugation, three layers occurred above the barrier: a plasma layer, the interphase containing the PBMCs and a small layer of Ficoll (Figure 9). The plasma layer was aspirated. The whitish buffy coat (PBMCs) formed in the interphase was carefully aspirated into the appropriate Falcon tubes. PBMCs were washed three times with PBS, centrifuged at 500 g at 20^{o}C for 10 min the procedure was repeated twice. The cells were checked for remaining red blood cells (RBCs). If RBCs were present (reddish pellet), freshly prepared RBCs lysing buffer was added ((Becton Dickinson) dilute to 1/10 with distilled water) to lyse the RBCs. The cells were mixed thoroughly and left for 10 min at room temperature; tubes were centrifuged at 500 g at 20^{o}C for 10 min and washed twice with PBS.

The cells were counted using the TC20 automated cell counter (BioRAD) and stained as described in 2.3.3. The remaining PBMC cells were lysed using 250 µL RNA lysis buffer as manufacturer recommendation (RNA lysis buffer; 1000 µL of RNA lysing (RL) buffer (Norgen Biotec) and 10 µL of β-Mercaptoethanol (MJ148-9m) using RNAse free tips applied in appropriate labelled Eppendorf tubes and stored at -20^{o}C for short-term (2-4 weeks) and at -80^{o}C for long-term storage (6 months).

2.4.3 RNA extraction: procedure for Purifying Total RNA using Norgen's Animal Tissue RNA Purification Kit

Figure 10 RNA extraction procedures

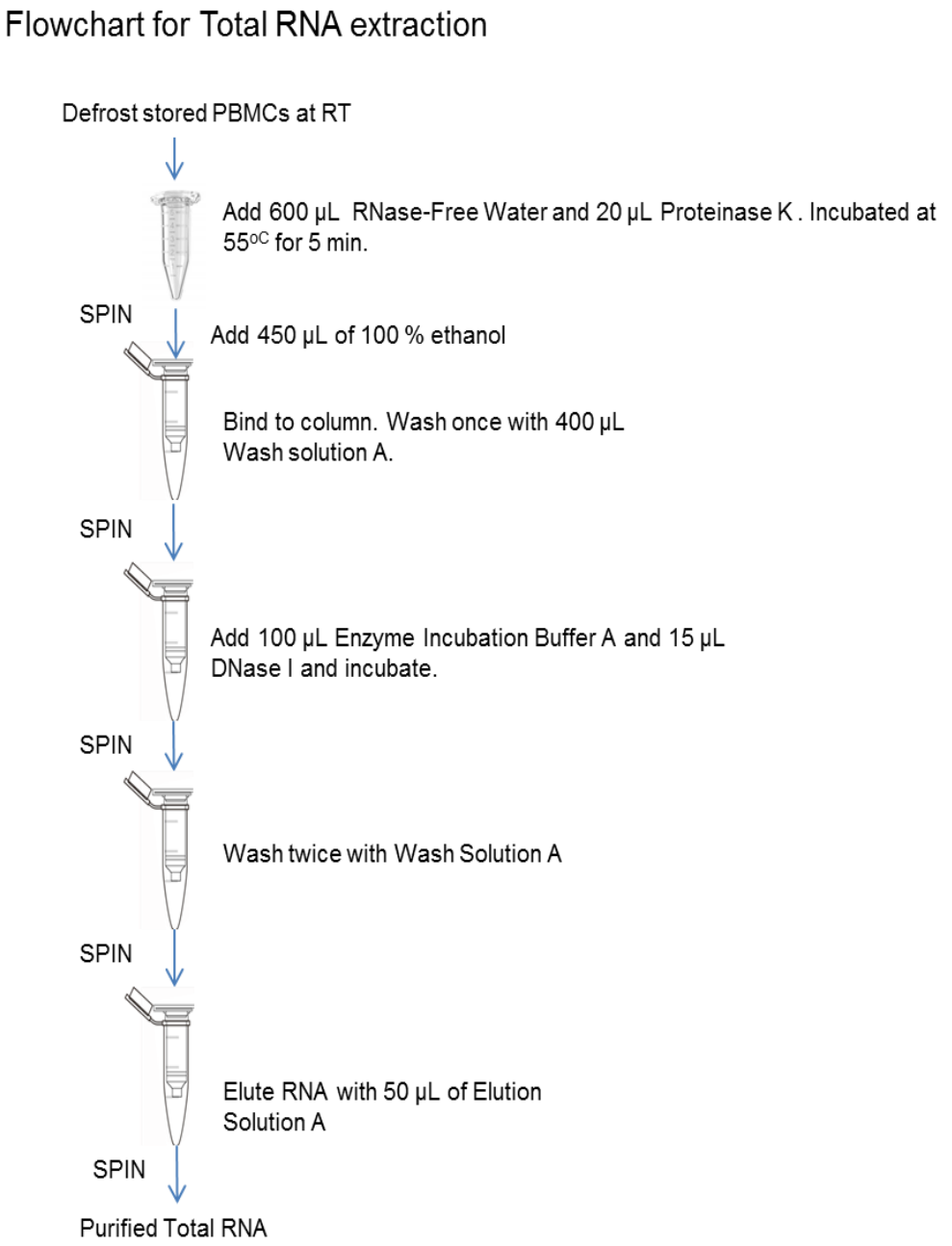


Fig10: Steps involved in RNA extraction. Norgen's Animal Tissue RNA Purification Kits were used. Norgen's Animal Tissue RNA Purification Kit provides a rapid method for the isolation and purification of total RNA.

As described in 2.4.2 the stored cells were defrosted and 600 µL of RNase-Free Water and 20 µL of reconstituted Proteinase K was added to the lysate, vortexed and was incubated at 55^{o}C for 5 min. The tubes were vortexed occasionally during incubation. The lysate was spun at 14,000 g for 1 min using a benchtop microcentrifuge. The supernatant was transferred into an RNase-free microcentrifuge tube and 450 µL of 100 % ethanol was added to the lysate and the tubes were vortexed. After cell lysate preparation RNA was bound to column by assembled a column for each sample with collection tubes; 650 µL of the lysate with the ethanol was added onto the column and centrifuged at 4000 g for 1 min. If the entire lysate volume had not passed, the tubes were spun for an additional min at 14,000 g. After centrifugation the flow-through was discarded and the spin column with its collection tube was reassembled. Depending on the lysate volume the above procedures were repeated.

Wash Solution A (400 µL) was added to the column and centrifuged at 4000 g for 2 min. The flow-through was discarded and the spin column with a new collection tube was assembled. After centrifugation, 100 µL of Enzyme Incubation Buffer A and 15 µL of DNase I was added to the column and the tubes were centrifuged at 14, 000 g for 1 min. (Note: if the entire 115 µL of DNase mix did not pass through the column it was centrifuged at 14,000 g for an additional min). After centrifugation the flow-through that was present in the collection tubes was pipetted back onto the top of the column and incubated at room temperature for 15 min.

After incubation, 400 µL of wash solution A was added to the column containing the DNase I mix and centrifuge at 14,000 g for 1 min. (Note: if the entire 115 µL of DNase mix did not pass through the column, it was centrifuged at 14,000 g

for an additional min). The flow-through was discarded and the spin column with its collection tubes was reassembled. The column wash was repeated. After the second wash the column was spun for 2 min in order to thoroughly dry the resin. The collection tubes were discarded. The columns were placed into a fresh 1.7 mL Elution tube provided with the kit. 50 µL of Elution Solution A was added to the column. The tubes were centrifuged at 200 g for 2 min, followed by 1 min at 14,000 g (Note: if the entire 50 µL has not been eluted, the column was centrifuged at 14,000 g for 1 additional minute).

2.4.4 Quantitation of Isolated RNA

The concentration and purity of the RNA samples were determined or assessed using the NanoDrop 1000 Spectrophotometer. The ratio absorbance of the RNA samples was measured at 260 nm and 280 nm against corresponding diluent buffer; the readings were obtained and recorded. The concentration of nucleic acid (RNA) was determined using the Beer-Lambert law, which predicts a linear change in absorbance with concentration. RNA purity was determined at A260/A280 of 2 at a ratio of 1.8 - 2.0. The purified RNA Samples were stored at -20^{o}C for a few days. Samples were placed at -80^{o}C for long term storage prior to use on a 96.96 chip (TaqMan).

2.4.5 Gene Expression PreAmp with Fluidigm® PreAmp Master Mix and TaqMan® Assays

This method was carried out according to the manufacturer (Fluidigm) user guide (PN 68000088 J1) instructions:

2.4.5.1 Reverse Transcription

The purified Total RNA obtained from RNA extraction (2.4.3) was converted in to complementary DNA (cDNA) by reverse transcription with 96.96 reactions. Reverse transcriptase (RT) master mix and RNase free water were mixed in a tube (Table 6) and 1 µL RNA was added. 4 µL of the mixed solution was added into each well of the microtiter plates and then incubated (Table 7).

RT mix component	**1 x (µL)**	**106**
RT master mix	1	106
RNase-free water	3	318
RNA	1	
Total volume	5	424
Volume of mix used per well	4	

Table 6: solutions used for reverse transcription

Below are the incubation temperatures and times involved for converting RNA into cDNA

Step	**ºC**	**Time**
	25	5 min
Reverse transcribe	42	30 min
Denature enzyme	85	5 min
Hold	4	Hold
Total (exclude hold)		45 min

Table 7: Incubation steps for transcribing RNA into cDNA (single stranded DNA).

2.4.5.2 Pooling the TaqMan Gene Expression Assays

In a microcentrifuge tube, equal volumes (2 µL) of each 20x Taqman gene expression assay were combined for the 96 assays chip. The pooled assays were diluted using 8 µL DNA Suspension Buffer (10 mM Tris, pH 8.0, 0.1 mM EDTA; TEKnova, PN T0221) to ensure each assay was at a final concentration of 0.2x (180 nM). These volumes were multiplied by three for the final working solutions.

2.4.5.3 Preparing Sample Pre-Mix and Samples

A pre-mix was prepared for the reactions as shown in the table below (Table 8). The pre-mix (3.75 µL) was aliquoted for each sample in a PCR plate and kept on ice. 1.25 µL of cDNA obtained from the step above (RT) was added into each well containing pre-mix. The plate was vortexed for 10 s and centrifuged at 500 g for 1 min.

Component	Volume / Reaction (µL)	Volume for 96 Reactions +10 (µL)
PreAmp Master Mix	1.00	106
Pooled Taqman assay mix (0.2x)	1.25	132.5
Water	1.50	159
cDNA (from RT step)	1.25	
Total Volume	**5.00**	397.5

Table 8**:** The highlighted are the Pre-Mix. 3.75 µL was added into each well.

2.4.5.4 Thermal Cycling

After centrifugation the plate was placed in the thermal cycler using the following steps as indicated Table 9. After cycling (PreAmp) 20 µL of 1xTE Buffer (10 mM

Tris- HCI, 1.0 mM EDTA, TEKnova, PN T0224) was added into each well of the 96 plate; and 2 mL of 1xTE solution was prepared per plate.

Condition	Hold	Cycle (14 cycles) Denaturation Annealing/Extension		Hold
Temperature	95°C	95°C	60°C	4°C
Time	2 min	15 S	4 min	∞

Table 9**:** Thermal cycler steps, total time period is 1 hr 30 min excluding hold

At this stage weak signals of cDNA are converted into strong signal that enables gene present to be detected.

2.4.5.5 Preparing 10X Assays

Aliquots of the 10X assays were prepared using the volumes in the table below in a DNA-free hood. All assay and sample solutions were vortexed thoroughly and centrifuged at 500 g for 1 min.

Component	Volume per Inlet (µL)	Volume per Inlet with Overage (µL)	Volume per 50 µL Stock
20X TaqMan Gene Expression Assay (Applied Biosystems)	2.5	3.0	25
2X Assay Loading Reagent (Fluidigm, PN 85000736)	2.5	3.0	25
Total Volume	**5.0**	**6.0**	**50**
Final Concentration at 10X	Primers: 9 µM	Probe: 2.5 µL	

Table 10**:** Aliquots of 10X assays, 5 µL was loaded on the chip

2.4.5.6 Preparing Sample Pre-Mix and Samples

A sample pre-mix solution was prepared containing the master mix and 20X gene expression (GE) sample loading reagent as shown in Table 11. The two sample pre-mix components in a 1.5 mL sterile tube were combined. The sample pre-mix (3.3 µL) was aliquoted for each sample. The aliquots were removed from the DNA-free hood and 2.7 µL of cDNA was added to each aliquot to make a total volume of 6 µL.

Component	Volume per Inlet (µL)	Volume per Inlet with Overage (µL)	Sample Pre-Mix for 96.96 (µL) (120 for ease of pipetting)
2X Master mix	2.50	3.0	360.0
20X GE Sample Loading Reagent (Fluidigm, PN 85000735, 85000746)	0.25	0.3	36.0
cDNA	2.25	2.7	
Total	**5.00**	**6.0**	

Table 11**:** Sample pre-mix

2.4.5.7 Priming and Loading the Dynamic Array integrated fluidics circuit (IFC)

For instructions on priming and loading the 96.96 Dynamic Array IFC, see the Fluidigm 96.96 Real-Time PCR Workflow Quick Reference (PN 68000130).

Before the assays and samples were loaded on the chip, the included syringes were used to inject 150 µL of the control line fluid into each accumulator on the 96.96 chip (Figure 11). The protective film from the bottom of the chip was removed and discarded. The 96 chip was placed into the IFC Controller HX and the Prime script (136x) was run for 20 min.

Figure 11 Taqman 96.96 chip

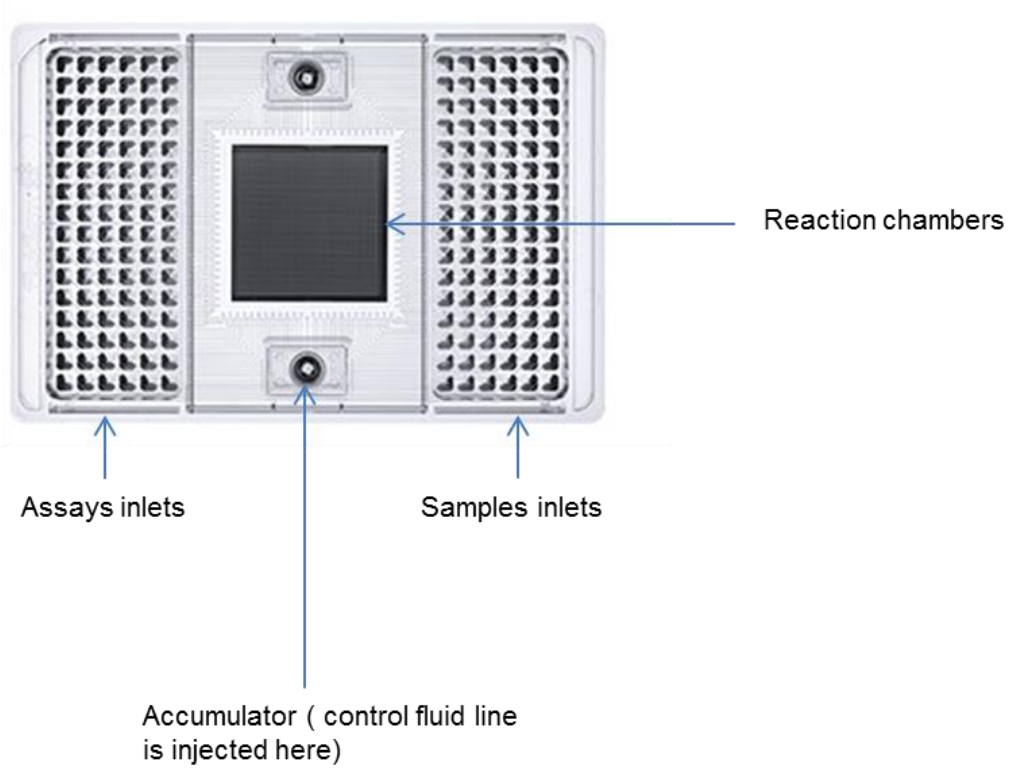


Fig11: The 96.96 chip, 96 assays by 96 samples gives 9216 reaction in 6 µL chambers.

2.4.5.8 Assay and Sample Loading

After the chip was primed, the assays as prepared above were loaded into the inlets and the samples as prepared above were loaded into the sample inlets. Pipetting was carefully carried out to prevent air bubbles. In each inlet (respective inlets) 5 µL of each assay or 5 µL of each sample was added. After

the chip was transfer to the loader, which pushes the inputs through the microfluidics and mixed each sample with each assay. The loader script (136x) was run for 1 hr 30min. The chip was then placed in the BioMark HD System; GE 96x96 standard v1.pcl was run for 3 hr. The data was collected on the BioMark HD System (Figure 13).

2.4.5.9 Using the Data Collection Software

The GE 96X96 standard v1.pcl in the GE folder was selected. The total program ran approximately 3 hr. The completed program is as follows see Figure 13.

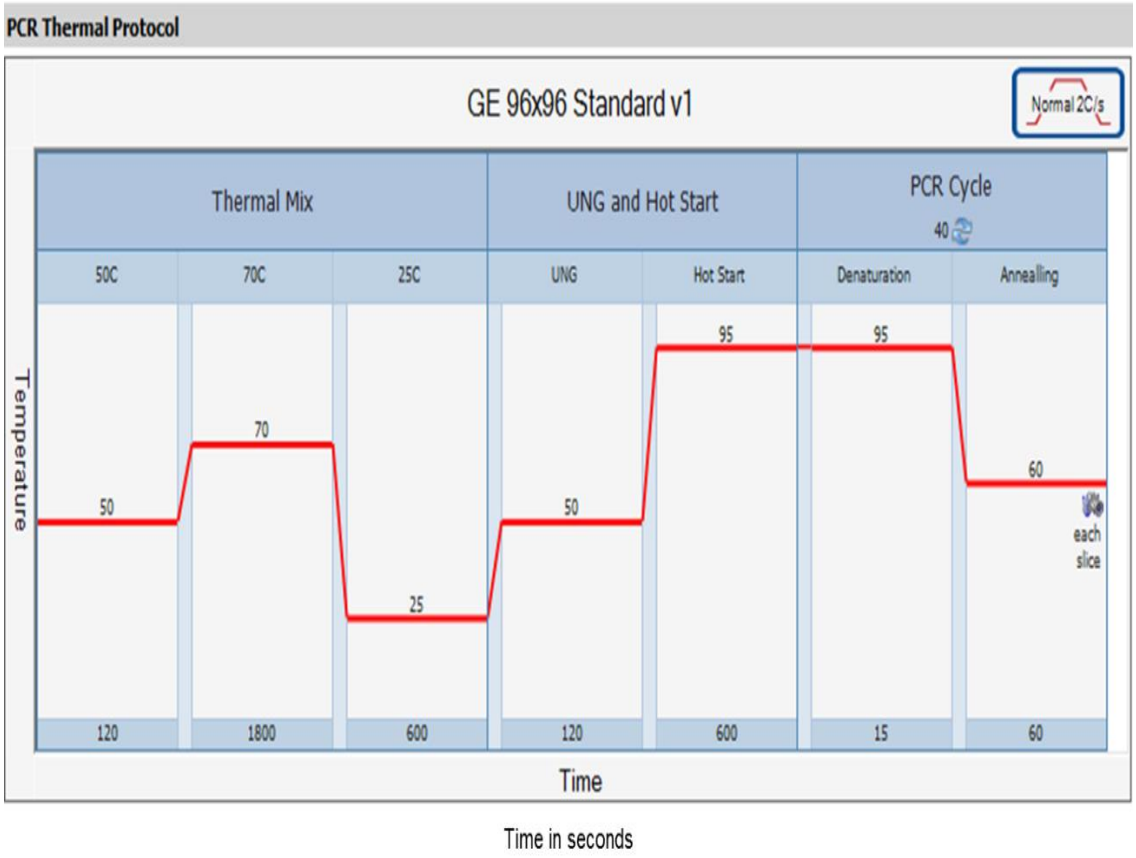


Figure 12: The cycling protocol for the 96.96 Dynamic Array IFC provided by Dr Claire Taylor

96.96 chip date representative

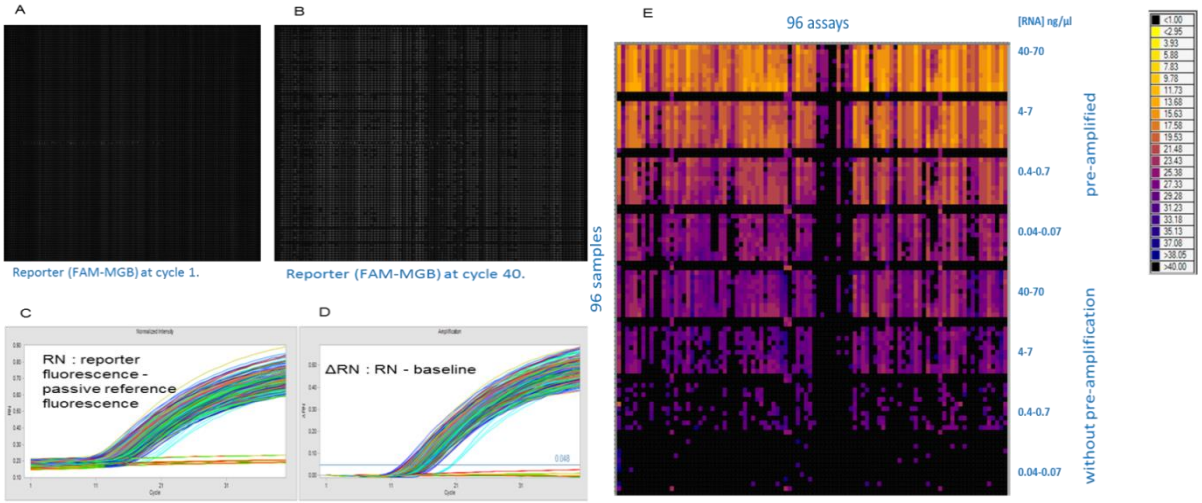


Figure 13: Representative of the Taqman chip data (shown with permission from Dr Claire Taylor).

Fig A shows cycle 1 when there is no signalling or the signalling is very low. Fig B shows cycle 40 when signalling is high, the genes present can easily be detected. Fig C and D shows the excitation of the signals. Fig E shows strong signals at the top (yellow) and weak or no signal at the bottom (black). On the right side of Fig E shows the RNA present.

Gene probe selection and gene expression

In our group 10 genes were selected from each IFN-annotated module (M1.2, M3.4, M5.12) (Chiche et al., 2014), with additional common ISGs (IFI27, IFI6). The selected genes were validated by meta-analysis of multiple GEO data biosets comparing PBMCs from SLE versus HC on Nextbio web engine. Peptidylprolyl isomerase A (PPIA) was used as a reference gene (confirmed not to respond to IFN-I). The housekeeping gene (PPIA) is considered to be the most stable and it is not fluctuated by IFN or IFN mediated disease compared to other reference genes such as, hypoxanthine-guanine phosphoribosyltransferase (HPRT1) and beta-glucuronidase (GUSB).

2.4.6 Flow method assessment

Representative scatter plots for staining

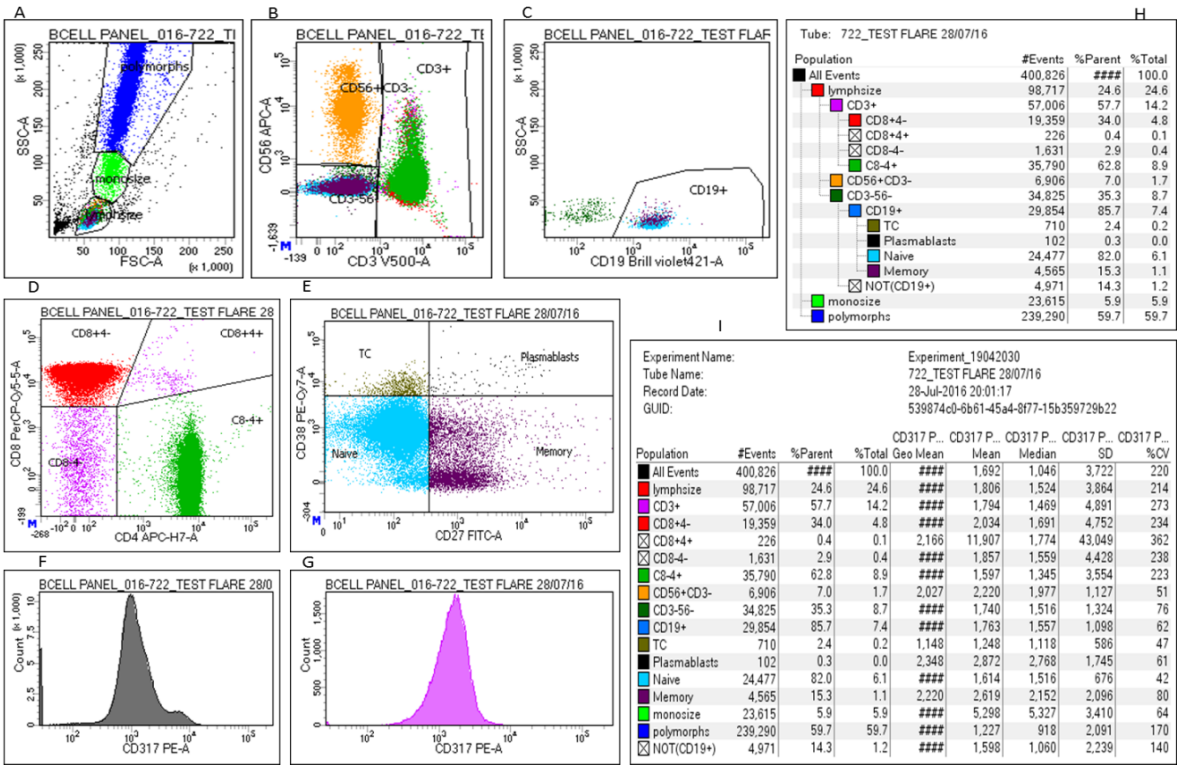


Figure 14: Representative scatter plots of whole blood and PBMC staining.

Cells were stained as described in 2.4.1 for whole blood staining and 2.4.2 for PBMC staining. The cells were surface stained and fixed. Tetherin expression was examined using blood samples cells with monoclonal antibody and analysed by flow cytometry. The intensity level of tetherin protein (CD317) expression was measured in each of these cell types by using the % parent, mean and median fluorescence intensity.

Lymphocytes, monocytes and neutrophils were gated from the forward side scatter (FSC) and side scatter (SSC) as shown in **fig A**. From the lymphocyte population $CD3^+$ T-Cells, NK-cells were identified (**see B**). From $CD3^+$ T-cells the sub population of T-Cells were differentiated as shown in **fig D**. $CD19^+$ B-Cells (**fig C**) were taken from the double negative population $CD3^-CD56^-$ in **fig B**. $CD19^+$ expression was further differentiated to naïve b-cell ($CD27^-CD38^-$),

memory b-cell ($CD27^{+}CD38^{-}$), transitional cells ($CD38^{+}CD27^{-}$) and plasmablasts ($CD27^{+}CD38^{hi}$) (**fig E**). The gates were adjusted for each sample. Isotypes were used as a control to adjust the tested samples. Each sample had an unstained, an isotype and a test tube. **Fig H** shows the population hierarchy of the cell types and **fig I** show the statistical review.

Definition of the cell types

Cell types	
$CD19^{+}$	B-cells
$CD38^{hi}CD27^{-}$	$CD19^{+}$ Transitional cells
$CD38^{+}CD27^{-}$	$CD19^{+}$ Naïve
$CD38^{-}CD27^{+}$	$CD19^{+}$ Memory
$CD28^{+}CD27^{+}$	$CD19^{+}$ Plasmablasts
$CD56^{+}CD3^{-}$	NK cells
$CD56^{+}CD3^{+}$	NKT cells
$CD56^{-}CD3^{-}$	CD19 cells by exclusion
$CD3^{+}CD8^{+}CD4^{+}$	T-cells
$CD3^{+}CD8^{+}CD4^{-}$	Cytotoxic T-cells
$CD3^{+}CD8^{-}CD4^{+}$	Helper T-cells
$CD3^{+}CD8^{-}CD4^{-}$	$CD3^{+}$ T-cells
$CD3^{+}$	$CD3^{+}$T-cells

Table 12: Cell types

Lymphocytes, monocytes and polymorphs are defined by forward scatter and side scatter. The above cells are defined by the antibody used. NK cells (Natural killer cells) NKT cells (Natural killer T cells).

2.4.7 British Isles Lupus Activity Group (BILAG) index

BILAG is a scoring system to evaluate the activity of lupus in clinical studies. It is valid, reliable and sensitive to change. BILAG is an organ-specific 86-question assessment that incorporates an evaluation of the change in patients' symptoms over the last month, combined with recent laboratory tests. The overall scores were calculated for each organ system (general, mucocutaneous, neurological, musculoskeletal, cardiorespiratory, vasculitis, renal and haematological) that relates to the activity of the disease. The resulting scores for each organ were stated as A through E, where A is very active disease, B is moderate activity, C is mild stable disease, D is resolved activity, and E indicates the organ was never involved. The BILAG index scoring is based upon the physician's intention to treat.

2.4.8 Data analysis

The BILAG index was used to determine the patients' disease activity. Descriptive statistics (mean and standard deviation, median and interquartile range) were used to measure the level of tetherin. Box and Whisker plots with 5-95 percentiles were used to demonstrate the distribution of tetherin among the groups. Statistical analysis was performed using SPSS statistics version 24 (IMB SPSS) and Graphpad Prism 7. All continuous variables were expressed as mean ± standard deviation unless stated otherwise. All continuous variables were compared across the groups using non-parametric test Kruskal-Wallis test. Dunn's multiple comparisons test was used to compare healthy control and remission and healthy control and flare. Differences in expression levels between patient groups (flare and remission) were investigated with non-parametric Mann-Whitney *U* tests. Cox regression analysis was used to determine whether patients in remission group with higher intensity of tetherin can predict flare on memory B cells and monocytes. Model fit was analysed by omnibus tests of model coefficients. Results were indicated as the hazard ratio (HR) with 95% confidence interval (95% CI). P value of less than 0.05 was considered significant.

ROC curve was used to determine sensitivity and specificity of the diagnostic value of tetherin on memory B cells of healthy control and patients (Remission and Flare). Score A was calculated as the median of delta CT (ΔCT) of the IFN stimulated genes, performed by gene expression (TaqMan).

3.0 Results

The expression level of tetherin (CD317) was determined in human blood using flow cytometry and gene expression (Taqman 96.96 chip method). Initial experiments compared peripheral blood mononuclear cells (PBMCs) staining with whole blood staining to determine the most effective in measuring tetherin levels. After the staining method was determined, healthy control, remission and flare groups were compared to determine the difference in tetherin levels between these groups. Flow cytometry measured the intensity of tetherin protein on blood cell surface using median MFI. Repeated measures Kruskal-Wallis test was used to compare the degree of significant difference between whole blood and PBMCs staining techniques and between healthy controls, remission and flare groups.

Gene expression by Taqman was analysed to detect IFN stimulated genes (ISGs) levels. A List of ISGs were selected to span over three annotated modules (M1.2, M3.4, and M5.12) (Chiche et al., 2014). The factor analysis; a special statistical analysis was carried out to reduce the number of variables (genes) into a continue score/s to categorise genes that behaved similarly. IFN-Score A (factor A) was identified as the best group of genes that distinguished SLE patients from healthy controls and rheumatoid arthritis patients. These genes were derived mainly from M1.2 gene module (Chiche et al., 2014). IFN-Score A was calculated as the median of ΔCt (delta cycle threshold) of the correspondent genes. PPIA was the housekeeping gene used for the gene expression method. PPIA was used as a reference gene to calculate IFN-Score A ΔCt of the corresponded ISGs.

3.1 Development of a whole blood assay for tetherin.

The results in this section test the following hypothesis:

Hypothesis 1: Cell specific tetherin can be measured accurately using a whole blood assay in a routine diagnostic laboratory.

Section methods

Venous samples were obtained from 16 SLE patients using EDTA vacutainer tubes. Whole blood staining required 4 mL blood, whereas PBMC isolation required 18 mL. Cells were stained as described in 2.4.1 for whole blood staining and as described in 2.4.2 for PBMC staining.

For both techniques, the level of tetherin was measured by the proportion of positively stained cells as a percentage of the parent gate (% parent) as well the median intensity of tetherin expression. The Kruskal-Wallis test was used to compare the expression of tetherin on the cell types for % parent and median fluorescence intensity (MFI) (Figure 15). Since the data was nonparametric and measured on a continuous scale. The cells that showed differences with the expression of tetherin between the staining techniques were compared using Wilcoxon matched-paired Signed rank test (Figure 16).

Section Results

Comparisons of PBMCs and whole blood staining technique

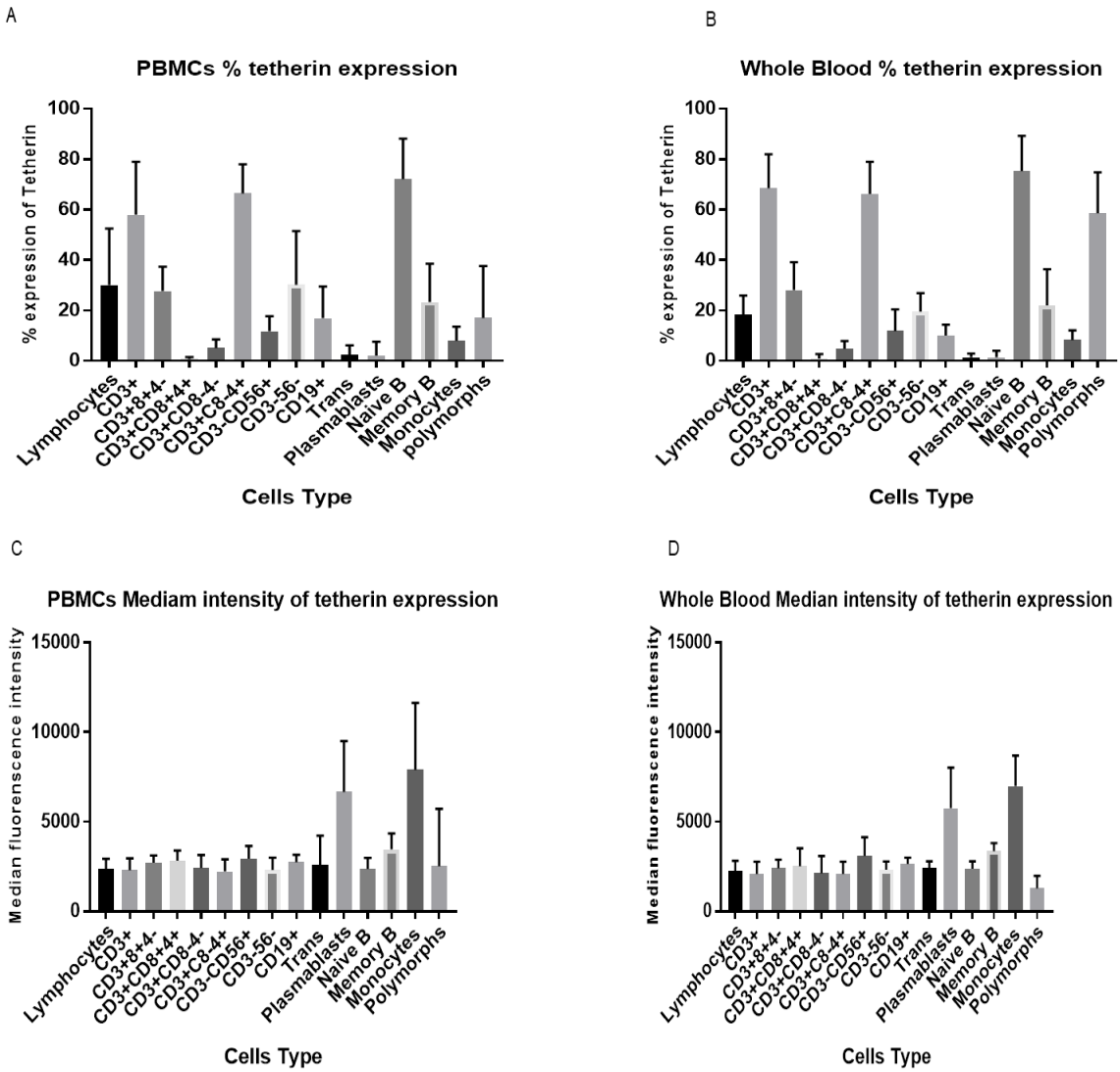


Figure 15: Representative bar charts of PBMCs and whole blood staining.

Cells were stained as described above for whole blood and PBMC staining and assessed using flow cytometry. The cellular subsets are listed on the x-axis including Trans (Transitional cells). Figure A-B represents the % parent with positive tetherin protein expression; Figure C-D represents the median fluorescence intensity (MFI) of tetherin on the cell surface. Figure A and B graphs were drawn with mean and standard deviation, whereas Figure C and D bars shown the median and interquartile range.

The intensity level of tetherin protein (CD317) was measured in each cell type by using the % parent (proportion of positive cells from the parent gate, (Figure 15A and B) and median MFI (Figure 15C and D). There were significant differences between the 15 cell types (P-value <0.0001). However there is no significant difference seen between the staining techniques (except for polymorphs as expected) as polymorphs were excluded from isolated PBMCs Figure 16).

Figure 15A-B (% expression of tetherin) show there is a high percentage expression of tetherin protein on the cell surface of T-cells ($CD3^+$), cytotoxic T-cells ($CD3^+CD8^+$), helper T-cells ($CD3^+CD4^+$), naïve B cells, memory B cells and polymorphs. A high median intensity of tetherin protein was demonstrated on the cell surface of NK cells ($CD56^+CD3^-$), plasmablasts, memory B and monocytes (Figure 15B-C). There was a significant difference between the medians of 15 cell types (the corresponded cell types are defined in Table 12). The data shows that regardless of the methodology used, a similar proportion of cells stained positive for tetherin, with PBMCs staining expressed slightly higher intensity of tetherin compared to whole blood staining.

3.1.1 Comparison between PBMCs and Whole Blood staining on % parent and median MFI

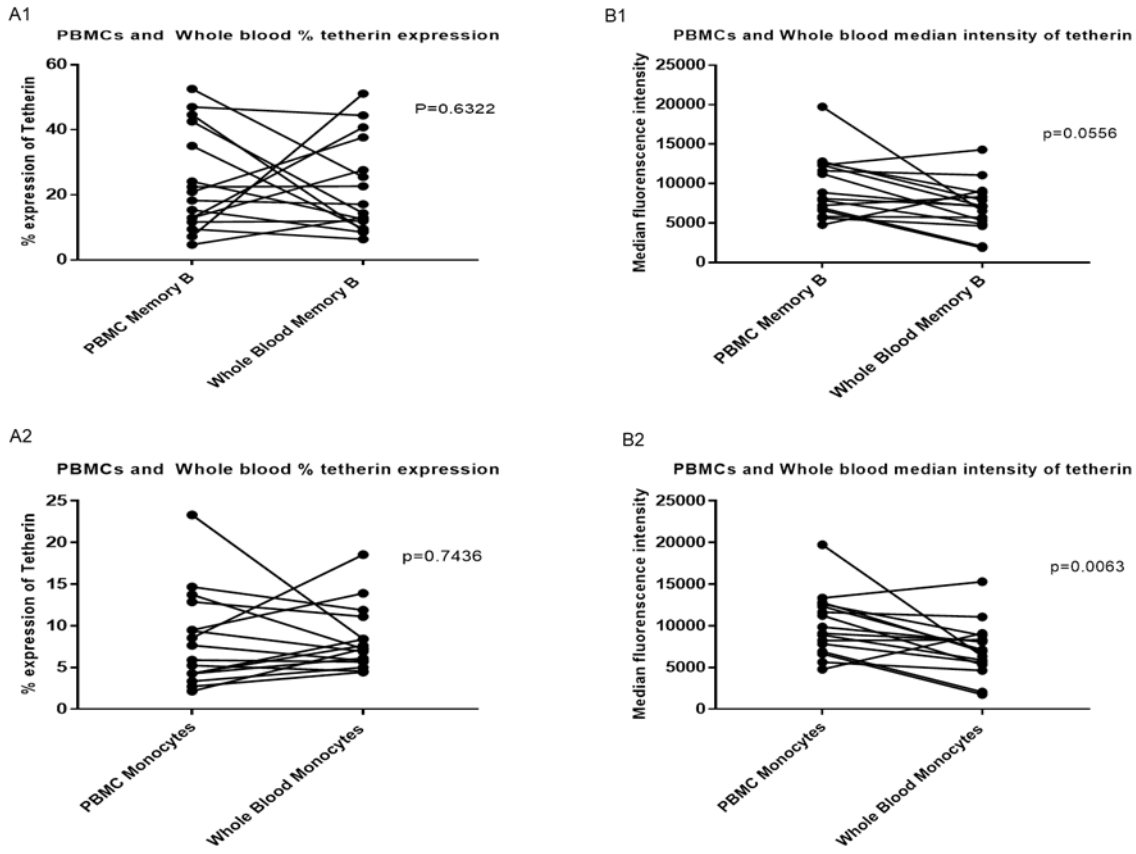


Figure 16: Comparison between PBMCs and Whole Blood staining on % parent and MFI.

The figure shows the cells that show differences with the level of tetherin protein on

Figure 15 with a P-value of <0.0001. The figures were drawn with symbols and lines. Wilcoxon matched-paired Signed rank test was used to assess the P-value and there is no statistically significant difference, except on monocytes measured with the MFI (Figure B2). However, tetherin is significantly more expressed with PBMC staining than whole blood staining.

Figure 16 compares the three cell types that had demonstrated differences in the level of tetherin protein expression between the staining techniques as previously shown in Figure 15.

The Wilcoxon matched-paired Signed rank test was used to compare the staining techniques in Figure 16; and no statistically significant differences were observed except on monocytes (Figure 16B2, P-value of 0.0063). Monocyte within PBMCs staining is statistically more significant in comparisons to whole blood staining. The Spearman outcome justifies the pairing were not significantly effective except on monocytes (Figure 16A2) with a Spearman of 0.5559. The above Figures (Figure 15 and Figure 16) show there is no significant differences in the expression level of tetherin with PBMCs compared to whole blood staining. The data shows that irrespective of the methodology used, a similar proportion of cells stained positive for tetherin. However the intensity of tetherin expression was slightly higher with PBMCs compared to whole blood lysis staining.

There is no statistical difference observed on memory B cells which is the focused (investigated) cell in this study; because our previous cross-sectional study (discovery cohort) found memory B cell tetherin had positive correlation with SLE disease activity (El-Sherbiny et al., 2015). Considering the costs of isolating PBMCs (purchasing Leucosep Barrier Ficoll Tubes), skills involved and time taken to process the samples, it was decided to proceed with whole blood staining technique for this study.

After determining the staining technique, whole blood staining was performed on remission patients, flare patients and healthy controls (healthy patients).

The figure below show a comparison of tetherin protein expression on memory B cells with PBMCs and whole blood staining techniques.

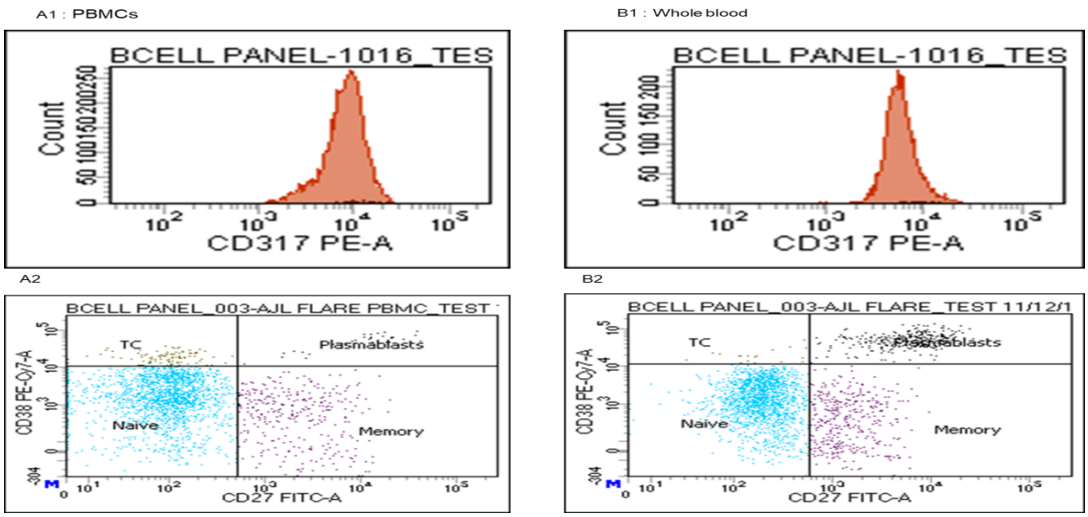


Figure 17: Comparison of tetherin expression on memory B cells with PBMCs and whole blood staining techniques.

The histograms are representative of tetherin protein intensity on memory B cells. Figure 17A represents PBMCs staining and B represent whole blood staining. The above analysis showed no statistically significant difference between the two staining techniques as mentioned above and the histograms (Figure 17) confirmed this finding.

Section Conclusion

It was concluded that tetherin could be measured using a whole blood assay under routine sample collection procedures with results of comparable accuracy to the discovery study performed in a university research laboratory with PBMCs. Furthermore, the results showed that tetherin has a continuous, skewed distribution so the median MFI for each patient group and non-parametric statistics in the subsequent analyses would be use. Monocytes had the highest expression of tetherin compared to memory B cells. However, previous work showed that memory B cell tetherin had the best correlation with disease activity. Therefore, tetherin level was measured on these two subsets for subsequent analyses (as well as a IFN score ((score A) as a comparator biomarker).

4.0 Comparing tetherin level between patient groups.

The results in this section test the following hypothesis:

Hypothesis 2: Tetherin will differentiate SLE from healthy control.

Section methods

Samples were obtained from 66 SLE patients in remission, 65 flaring SLE patients and 20 healthy controls. Whole blood flow cytometry for tetherin MFI was performed on each sample as described in 2.4.1. Using the same samples, gene expression was performed as described in 2.4.5 and IFN stimulated genes were used to calculate IFN-score A.

The Kruskal-Wallis test was used to compare the groups. The Dunn's post hoc for multiple comparisons test was used to compare healthy control with remission and healthy control with flare. The Dunn's post hoc for multiple comparisons test was used to distinguish which group differed. Mann-Whitney *U* test was used to compare flare and remission within the groups.

Gene expression (TaqMan) method (IFN score A) was compared with flow cytometry to measure tetherin.

Receiver operator curve (ROC) analysis was used to select the optimum sensitivity and specificity of the assay.

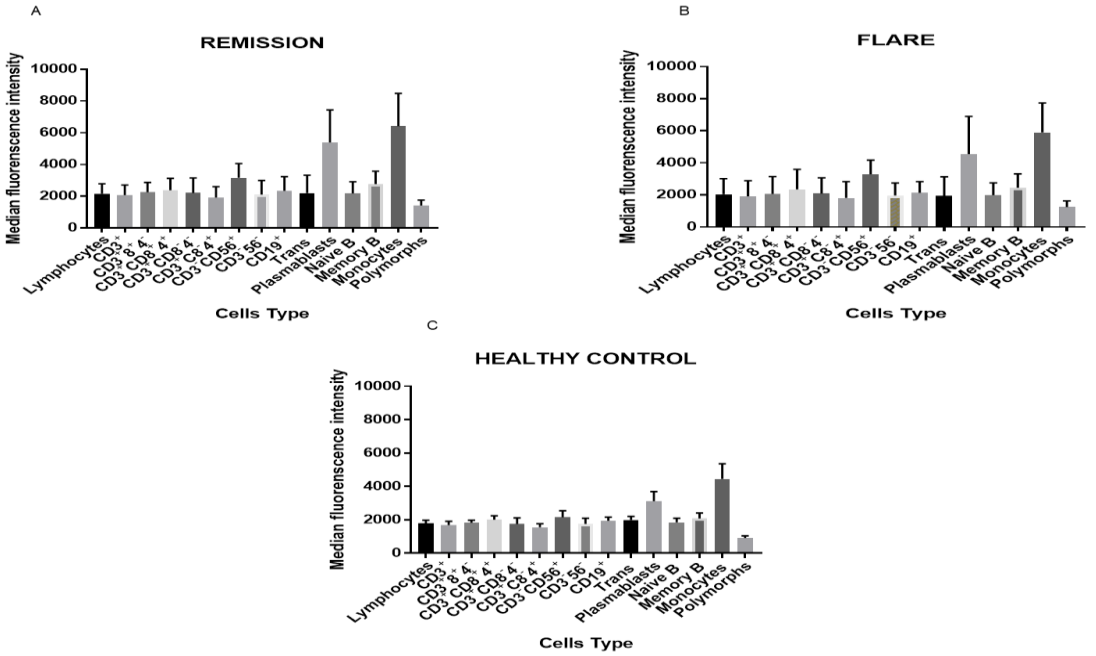


Figure 18: Representative bar charts of whole blood staining on patient groups (Remission n=66, Flare n=65 and healthy control n=20).

Cells were stained as described in 2.4.1. The cells were surface stained, fixed and assessed by using flow cytometry. The cellular subsets are listed on the x-axis including Trans (Transitional cells). Figure A-C represents the median fluorescence intensity of tetherin protein on the cell surface. The bars show the median and interquartile range. The Kruskal-Wallis test was used to compare the groups.

The intensity level of tetherin protein was measured in each of these cell types by using the median fluorescence intensity (Figure 18). The patient groups (remission and flare) expressed higher level of tetherin compared to the healthy control group. There was higher intensity of tetherin protein on the cell surface of NK cells, plasmablasts, memory B and monocytes, with a P-valve < 0.0001. The data showed that the remission group expressed highest intensity of tetherin compared to flare and healthy control groups. The cell types that showed highest median intensity of tetherin protein with statistically significant differences were then presented on box plots, as shown in Figure 19.

4.1 Comparison of level of tetherin on healthy control, remission and flare patients of the median intensity.

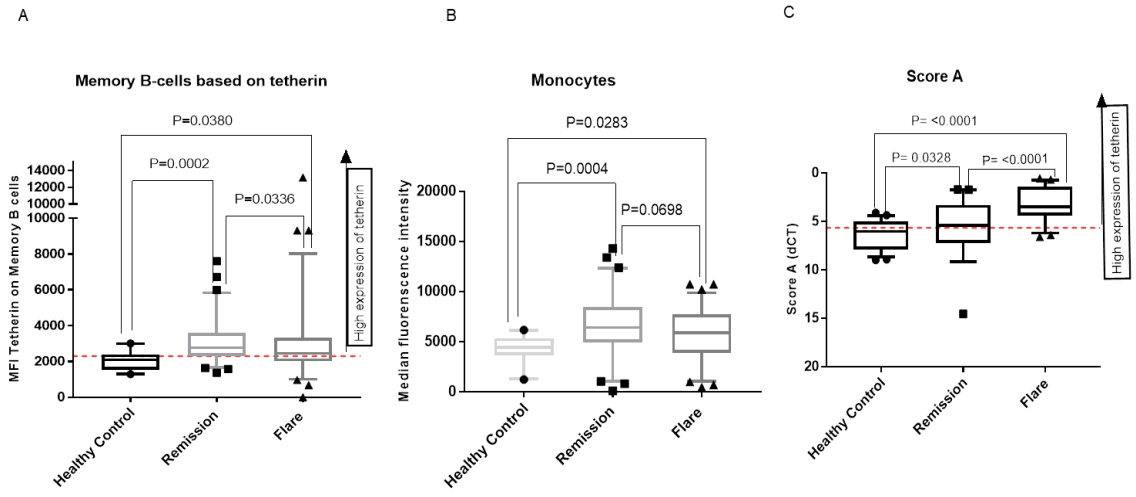


Figure 19: Determining the significance difference between flow cytometry and gene expression of tetherin expression on memory B cells.

Figures A-B shows the cells from Figure 18. Figures A and C show a comparison of tetherin protein expression based on tetherin and IFN score A. The tetherin reference range was determined by taking the upper confidence interval (CI = 2300) on memory B cells of the healthy controls. The IFN score A, reference range was determined by taking the lower bound limit (5.677632) of the healthy control IFN genes expression. The orange lines indicate the reference ranges. Box and Whisker plots are drawn with 5-95 percentiles. The darker shapes are outliers and were observed in all of the groups. The groups were compared using Kruskal-Wallis test. Dunn's post hoc for multiple comparisons test are used to compare healthy control with remission and healthy control with flare. Mann-Whitney *U* test was used to compare flare and remission groups.

Figure 19 shows there is statistically significant difference when comparing healthy controls against flare and remission groups. The healthy control group expressed the lowest level of tetherin compared to the patients groups. The

remission group in Figure 19A had greater median value (2770) compare to flare (2443) and healthy control group (2076). In Figure 19A there is a statistically significant difference between healthy control and remission with a P-value of 0.0002. When healthy control and flare groups are compared, there is no statistical difference with a mean rank difference -26.23 and a P-value of 0.0380 (according to the Dunn's post hoc for multiple comparisons test) 0.03 (see 8.0 statistical analyses).

Figure 19A-B shows that patients in remission group expressed slightly higher intensity of tetherin compared to the flare group and a statistical difference was shown (Figure 19A).

Figure 19B (monocytes) shows there is statistical difference between the three groups with a P-value 0.0008. When healthy control and remission groups are compared they give a mean rank difference of -41.5 and a P-value 0.0004. There is a statistical difference between the groups. The healthy control group is statistically less significant in comparison to the remission group. When healthy control and flare groups are compared; there is a statistical difference with a mean rank difference -27.44 and a P-value 0.0283. The healthy control group had the lowest median value (4449) compared to the patients groups, flare (5898) and remission (64114) in expressing tetherin. Mann-Whitney *U* test was used to compare Flare and Remission groups. There is no statistical difference with a P-value 0.0698.

Figure 19C (score A (measured IFN stimulated genes)) shows there is a statistically significant difference when comparing the healthy control against flare and remission groups (P-value of <0.0001). There is statistically more significant difference between healthy control and flare group with a mean rank difference of 57.95 and a P-value <0.0001 and between remission and flare group with a P-value of <0.0001 compare to Figure 19A. Figure 19A (memory B based on tetherin) shows patients in the remission group expressed higher intensity of tetherin compared to the flare group. However, the opposite is observed in Figure 19C and it shows greater statistical differences in comparison to Figure 19A. In Figure 19A, tetherin expression equal to or greater than the upper CI (2300) indicated higher expression of tetherin on memory B cells by flow cytometry. However, in Figure 19C score A equal to or lower than the lower bound limit (5.67732) indicates higher expression of score A (IFN gene signatures) by gene expression. The flare group had the highest median value (3.477) compared to the remission (5.386) and healthy control groups (6.02) in expressing tetherin protein. IFN score A (gene expression method) is statistically more significant in comparison to tetherin expression using flow cytometry. The above analyses also shows that overall, SLE patients have higher level of tetherin compared to the healthy control group.

The genes selected for IFN score A factorial analysis were ISG15, IFI44, IFI27, CXCL10, RSAD2, IFIT1, IFI44L, CCL8, XAF1, GBP1, IRF7 and CEACAM1. Score A was calculated as a median of the above genes ΔCt. PPIA was used as the reference gene (housekeeping gene).

4.2 Sensitivity and specificity of memory B cell tetherin for diagnosis of SLE

Figure 20 below shows how sensitivity and specificity of the diagnostic value of tetherin of healthy control and patients was determined from median intensity of tetherin protein on memory B cells (Figure 20A1-A2) in comparison to gene expression (IFN-score A) (Figure 20B1-B2). The ROC Curve was used to assess the P-values, sensitivity and specificity values.

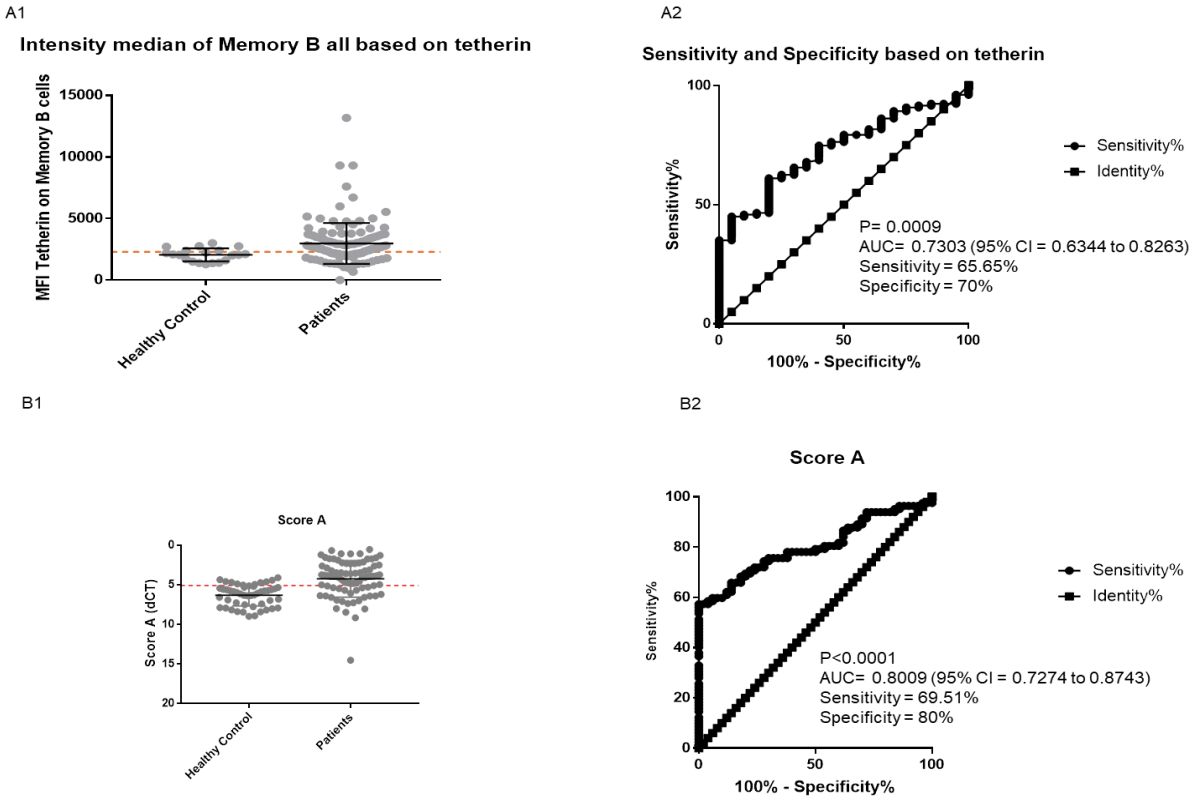


Figure 20: Determining sensitivity and specificity of the diagnostic value of tetherin and score A of healthy control and patients (including Flare and Remission).

Figures A1 and B1 graphs were drawn with mean and standard deviation. Individual values for each patient are shown along with mean errors bars that represent standard error mean (SEM). Figures A2 and B2 graphs were used to determine the sensitivity and specificity of the assays. It was also used to assess the area under the curve and the P-value. The orange line was drawn to indicate the diagnostic values predicted by the ROC Curve.

Figure 20A1-A2, the patients group (n=131) have greater tested values compared to healthy control group (n=20) and Figure 20B1-B2 patients group (n=82) and healthy control group (n=50). The Figure shows the healthy control group values are closer in range compared to the patient groups.

Figure 20A2 has an area under the curve of 0.7303, P-value 0.0009, standard deviation error 0.04893 and 95% confident interval 0.6344 to 0.8263. For >2288 median intensity of tetherin gives 65.65 % sensitivity and 70% specificity with a likelihood ratio of 2.188. Figure 20B2 has an area under the curve of 0.8009, P-value <0.0001, standard deviation error 0.03749 and 95% confident interval 0.7274 to 0.8743. For <5.124 IFN score A, gives 69.51% sensitivity and 80% specificity with a likelihood ratio of 3.476. Gene expression method (IFN score A) gives a greater specificity and it statistically more significant compared to flow cytometry tetherin measurement.

The area under a ROC curve provides the overall ability of the test to discriminate between those individuals with the disease and those without the disease. A poor test has an area of 0.5 and a perfect test has an area of 1.0. Looking at the area under the curves and the P-values from Figure 20 we could conclude by stating the test does discriminate between abnormal patients and normal controls even though it is not a perfect test. The assay did not produce higher sensitivity or higher specificity when discriminating between clinically normal and clinically abnormal laboratory values. The values of sensitivity and specificity would have been great if they were close to 100%. However, our

assay and propose biomarker (tetherin) seems to be better in comparison to current C3 and C4 assays (Heidenreich et al., 2009).

Section Conclusion

From these results tetherin is significantly higher in patients with SLE, as expected. The sensitivity and specificity of tetherin appeared lower than expected. However, the SLE patient samples were obtained from patients with established disease. These patients may have received glucocorticoids, immunosuppressants or rituximab, which may have affected tetherin levels. A diagnostic test for SLE is more likely to be applied to patients who are therapy-naïve at the time of testing. This question could be explored further by testing the effects of therapies on tetherin levels as well as recruiting a cohort of newly diagnosed SLE patients. Another unexpected finding in this analysis was that tetherin levels were not higher in patients with flares. This contradicts the results in the discovery cohort. The most obvious difference between this study results and those in the discovery cohort is that the treating physician's opinion of flare was used rather than BILAG-determined flare. Patients presenting with features such as joint pain without swelling might be judged by their physician to have a flare but do not have confirmed clinical disease activity that would score on BILAG. The results in the next section were analysed according to BILAG disease activity and indeed show a relationship between tetherin levels and flare. This discrepancy emphasises the importance of accurate clinical assessment to optimise the treatment of SLE as well as biomarkers.

5.0 Determining whether memory B cell tetherin predicts flare in patient in remission.

The results in this section test the following hypothesis:

Hypothesis 3: SLE patients in remission with higher level of tetherin on memory B cells have a significantly higher risk of flare compare to patients in remission with lower or normal level of tetherin.

Section methods

Cells were processed and stained as described in 2.4.1 and flow cytometry panel was performed on each sample.

The level of tetherin was measured by median fluorescence intensity of tetherin protein. Patients with higher expression of tetherin protein from remission group were selected. Cox Regression and Cox regression with a time-dependent covariate were used to determine the degree of significant difference between the groups. The groups were determined whether flare (Y) or no flare (N).

The table below (Table 13) outlined the 36 SLE remission patients that were selected for the prediction of flare, including their disease characteristics, demographic and their treatments. These patients expressed higher level of tetherin compared to the rest of SLE remission patients' samples processed.

Patient	Age	Gender	Demographic	Disease characteristics (BILAG)		Medication / Treatment			
				Muco	MSK	RTX	Steroid	Hydro	MMF MTX AZA
1	52	F	White British	D	D	N	N	Y	Y
2	34	F	White British	D	D	N	Y	Y	N
3	49	F	White British	E	D	N	Y	Y	N
4	18	F	Bangladeshi	D	E	N	N	Y	Y
5	59	F	Caribbean	D	D	N	N	Y	Y
6	57	M	White British	E	E	N	N	N	N
7	37	F	White British	E	E	N	N	Y	N
8	39	F	White British	E	E	N	N	Y	Y
9	66	F	Caribbean	D	E	N	N	N	Y
10	46	F	Pakistani	D	E	N	Y	Y	Y
11	33	F	Caribbean	E	E	N	N	Y	Y
12	41	F	White British	D	D	N	N	Y	Y
13	45	F	Indian	C	C	N	N	Y	Y
14	32	F	Indian	E	E	N	N	Y	N
15	74	F	White British	D	D	N	Y	Y	Y
16	36	F	White British	D	D	N	Y	Y	Y
17	66	F	Mixed race	D	E	N	N	Y	N
18	19	F	White British	D	E	N	N	Y	N
19	36	M	Black African	E	E	N	N	N	Y
20	63	F	Indian	D	D	N	Y	N	N
21	33	F	Indian	D	C	N	N	Y	Y
22	63	F	White British	D	D	N	Y	N	N
23	73	F	White British	D	D	N	Y	Y	Y
24	27	F	White British	E	E	N	Y	N	N
25	31	F	White British	D	E	N	N	Y	N
26	60	F	White British	D	D	N	Y	N	Y
27	67	F	White British	C	B	N	Y	N	N
28	37	F	White British	D	D	N	Y	Y	N
29	63	F	White British	E	D	N	N	Y	N
30	29	F	Caribbean	D	D	N	N	Y	Y
31	46	F	White British	D	D	N	N	Y	Y
32	46	F	Chinese	D	D	N	N	Y	Y
33	47	F	Chinese	D	D	N	N	Y	Y
34	28	F	Indian	D	D	N	Y	Y	N
35	51	M	Black African	D	C	N	N	Y	Y
36	56	M	White British	D	D	N	Y	Y	N

Table 13: SLE remission patients selected for the prediction of flare.

Majority of these patients are female within the child bearing age. Muco = Mucocutaneous (the involvement of skin and mucosal), MSK = musculoskeletal, RTX = Rituximab, Hydro = Hydroxychloroquine, MMF = Mycophenolate mofetil, MTX = Methotrexate and AZA = Azathioprine. N = No and Y = Yes.

Section Results

The diagram below (Figure 21) shows memory B and monocytes cells with higher median intensity of tetherin protein. These cell types were compared to determine which is most likely to predict flare in SLE patients especially for those in remission.

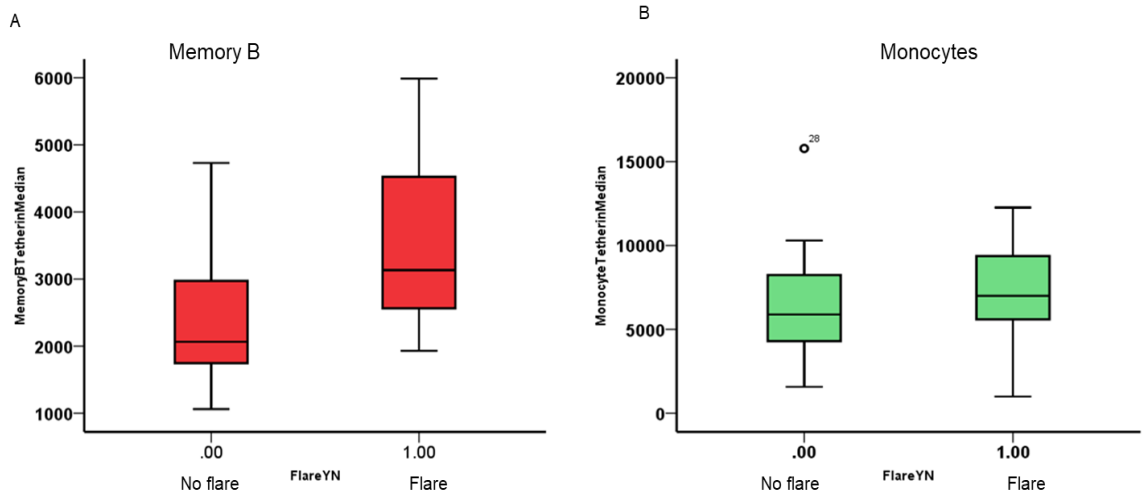


Figure 21: Determining the prediction of flare in SLE patients

Figures A-B shows memory B cells and monocytes with higher median fluorescence intensity of tetherin protein. These are presented in Box and Whisker plots with 5-95 percentiles. The proportions were compared by chi-square. Cox Regression and Cox regression with a time-dependent covariate were used to determine the degree of significant difference. The groups were determined whether flare (Y) or no flare (N) 0.00= No Flare (n=28) and 1.00= Flare (n=8) (Y=yes and N=no).

Figure 21A (memory B cells) shows there is a significant difference between the two groups in the expression of tetherin protein on memory B cell surface. The flare group is statistically more significant compared to the remission group. The flare group have a smaller number of patients (n=8) compared to the remission group (n=28) but shows higher intensity of tetherin on memory B compared to the remission group. The Cox Regression gives a Chi-square of 5.623 and a P-

value 0.018 overall score. There is a statistical difference between these groups (Table 14) and Table 15 confirms the statistical difference. This shows that memory B is a better predictor of flare compared to monocytes.

Figure 21B (monocytes) shows the remission group demonstrates similar distribution of the intensity of tetherin protein compared to the flare group. The flare group expressed slightly higher intensity of tetherin compared to the remission group; however, there is no statistical difference. Cox Regression gives a Chi-square of 0.580 and no statistical difference with a P-value 0.446 for the overall score (Table 14).

The tables below showed the significant differences of both cells type.

Cells Type	- 2 Log Likelihood	Overall (score)			Change From Previous Step		
		Chi-square	df	Sig.	Chi-square	df	Sig.
Monocytes	51.625	0.580	1	0.446	0.535	1	0.465
Memory B	47.828	5.623	1	0.018	4.332	1	0.037

Change From Previous Block		
Chi-square	df	Sig
0.535	1	0.465
4.332	1	0.037

Table 14: Omnibus Tests of Modal Coefficients.

The Omnibus Tests of Modal Coefficients generated -2 Log Likelihood 52.160. From Table 14, monocytes have an omnibus tests of modal coefficient (51.625) close to 52.160 compared to the Memory B (46.377). The P-values shows that memory B is statistically more significant in predicting flare compared to monocyte.

	B	SE	Wald	df	Sig.	EXP (B)	95% Cl for Exp (B)		Covariate Mean
							Lower	Upper	
Monocytes Tetherin 1000s	0.077	0.101	0.578	1	0.447	1.080	0.886	1.316	6.402
Memory B Tetherin 1000s	0.5	0.225	4.949	1	0.026	1.649	1.061	2.562	2.605

Table 15: Variables in the equation

The above table shows memory B is statistiscally significant in predicting flare compared to monocytes. Memory B cells have a lower P-value, a higher hazared ratio (Exp (B)) and a lower convariate mean compared to monocytes. Because the hazard ratio for memory B was above 1 and confidence interval (95% for Exp (B)) is above 1.0, these findings indicate that having high level of tetherin protein on memory B cells increases the risk of flare. This is because it increases the risk of the flare with a higher hazard ratio and higher confidence interval (95% for Exp (B)) compared to monocytes.

Section conclusion

These results confirmed the hypothesis that, when patients are in clinical remission, the presence of high memory B cell tetherin predicts a higher rate of clinical flare in follow up. This is clinically useful, as when patients are in remission physicians may want to reduce therapies such as glucocorticoids if the risk of flare is low. However, they may even want to increase therapy (or repeat a cyclic treatment such as rituximab) if risk of flare is high. The main limitation of these results is that covariates were not adjusted such as other therapies. This would require a larger sample with more detailed clinical.

6.0 Determining whether tetherin level falls at follow up of flare patients.

The results in this section test the following hypothesis:

Hypothesis 4: SLE patients who are flaring will show a reduction in tetherin level following treatment.

Section methods

Sample was obtained from 15 SLE flaring patients according to BILAG score. Flow cytometry panel was performed on each sample. Cells were stained as described in 2.4.1 and level of tetherin was measured by median fluorescence intensity of tetherin protein. Tetherin intensity was measured at baseline and at follow up visit. Wilcoxon matched-paired Signed rank test was used to compare the groups. The table below gives a snap shot of the 15 patients, with their condition, treatments and demographic.

Patient	Age	Gender	Demographic	Disease characteristics (BILAG)		Medication / Treatment			
				Muco	MSK	RTX	Steroid	Hydro	MMF MTX AZA
1	62	F	White British	A	B	Y	Y	N	Y
2	40	F	Indian	B	D	Y	Y	Y	Y
3	48	F	Black	C	D	Y	Y	Y	Y
4	30	F	Pakistani	B	B	Y	Y	Y	Y
5	34	F	White British	B	D	Y	Y	N	Y
6	43	F	White British	B	B	Y	N	Y	Y
7	47	M	Caribbean	B	C	N	Y	Y	Y
8	61	F	White British	C	D	N	N	Y	Y
9	42	F	N/A	D	C	N	N	Y	N
10	34	F	White British	B	C	N	Y	Y	Y
11	32	F	N/A	D	A	Y	Y	Y	Y
12	42	F	White British	A	B	Y	Y	N	Y
13	69	F	White British	C	D	Y	Y	Y	Y
14	33	F	Indian	B	C	N	Y	Y	Y
15	46	F	White British	B	C	Y	N	Y	Y

Table 16: SLE BILAG flare patients used to determine the effect of treatments.

Muco = Mucocutaneous, MSK = musculoskeletal, RTX = Rituximab, Hydro = Hydroxychloroquine, MMF = Mycophenolate mofetil, MTX = Methotrexate and AZA = Azathioprine. N = No and Y = Yes.

Section Results

Figure 22 below, shows the median intensity of tetherin protein on memory B cell surface in flare patients. The median intensity of tetherin protein was compared between the first and second visit. This is to determine whether the level of tetherin decreases with treatment, and whether this was associated with patients going into remission.

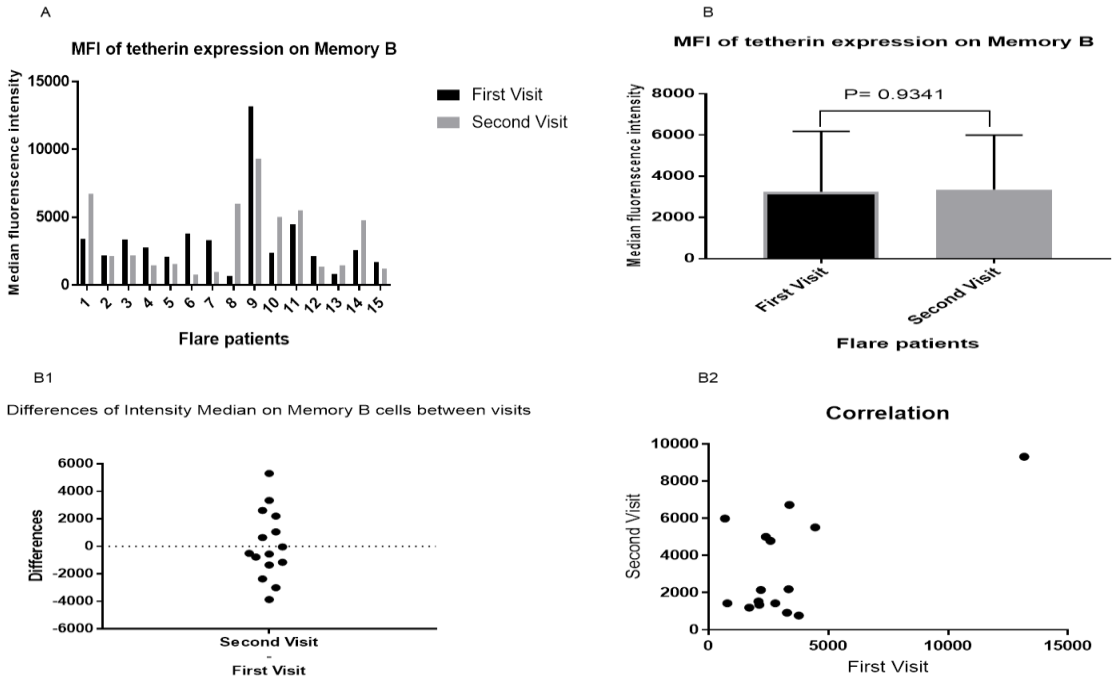


Figure 22: 15 repeat measures of median fluorescence intensity of memory B tetherin expression on Flare patients

The Figure represents the median fluorescence intensity of tetherin protein on memory B cell surface on SLE flare patients. Figure B was drawn with mean and standard deviation. Wilcoxon matched-paired Signed rank test was used to assess the P-value and there is no significant difference. Figure 22B1 shows there is no significant difference between the first and second visit. Figure 22B2 shows there is no or poor correlation between the visits.

Figure 22A shows there is a difference between the visits. Patient 3, 4, 5, 6, 7, 9, 12 and 15 have higher median intensity of tetherin in the first visit compared to the rest of the patients. Patient 1, 8,10,11,13, and 14 have higher median intensity of tetherin in the second visit compared to the rest of the patients. A significant difference was noted from both visits with patient 1, 6, 7, 8, 9, 10 and 14. Patient 2 shows no difference. There is a significant decrease of tetherin expression level noted on patients 5 and 7 on the second visit compared to the first visit. Patient 1, 8, 10 and 14 shows a significant increase in the level of tetherin in the second visit compared to the first visit. Patient 9 is shown to express the highest level of tetherin on both visits compared to the rest to the patients.

Figure 22B, the intensity of tetherin protein increased slightly in the second visit compared to the first visit. However there is no statistically significant difference with the intensity of tetherin on memory B in both visits as shown by Wilcoxon matched-paired Signed rank test, P-value 0.9341. The statistical analysis gives a sum of signed ranks (W) -4 and median difference of -507. Spearman 0.2449 outcome justifies the pairing was not significantly effective.

Section Conclusions

These results did not support the hypothesis, there is no change observed in tetherin level in follow up. Unfortunately, however, there were major limitations to this analysis. The number of follow up samples received was much lower than expected. This prevented analysis to be performed in patients who improved to remission in follow up. We were also unable to test whether

different levels of flare at baseline and different types of therapy would have more or less effect on change in tetherin levels. Therefore, no conclusion is drawn for this hypothesis, but these results would help to design a definitive study with larger numbers in future.

7.0 Use of tetherin as a biomarker to select targeted therapy.

The results in this section test the following hypothesis:

Hypothesis 5: Tetherin may have value as part of a biomarker panel to select patients for targeted therapy.

Section methods

Interferon-blocking biologic therapy (anifrolumab) is currently in phase III clinical trials and phase II data suggest it should only be prescribed to patients who have evidence of high interferon activity. Tetherin might be useful for this purpose. Another biologic is already licensed for SLE: the anti BAFF biologic belimumab. In England, belimumab is only commissioned for patients who have “B cell biomarkers” that have raised levels of anti-dsDNA antibodies and low levels of complement C3 or C4. There was no access available to samples from patients treated with anifrolumab. However, tetherin results from this study was used to test how many patients would be judged to be eligible for each of these agents if both were available. This judgement depends on the relationship between the tetherin and B cell biomarkers.

Section Results: illustration of patients that could be eligible for IFN blocking therapy.

A: based on tetherin expression

		Anifrolumab Y	Anifrolumab N
Belimumab	Y	Valid for both drugs 7 (13.73%)	Belimumab only 2 (2.92%)
	N	Anifrolumab only 25 (49.02%)	Invalid for both drugs 17 (33.35%)

B: based on IFN score A

		Anifrolumab Y	Anifrolumab N
Belimumab	Y	Valid for both drugs 9 (17.65%)	Belimumab only 0 (0%)
	N	Anifrolumab only 31 (60.78%)	Invalid for both drugs 11 (21.57%)

Table 17: SLE patients that would be appropriate for either anifrolumumab or belimumab or for both drugs and not suitable for either (Y= yes and N= no).

Table 17A was created based on tetherin expression. It only includes the primary or single visit (n=51). Table 17B was created based on interferon (IFN) score A, only includes the primary score A (n=51). Tetherin reference range was determined by taking the upper confidence interval (CI = 2300) of memory B cells of the healthy controls. IFN score A, reference range was determined by taking the lower bound limit (5.677632) of the healthy control ISGs expression. % was determined by the number of patients divided by 'n' multiple by 100.

Table 17A gives an indication of flare patients that would be eligible for either anifrolumab or belimumab or for both drugs. It also shows patients that were not eligible for either drug based on tetherin expression. The tetherin reference range was determined by taking the upper confidence interval (CI = 2300) of the intensity of tetherin expression on memory B cells in healthy controls. Tetherin expression above the CI value indicates high expression of tetherin. Table 17B gives an indication of flare patients that would be appropriate for either anifrolumab or belimumab or for both drugs, and patients that would not be suitable for either drugs based on interferon (IFN) gene expression score A. IFN score A, reference range was determined by taking the lower bound limit (5.677632) of the healthy control IFN genes expression on memory B cells. Score below the lower bound value (reference range) indicate high expression of tetherin. Majority of the patients are eligible for anifrolumab. Table 17 was presented on graphs format and healthy controls are included to illustrate the data better, see Figure 23 below.

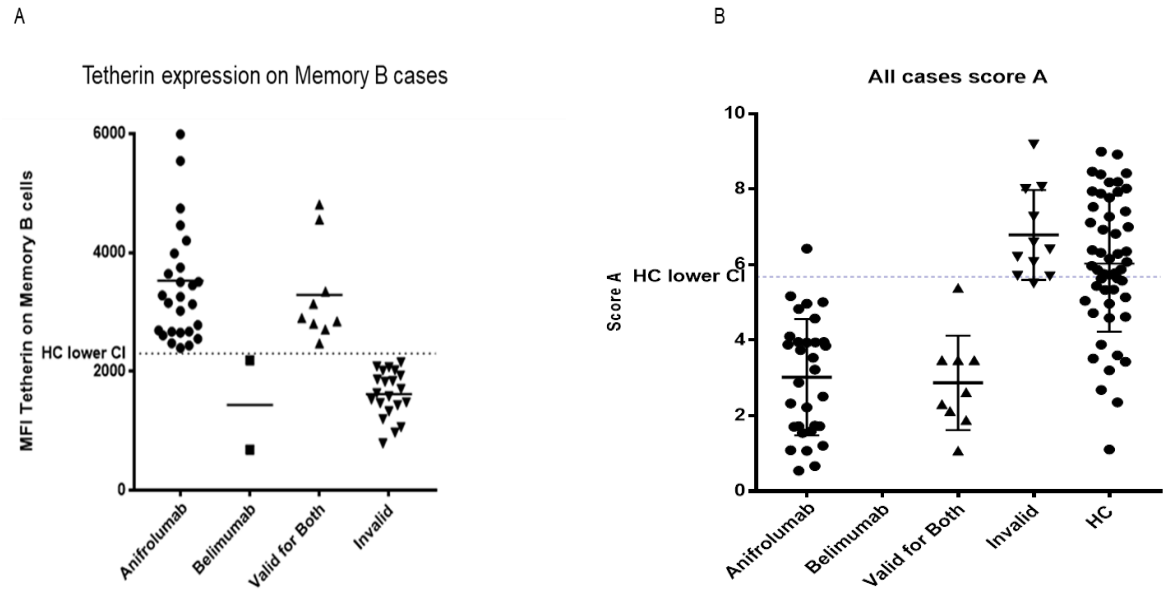


Figure 23: illustration indicating SLE flare patients eligible for either IFN blocking therapy or anti-BAFF therapy or eligible for both and not eligible for either therapy.

Figure 23A-B shows patients that are below and above the mean. Figure 23A was created based on tetherin expression on memory B cells. Figure 23B was created based on IFN score A. The dotted line gives an indication of the reference range. Tetherin reference range was determined by taking the upper confidence interval (CI = 2300) of memory B cells of the healthy controls. IFN score A, reference range was determined by taking the lower bound limit (5.677632) of the healthy control IFN genes expression.

Figure 23A shows patients eligible for anifrolumab and eligible for both therapy expressed tetherin that is equal or greater than the upper CI value (CI=2300). It also showed that patients eligible for belimumab and those that were not eligible for both therapy expresses lower intensity of tetherin, that is below the upper CI value.

Figure 23B shows IFN score A (lower bound limit = 5.677632) was calculated based on the IFN genes expression. Scores that are equal or below the reference score A (5.677632) expressed higher level of IFN compered to scores

above (5.677632). Figure 23B1-B2 shows no patients in our cohort is eligible for belimumab based on the IFN score A. Majority of the patients are eligible of anifrolumab and fewer eligible for both treatments.

Section conclusion

These results illustrate how a convenient interferon biomarker could be applied in routine clinical practice. The results shows that the majority of flaring patients have evidence of increased interferon activity using tetherin, a proportion that is consistent with other population data using gene expression assays. For the first time we show the relationship between the biomarker criteria for these two biologic therapies. These data show that the population of patients eligible for anifrolumab is larger and further includes almost all the patients eligible for belimumab.

8.0 Statistical Analysis

The Kruskal-Wallis test was used to compare the groups. This test is an *omnibus* test statistic and it cannot tell which specific groups of the independent variable are statistically significantly different from each other; it only tells at least two groups were different. When comparing three groups (healthy control, flare and remission groups) determining which of these groups differ from each other, Dunn's multiple comparisons test was used.

Dunn's multiple comparisons test was used to determine the level of significance where 0.05 was divided by the number of groups compared, so 0.05 was divided by 2 = 0.025, therefore the level of significant is 0.03. This was used to determine which groups differ. Dunn's multiple comparisons test are multiplicity adjusted P values.

The Kruskal-Wallis test analysis indicates significant differences between the medians. Significant differences (P-values) were less than 0.05.

Cox regression, the omnibus tests of modal coefficients (Chi-square) test did not distinguish which group differ, the variables in the equation determined which group differ.

9.0 Discussion

This study investigated several aspects of tetherin as a biomarker in SLE. We showed several areas in which there is potential utility; tetherin functioned as a cell-specific interferon biomarker as expected, correlated with diagnosis and predicted flares. Our results also suggest how tetherin could be used in future to select patients for interferon-blocking therapy. Below covers the discussion of the positive and negative findings, limitations, and the potential for future work.

Tetherin was initially discovered as a cell surface protein that was highly expressed on multiple myeloma cells (Wang et al., 2009). This anti-viral gene has also been identified as being overexpressed in many solid organ tumours, including breast cancer (Mahauad-Fernandez et al., 2015). It has been noted to be critical for the invasiveness of breast cancer cells and the formation of metastasis in *vivo* (Mahauad-Fernandez et al., 2015). Several studies have been carried out to determine the effect and impact of tetherin in cancer and HIV studies.

In 2014 Mahauad-Fernandez *et al* performed a Meta-analysis of tumours from breast cancer patients obtained from the Gene Expression Omnibus (GEO) and The Cancer Genome Atlas (TCGA) data-sets and they were evaluated for levels of BST-2 (tetherin) expression and for tumour aggression. In humans, they found tetherin mRNA was elevated in metastatic and invasive breast tumours (Mahauad-Fernandez et al., 2014a). Their findings demonstrated that tetherin contributes to the emergence of neoplasia and malignant progression of breast cancer. The group concluded tetherin may serve as a biomarker for aggressive

breast cancers, and may also be a novel target for breast cancer therapeutics (Mahauad-Fernandez et al., 2014a).

A comprehensive meta-analyses of BST-2 gene expression and BST-2 DNA methylation in TCGA and various GEO datasets (Mahauad-Fernandez et al., 2015) was carried out to compare tetherin expression levels and tetherin DNA methylation status at specific CpG sites on the tetherin (BST-2) gene by Mahauad-Fernandez et al (2015). The findings show that tetherin gene expression was associated with the methylation status at specific CpG sites in primary breast cancer specimens and breast cancer cell lines. They found out that, tetherin demethylation was significantly more predominant in primary tumours and cancer cells than in normal breast tissues or normal epithelial cells. These findings suggested that DNA methylation pattern and expression of tetherin may play a role in disease pathogenesis and also suggested it could serve as a biomarker for the diagnosis of breast cancer.

Tetherin is an interferon-inducible protein and has vital roles in anti-viral immunity. HIV studies have shown tetherin to impair the release of mature HIV-1 particles from infected cells. Tetherin has been found to be an innate restriction factor limiting HIV cell-to-cell spread. It acts by impairing viruses in donor cells, and significantly reducing their infectious potential once they have been transferred to target cells (Casartelli et al., 2010). Other HIV studies have shown that tetherin is upregulated in infected patients compared to healthy controls. A study carried out by (Homann et al., 2011) showed the detection of BST-2 by flow cytometry on the surface of subsets of peripheral blood leukocytes, including $CD4^+$ T-cells from healthy and HIV infected individuals. They found out BST-2 was upregulated by 2 to 3 fold on cells of infected patients

(Homann et al., 2011). It was shown the upregulation occurs on $CD4^+$ T-cells and appears to be part of an innate response to the virus itself. However, they later observed that elevated levels decreased slightly during chronic HIV infection by effective antiretroviral therapy (Homann et al., 2011). It is because of the interferon-inducible character of tetherin that makes it have the potential value as an SLE biomarker. The unique property of this protein is that most other interferon inducible proteins are intracellular or, in the case of sialic acid binding Ig-like lectin 1 (SIGLEC-1), highly expressed on monocytes (Xiong et al., 2017). A limitation of gene expression assays for interferon, apart from logistical difficulty of using gene expression in routine clinical practice, is that they may be affected by changes in the cellular composition of the sample rather than a change in secretion of the interferon ligand. Flow cytometry removes this problem, and also allows analysis of specific cell subsets (such as B cells) that have a more prominent role in models of SLE pathogenesis.

9.1 Evaluation of the comparison between PBMC and Whole Blood staining.

The first section addresses basic requirements for the measurement of tetherin. Tetherin expression was similar between PBMCs and whole blood staining. The findings demonstrated that the staining protocols produced similar levels of tetherin protein on the cell surface for most subsets. PBMC staining showed a slightly higher median intensity of tetherin protein compared to whole blood staining but this was not statistically significant except for monocytes. We previously found that, although tetherin MFI was high on monocytes, this did not correlate well with clinical characteristics of SLE. Memory B cells tetherin

was previously found to best correlate with clinical outcomes and was, therefore, the priori focus of my study.

The use of whole blood to perform lymphocyte subsets analysis is a straightforward procedure compared to the laborious preparation for the analysis of isolated PBMC with cell enumeration (Appay et al., 2006). Additionally, it has the advantage to the patient of requiring only small amounts of blood 4 mL compared to PBMCs which requires 18 mL (Marits et al., 2014). The results are therefore essential if memory B cells tetherin is to be analysed in a routine diagnostic laboratory setting. After considering the disadvantages of PBMC (PBMC density gradient separation) isolated by Ficoll, it was decided to proceed with whole blood staining technique for this study.

Our results also demonstrated that tetherin MFI is a better measure of expression than proportion of “tetherin positive” cells. The skewed distribution of tetherin MFI indicates that median MFI should be reported in clinical use, and that non-parametric statistics should be used in research. We understand the difficulties of using MFI based cytometric measurement technique across different laboratories however; these can be minimised by using the same batch and clone of monoclonal antibodies. Set the voltages and perform compensation using cells instead of beads. Perform baseline check every 6 months, Calibrate and run quality control (CS&T beads) daily to ensure the lasers are set. We could also use standardised MFI reference beads. Standardised MFI beads would reduce changes between different Flow cytometry. We believe reproducibility would be achieved through utilisation of standard operating procedures, common reagents and normalisation algorithms.

Flow cytometry is routinely used in other clinical settings such as HIV monitoring, immunodeficiencies and, in rheumatology, monitoring B cell numbers after anti-CD20 therapy. Most of these applications rely on enumerating cell populations rather than fluorescence intensity.

9.2 Comparing tetherin expression between different patient groups.

The SLE patient groups and healthy control group level of tetherin were compared to determine which group produced the highest level of tetherin. We confirmed that, as expected, the SLE patients exhibited a higher level of tetherin compared to the healthy controls. Surprisingly, when flare and remission patients were compared, remission group expressed higher level of tetherin compared to flare.

Previous studies have sought to compare interferon activity using gene expression with disease activity. Although some showed a correlation, others did not (Niewold et al., 2010, Blanco et al., 2001, Chiche et al., 2014). We had hypothesised that tetherin would clarify this and indeed my group's previous work showed a closer correlation of memory B cells tetherin with diagnosis and disease activity than gene expression. Although this study did not reproduce that finding, there are several limitations to the study that may have affected the findings and would need further research to explore. First, this study was designed to mimic routine care and we therefore asked requesting physicians to label samples as either "flare" or "remission". Assessment of disease activity in SLE is difficult. Physicians may decide that a patient is likely to be flaring based on subjective symptoms such as joint pain without objective evidence, and treat them accordingly. In my group's previous research we avoided this

problem by using BILAG scores, which require objective evidence to score highly for disease activity. Second, this setting, as well as the patient numbers in the study, did not allow us to control other characteristics such as organ involvement and therapy. In our group's previous research interferon activity was found to be more closely associated with certain organs (such as skin disease), with no association with musculoskeletal disease. Immunosuppressant therapies, prescribed in flare patients, modify biological parameters as well as clinical disease activity and may have affected my findings (Homann et al., 2011). The effect of rituximab may be particularly important since it depletes memory B cells as well as lowering the tetherin expression in the small numbers of repopulating B cells (Vital et al., 2011). A new study in Leeds (DEFINITION) has been designed to overcome these problems and my results were used to design that study.

2.1 Evaluating the sensitivity and specificity of memory B cell tetherin for diagnosis of SLE using flow cytometry in comparison to gene expression.

We determined the sensitivity and specificity of the diagnostic value of tetherin on memory B cells by comparing healthy controls and patients (both Flare and Remission). We found that gene expression (IFN score A) produced slightly higher sensitivity (69.51%) and better specificity (80%) in comparison to flow cytometry sensitivity (65.65 %) and specificity (70%). We concluded by stating the test does discriminate between abnormal patients and normal controls even though the assay level of sensitivity and specificity of this test in isolation of other clinical criteria and biomarkers is suboptimal.

Producing an accurate assay with high sensitivity and high specificity would aid in the diagnosing, stratifying and monitoring of the SLE disease. It would also ensure that SLE patients receive appropriate treatment promptly. However, for a test to be clinically useful and to be introduced into routine clinical practice, the assay must be sensitive enough to detect the analyte of interest (Powers and Palecek, 2012), in our case tetherin protein. The assay must be specific to the protein that it aims to detect as there is less protein concentration in blood and our assay produced 70% specificity. Interferon activity is a marked feature of SLE but is not unique to this disease, as discussed above.

Alternative biomarkers commonly used in diagnosis of SLE are autoantibody tests. A study carried out by Heidenreich *et al* (2009) investigated the diagnostic value of nine kits for autoantibody against ds-DNA, ANA, circulating ICs, C1q, histones, nucleosomes, Sm, C3 and C4 levels. These kits were evaluated in 39 patients with biopsy-proven lupus nephritis in comparison to 43 patients suffering from other forms of glomerulonephritis (Heidenreich et al., 2009). They only found one test to be useful which was anti-nucleosome antibody performed by enzyme-linked immunosorbent assay (ELISA) with a sensitivity of 90% and a specificity of 88% (Heidenreich et al., 2009). This study proves that our assay would not be a useful test in routine clinical practice as it produced less sensitivity and less specificity. The tests for anti-dsDNA antibodies performed with Crithidia luciliae Anti-dsDNA and BINDAZYME Anti-dsDNA were found to be of moderate sensitivity and good specificity. However, anti-C1q and ICs performed worse than the anti-dsDNA tests, with less sensitivity and less specificity (Heidenreich et al., 2009).

9.3 Determining whether Tetherin can predict flare in SLE patients.

Because SLE has a relapsing-remitting disease course, prediction of flare is an unmet need for rheumatologists and immunologists. The level of tetherin on Memory B cells and monocytes were therefore investigated to identify whether it could predict flare, as well as which cell type would be best at predicting flare. We found that, although the flare group consisted of only 8 patients compared to the remission group (n=28), the flare group showed a significantly higher intensity of tetherin on Memory B cells. In contrast, when analysing monocytes there was no statistical difference between the groups. These findings are of great interest as, not only does tetherin appear clinically useful, they also underline the central hypothesis that interferon response in particular cell subsets may be more clinically significant than others.

Patient numbers were too low to create an entirely adjusted Cox regression model, that accounts for the other co-factors that can affect flare-free survial, for example medication (immunosupressants) ethnic background and age, C3, C4 and anti-DNA levels.

Other studies have investigated biomarkers predictive of flare in SLE patients, and they have discovered that increased IFN levels correlate with disease flares (Landolt-Marticorena et al., 2009, Niewold et al., 2010). A cross-sectional study showed that IFN-induced chemokines correlated with disease activity as the expression increased at the time of flare, and decreased as the disease remitted (Landolt-Marticorena et al., 2009), suggesting that these chemokine could be used as a potential biomarker during the study. This is consistent with our finding.

9.4 Determining whether the Median intensity of tetherin protein on Memory B cells decreased in the second visit with Flare patients.

The level of tetherin on memory B cells was measured by flow cytometry at both the baseline visit and a follow up visit on 15 SLE flare patients. A significant difference was noted between both visits for some with patients. As all patients received treatment at first visit, the decrease in the expression of tetherin could be a positive response to treatment. However, there was no overall statistical difference in the intensity of tetherin protein on memory B cells at follow up, as we had hypothesised.

Apart from the limited patient numbers, this could be due to varying treatments prescribed, types of flare, and variable response to treatment (in most trials clinical response rates are around 50%). We were able to confirm objective flare in our 15 patients but these issues could be resolved in a larger study.

9.5 Evaluating the use of tetherin as a biomarker to select targeted therapy.

As there are now interferon-blocking studies in phase III trials, an interferon-specific biomarker is likely to be of value in selecting patients for therapy. SLE is clinically and immunologically heterogeneous, and biomarkers may by valuable to stratify patients according to the most appropriate therapeutic target (Psarras et al., 2017). Anifrolumab (an interferon-blocking biologic) appears to be most useful in patients with high interferon activity. Meanwhile, the B cell targeted agent belimumab is most effective, and routinely commissioned, in patients with B cell biomarkers of activity (anti-dsDNA titres and low

complement). We therefore used my results to estimate how many flaring patients would be appropriate for one, both or neither of these therapies.

We found that a larger number of flare patients appeared eligible for IFN blocking therapy rather than anti-BAFF therapy (belimumab). Almost all patients eligible for belimumab would also be eligible for anifrolumab. This illustrates how a convenient interferon biomarker could be applied in routine clinical practice, allowing the tailoring of medication to each individual patient. Blocking the production of IFN (IFN pathway) could, in patient with high tetherin expression, potentially improve prognosis and consequently decrease mortality and morbidity. Further work would involve the use of tetherin levels in patients receiving anifrolumab and belimumab therapy when these are more widely available.

In conclusion, this study has evaluated tetherin expression using two complementary methods, flow cytometry and gene expression using qRT-PCR. We have identified that tetherin expression is significantly higher in patients with SLE in comparison to healthy controls, with sensitivity of 65.65% and specificity of 70% by flow cytometry. Additionally we found that in patients with clinical remission, the presence of high memory B cell tetherin predicted a higher rate of flare in follow up. This has the potential to be clinically useful, as memory B cells tetherin may guide physicians in the adjustment of maintenance therapies based on the predictive risk of flare. Unfortunately we did not observe a change in tetherin level in follow up, when compared tetherin expression on memory B cells on baseline and follow up visits with flare patients. The number of follow up samples received was much lower than anticipated. We also examined how

convenient interferon biomarker could be applied in routine clinical practice. We found that the majority of flaring patients have evidence of increased interferon activity using tetherin (flow cytometry), a proportion that is consistent with using gene expression assay. We found out that the population of patients' eligible for anifrolumab is larger than, and inclusive of, the patients eligible for belimumab. Tetherin has shown to have potential factors that would make it to be a reliable biomarker for SLE patients in comparison to the current biomarkers (e.g. anti-dsDNA, C4, C3). Due to the limitations experienced in this study, it would be useful to explore these limitations in a future study, which would help to generalise the findings and draw accurate conclusions.

10.0 Proposal for future work

This study aimed to create an accurate assay and to identify a reliable biomarker for SLE patients using limited sample sizes. In light of the findings, a definitive study has been designed. A sample size using 450 patients will be processed in a future multicentre trial with the same analytic techniques established. This will allow adequate power and clinical phenotyping to allow a final conclusion as to whether tetherin (or other interferon biomarkers) are valuable for the diagnosis, monitoring and treatment stratification of SLE patients.

References

ALARCON-SEGOVIA, D., ALARCON-RIQUELME, M. E., CARDIEL, M. H., CAEIRO, F., MASSARDO, L., VILLA, A. R. & PONS-ESTEL, B. A. 2005. Familial aggregation of systemic lupus erythematosus, rheumatoid arthritis, and other autoimmune diseases in 1,177 lupus patients from the GLADEL cohort. *Arthritis Rheum,* 52**,** 1138-47.

ALARCON, G. S., MCGWIN, G., BERTOLI, A. M., FESSLER, B. J., CALVO-ALEN, J., BASTIAN, H. M., VILA, L. M. & REVEILLE, J. D. 2007. Effect of hydroxychloroquine on the survival of patients with systemic lupus erythematosus: data from LUMINA, a multiethnic US cohort (LUMINA L). *Ann Rheum Dis,* 66**,** 1168-72.

ANDREW, A. J., KAO, S. & STREBEL, K. 2011. C-terminal hydrophobic region in human bone marrow stromal cell antigen 2 (BST-2)/tetherin protein functions as second transmembrane motif. *J Biol Chem,* 286**,** 39967-81.

ANDREW, A. J., MIYAGI, E., KAO, S. & STREBEL, K. 2009. The formation of cysteine-linked dimers of BST-2/tetherin is important for inhibition of HIV-1 virus release but not for sensitivity to Vpu. *Retrovirology,* 6**,** 80.

ANOLIK, J. H. 2007. B cell biology and dysfunction in SLE. *Bull NYU Hosp Jt Dis,* 65**,** 182-6.

APPAY, V., REYNARD, S., VOELTER, V., ROMERO, P., SPEISER, D. E. & LEYVRAZ, S. 2006. Immuno-monitoring of CD8+ T cells in whole blood versus PBMC samples. *J Immunol Methods,* 309**,** 192-9.

ARECHIGA, A. F., HABIB, T., HE, Y., ZHANG, X., ZHANG, Z. Y., FUNK, A. & BUCKNER, J. H. 2009. Cutting edge: the PTPN22 allelic variant associated with autoimmunity impairs B cell signaling. *J Immunol,* 182**,** 3343-7.

ARMSTRONG, D. L., REIFF, A., MYONES, B. L., QUISMORIO, F. P., JR., KLEIN-GITELMAN, M., MCCURDY, D., WAGNER-WEINER, L., SILVERMAN, E., OJWANG, J. O., KAUFMAN, K. M., KELLY, J. A., MERRILL, J. T., HARLEY, J. B., BAE, S. C., VYSE, T. J., GILKESON, G. S., GAFFNEY, P. M., MOSER, K. L., PUTTERMAN, C., EDBERG, J. C., BROWN, E. E., ZIEGLER, J., LANGEFELD, C. D., ZIDOVETZKI, R. & JACOB, C. O. 2009. Identification of new SLE-associated genes with a two-step Bayesian study design. *Genes Immun,* 10**,** 446-56.

BANCHEREAU, J. & PASCUAL, V. 2006. Type I interferon in systemic lupus erythematosus and other autoimmune diseases. *Immunity,* 25**,** 383-92.

BARTEE, E., MANSOURI, M., HOVEY NERENBERG, B. T., GOUVEIA, K. & FRUH, K. 2004. Downregulation of major histocompatibility complex class I by human ubiquitin ligases related to viral immune evasion proteins. *J Virol,* 78**,** 1109-20.

BARTEE, E., MCCORMACK, A. & FRUH, K. 2006. Quantitative membrane proteomics reveals new cellular targets of viral immune modulators. *PLoS Pathog,* 2**,** e107.

BERTSIAS, G., IOANNIDIS, J. P., BOLETIS, J., BOMBARDIERI, S., CERVERA, R., DOSTAL, C., FONT, J., GILBOE, I. M., HOUSSIAU, F., HUIZINGA, T., ISENBERG, D., KALLENBERG, C. G., KHAMASHTA, M., PIETTE, J. C., SCHNEIDER, M., SMOLEN, J., STURFELT, G., TINCANI, A., VAN VOLLENHOVEN, R., GORDON, C. & BOUMPAS, D. T. 2008. EULAR recommendations for the management of systemic

lupus erythematosus. Report of a Task Force of the EULAR Standing Committee for International Clinical Studies Including Therapeutics. *Ann Rheum Dis,* 67**,** 195-205.

BLANCO, P., PALUCKA, A. K., GILL, M., PASCUAL, V. & BANCHEREAU, J. 2001. Induction of dendritic cell differentiation by IFN-alpha in systemic lupus erythematosus. *Science,* 294**,** 1540-3.

BLANCO, P., PITARD, V., VIALLARD, J. F., TAUPIN, J. L., PELLEGRIN, J. L. & MOREAU, J. F. 2005. Increase in activated CD8+ T lymphocytes expressing perforin and granzyme B correlates with disease activity in patients with systemic lupus erythematosus. *Arthritis Rheum,* 52**,** 201-11.

BLASIUS, A. L., GIURISATO, E., CELLA, M., SCHREIBER, R. D., SHAW, A. S. & COLONNA, M. 2006. Bone marrow stromal cell antigen 2 is a specific marker of type I IFN-producing cells in the naive mouse, but a promiscuous cell surface antigen following IFN stimulation. *J Immunol,* 177**,** 3260-5.

BLOMBERG, S., ELORANTA, M. L., CEDERBLAD, B., NORDLIN, K., ALM, G. V. & RONNBLOM, L. 2001. Presence of cutaneous interferon-alpha producing cells in patients with systemic lupus erythematosus. *Lupus,* 10**,** 484-90.

CAO, W., BOVER, L., CHO, M., WEN, X., HANABUCHI, S., BAO, M., ROSEN, D. B., WANG, Y. H., SHAW, J. L., DU, Q., LI, C., ARAI, N., YAO, Z., LANIER, L. L. & LIU, Y. J. 2009. Regulation of TLR7/9 responses in plasmacytoid dendritic cells by BST2 and ILT7 receptor interaction. *J Exp Med,* 206**,** 1603-14.

CAPPIONE, A., 3RD, ANOLIK, J. H., PUGH-BERNARD, A., BARNARD, J., DUTCHER, P., SILVERMAN, G. & SANZ, I. 2005. Germinal center exclusion of autoreactive B cells is defective in human systemic lupus erythematosus. *J Clin Invest,* 115**,** 3205-16.

CASARTELLI, N., SOURISSEAU, M., FELDMANN, J., GUIVEL-BENHASSINE, F., MALLET, A., MARCELIN, A. G., GUATELLI, J. & SCHWARTZ, O. 2010. Tetherin restricts productive HIV-1 cell-to-cell transmission. *PLoS Pathog,* 6**,** e1000955.

CHICHE, L., JOURDE-CHICHE, N., WHALEN, E., PRESNELL, S., GERSUK, V., DANG, K., ANGUIANO, E., QUINN, C., BURTEY, S., BERLAND, Y., KAPLANSKI, G., HARLE, J. R., PASCUAL, V. & CHAUSSABEL, D. 2014. Modular transcriptional repertoire analyses of adults with systemic lupus erythematosus reveal distinct type I and type II interferon signatures. *Arthritis Rheumatol,* 66**,** 1583-95.

COXON, A., RIEU, P., BARKALOW, F. J., ASKARI, S., SHARPE, A. H., VON ANDRIAN, U. H., ARNAOUT, M. A. & MAYADAS, T. N. 1996. A novel role for the beta 2 integrin CD11b/CD18 in neutrophil apoptosis: a homeostatic mechanism in inflammation. *Immunity,* 5**,** 653-66.

CRISPIN, J. C., KYTTARIS, V. C., TERHORST, C. & TSOKOS, G. C. 2010. T cells as therapeutic targets in SLE. *Nat Rev Rheumatol,* 6**,** 317-25.

DANCHENKO, N., SATIA, J. A. & ANTHONY, M. S. 2006. Epidemiology of systemic lupus erythematosus: a comparison of worldwide disease burden. *Lupus,* 15**,** 308-18.

DE ANDREA, M., RAVERA, R., GIOIA, D., GARIGLIO, M. & LANDOLFO, S. 2002. The interferon system: an overview. *Eur J Paediatr Neurol,* 6 Suppl A**,** A41-6; discussion A55-8.

DE WEERD, N. A., SAMARAJIWA, S. A. & HERTZOG, P. J. 2007. Type I interferon receptors: biochemistry and biological functions. *J Biol Chem,* 282**,** 20053-7.

DEAPEN, D., ESCALANTE, A., WEINRIB, L., HORWITZ, D., BACHMAN, B., ROY-BURMAN, P., WALKER, A. & MACK, T. M. 1992. A revised estimate of twin concordance in systemic lupus erythematosus. *Arthritis Rheum,* 35**,** 311-8.

DENG, Y. & TSAO, B. P. 2010. Genetic susceptibility to systemic lupus erythematosus in the genomic era. *Nat Rev Rheumatol,* 6**,** 683-92.

DI PUCCHIO, T., PILLA, L., CAPONE, I., FERRANTINI, M., MONTEFIORE, E., URBANI, F., PATUZZO, R., PENNACCHIOLI, E., SANTINAMI, M., COVA, A., SOVENA, G., ARIENTI, F., LOMBARDO, C., LOMBARDI, A., CAPORASO, P., D'ATRI, S., MARCHETTI, P., BONMASSAR, E., PARMIANI, G., BELARDELLI, F. & RIVOLTINI, L. 2006. Immunization of stage IV melanoma patients with Melan-A/MART-1 and gp100 peptides plus IFN-alpha results in the activation of specific CD8(+) T cells and monocyte/dendritic cell precursors. *Cancer Res,* 66**,** 4943-51.

DOUGLAS, J. L., VISWANATHAN, K., MCCARROLL, M. N., GUSTIN, J. K., FRUH, K. & MOSES, A. V. 2009. Vpu directs the degradation of the human immunodeficiency virus restriction factor BST-2/Tetherin via a {beta}TrCP-dependent mechanism. *J Virol,* 83**,** 7931-47.

EGNER, W. 2000. The use of laboratory tests in the diagnosis of SLE. *J Clin Pathol,* 53**,** 424-32.

EL-SHERBINY, Y., MOHAMED, A. A., YUSOF, Y. M., VITAL, E. & EMERY, P. 2015. Flow-Based Cell-Specific Interferon Signature as a Biomarker in Systemic Lupus Erythematosus. *Annals of the Rheumatic Diseases,* 74**,** 562-563.

EL-SHERBINY, Y., YUSOF, M. Y. M., HENSOR, E., WITTMANN, M., EMERY, P. & VITAL, E. 2016. Surface Tetherin Is a Novel Cell-Specific Biomarker for Interferon Response in Systemic Lupus Erythematosus. *Annals of the Rheumatic Diseases,* 75**,** 546-546.

ELKON, K. B. & STONE, V. V. 2011. Type I interferon and systemic lupus erythematosus. *J Interferon Cytokine Res,* 31**,** 803-12.

FAIRHURST, A. M., HWANG, S. H., WANG, A., TIAN, X. H., BOUDREAUX, C., ZHOU, X. J., CASCO, J., LI, Q. Z., CONNOLLY, J. E. & WAKELAND, E. K. 2008. Yaa autoimmune phenotypes are conferred by overexpression of TLR7. *Eur J Immunol,* 38**,** 1971-8.

FRASER, D. A., LAUST, A. K., NELSON, E. L. & TENNER, A. J. 2009. C1q differentially modulates phagocytosis and cytokine responses during ingestion of apoptotic cells by human monocytes, macrophages, and dendritic cells. *J Immunol,* 183**,** 6175-85.

GALAO, R. P., LE TORTOREC, A., PICKERING, S., KUECK, T. & NEIL, S. J. 2012. Innate sensing of HIV-1 assembly by Tetherin induces NFkappaB-dependent proinflammatory responses. *Cell Host Microbe,* 12**,** 633-44.

GATEVA, V., SANDLING, J. K., HOM, G., TAYLOR, K. E., CHUNG, S. A., SUN, X., ORTMANN, W., KOSOY, R., FERREIRA, R. C., NORDMARK, G., GUNNARSSON, I., SVENUNGSSON, E., PADYUKOV, L., STURFELT,

G., JONSEN, A., BENGTSSON, A. A., RANTAPAA-DAHLQVIST, S., BAECHLER, E. C., BROWN, E. E., ALARCON, G. S., EDBERG, J. C., RAMSEY-GOLDMAN, R., MCGWIN, G., JR., REVEILLE, J. D., VILA, L. M., KIMBERLY, R. P., MANZI, S., PETRI, M. A., LEE, A., GREGERSEN, P. K., SELDIN, M. F., RONNBLOM, L., CRISWELL, L. A., SYVANEN, A. C., BEHRENS, T. W. & GRAHAM, R. R. 2009. A large-scale replication study identifies TNIP1, PRDM1, JAZF1, UHRF1BP1 and IL10 as risk loci for systemic lupus erythematosus. *Nat Genet,* 41**,** 1228-33.

GERGELY, P., JR., GROSSMAN, C., NILAND, B., PUSKAS, F., NEUPANE, H., ALLAM, F., BANKI, K., PHILLIPS, P. E. & PERL, A. 2002. Mitochondrial hyperpolarization and ATP depletion in patients with systemic lupus erythematosus. *Arthritis Rheum,* 46**,** 175-90.

GILLIET, M., CAO, W. & LIU, Y. J. 2008. Plasmacytoid dendritic cells: sensing nucleic acids in viral infection and autoimmune diseases. *Nat Rev Immunol,* 8**,** 594-606.

GOTO, T., KENNEL, S. J., ABE, M., TAKISHITA, M., KOSAKA, M., SOLOMON, A. & SAITO, S. 1994. A novel membrane antigen selectively expressed on terminally differentiated human B cells. *Blood,* 84**,** 1922-30.

GRAHAM, R. R., KYOGOKU, C., SIGURDSSON, S., VLASOVA, I. A., DAVIES, L. R. L., BAECHLER, E. C., PLENGE, R. M., KOEUTH, T., ORTMANN, W. A., HOM, G., BAUER, J. W., GILLETT, C., BURTT, N., CUNNINGHAME GRAHAM, D. S., ONOFRIO, R., PETRI, M., GUNNARSSON, I., SVENUNGSSON, E., RÖNNBLOM, L., NORDMARK, G., GREGERSEN, P. K., MOSER, K., GAFFNEY, P. M., CRISWELL, L. A., VYSE, T. J., SYVÄNEN, A.-C., BOHJANEN, P. R., DALY, M. J., BEHRENS, T. W. & ALTSHULER, D. 2007. Three functional variants of IFN regulatory factor 5 (IRF5) define risk and protective haplotypes for human lupus. *Proceedings of the National Academy of Sciences,* 104**,** 6758-6763.

GRAMMER, A. C. & LIPSKY, P. E. 2003. B cell abnormalities in systemic lupus erythematosus. *Arthritis Res Ther,* 5 Suppl 4**,** S22-7.

GRONDAL, G., GUNNARSSON, I., RONNELID, J., ROGBERG, S., KLARESKOG, L. & LUNDBERG, I. 2000. Cytokine production, serum levels and disease activity in systemic lupus erythematosus. *Clin Exp Rheumatol,* 18**,** 565-70.

HAQUE, S., MIRJAFARI, H. & BRUCE, I. N. 2008. Atherosclerosis in rheumatoid arthritis and systemic lupus erythematosus. *Curr Opin Lipidol,* 19**,** 338-43.

HARLEY, I. T., KAUFMAN, K. M., LANGEFELD, C. D., HARLEY, J. B. & KELLY, J. A. 2009. Genetic susceptibility to SLE: new insights from fine mapping and genome-wide association studies. *Nat Rev Genet,* 10**,** 285-90.

HARLEY, J. B., KELLY, J. A. & KAUFMAN, K. M. 2006. Unraveling the genetics of systemic lupus erythematosus. *Springer Semin Immunopathol,* 28**,** 119-30.

HEIDENREICH, U., MAYER, G., HEROLD, M., KLOTZ, W., STEMPFL AL-JAZRAWI, K. & LHOTTA, K. 2009. Sensitivity and specificity of autoantibody tests in the differential diagnosis of lupus nephritis. *Lupus,* 18**,** 1276-80.

HOCHBERG, M. C. 1997. Updating the American College of Rheumatology revised criteria for the classification of systemic lupus erythematosus. *Arthritis Rheum,* 40**,** 1725.

HOFFMAN, R. W. 2004. T cells in the pathogenesis of systemic lupus erythematosus. *Clin Immunol,* 113**,** 4-13.

HOMANN, S., SMITH, D., LITTLE, S., RICHMAN, D. & GUATELLI, J. 2011. Upregulation of BST-2/Tetherin by HIV infection in vivo. *J Virol,* 85**,** 10659-68.

HONDA, K., YANAI, H., NEGISHI, H., ASAGIRI, M., SATO, M., MIZUTANI, T., SHIMADA, N., OHBA, Y., TAKAOKA, A., YOSHIDA, N. & TANIGUCHI, T. 2005. IRF-7 is the master regulator of type-I interferon-dependent immune responses. *Nature,* 434**,** 772-7.

HOOKS, J. J., MOUTSOPOULOS, H. M., GEIS, S. A., STAHL, N. I., DECKER, J. L. & NOTKINS, A. L. 1979. Immune interferon in the circulation of patients with autoimmune disease. *N Engl J Med,* 301**,** 5-8.

ILLEI, G. G., TACKEY, E., LAPTEVA, L. & LIPSKY, P. E. 2004a. Biomarkers in systemic lupus erythematosus. I. General overview of biomarkers and their applicability. *Arthritis Rheum,* 50**,** 1709-20.

ILLEI, G. G., TACKEY, E., LAPTEVA, L. & LIPSKY, P. E. 2004b. Biomarkers in systemic lupus erythematosus: II. Markers of disease activity. *Arthritis Rheum,* 50**,** 2048-65.

IMANISHI, J. 1994. [Interferon-alpha, beta, gamma]. *Gan To Kagaku Ryoho,* 21**,** 2853-8.

ISENBERG, D. A., RAVIRAJAN, C. T., RAHMAN, A. & KALSI, J. 1997. The role of antibodies to DNA in systemic lupus erythematosus--a review and introduction to an international workshop on DNA antibodies held in London, May 1996. *Lupus,* 6**,** 290-304.

ISHIKAWA, J., KAISHO, T., TOMIZAWA, H., LEE, B. O., KOBUNE, Y., INAZAWA, J., ORITANI, K., ITOH, M., OCHI, T., ISHIHARA, K. & ET AL. 1995. Molecular cloning and chromosomal mapping of a bone marrow stromal cell surface gene, BST2, that may be involved in pre-B-cell growth. *Genomics,* 26**,** 527-34.

JAHNSEN, F. L., LUND-JOHANSEN, F., DUNNE, J. F., FARKAS, L., HAYE, R. & BRANDTZAEG, P. 2000. Experimentally induced recruitment of plasmacytoid (CD123high) dendritic cells in human nasal allergy. *J Immunol,* 165**,** 4062-8.

KAVAI, M. 2008. Immune complex clearance by complement receptor type 1 in SLE. *Autoimmun Rev,* 8**,** 160-4.

KIROU, K. A., LEE, C., GEORGE, S., LOUCA, K., PETERSON, M. G. & CROW, M. K. 2005. Activation of the interferon-alpha pathway identifies a subgroup of systemic lupus erythematosus patients with distinct serologic features and active disease. *Arthritis Rheum,* 52**,** 1491-503.

KUPZIG, S., KOROLCHUK, V., ROLLASON, R., SUGDEN, A., WILDE, A. & BANTING, G. 2003. Bst-2/HM1.24 is a raft-associated apical membrane protein with an unusual topology. *Traffic,* 4**,** 694-709.

KYTTARIS, V. C., JUANG, Y. T. & TSOKOS, G. C. 2005. Immune cells and cytokines in systemic lupus erythematosus: an update. *Curr Opin Rheumatol,* 17**,** 518-22.

LANDOLT-MARTICORENA, C., BONVENTI, G., LUBOVICH, A., FERGUSON, C., UNNITHAN, T., SU, J., GLADMAN, D. D., UROWITZ, M., FORTIN,

P. R. & WITHER, J. 2009. Lack of association between the interferon-alpha signature and longitudinal changes in disease activity in systemic lupus erythematosus. *Ann Rheum Dis,* 68**,** 1440-6.
LATEEF, A. & PETRI, M. 2010. Biologics in the treatment of systemic lupus erythematosus. *Curr Opin Rheumatol,* 22**,** 504-9.
LE BON, A., SCHIAVONI, G., D'AGOSTINO, G., GRESSER, I., BELARDELLI, F. & TOUGH, D. F. 2001. Type i interferons potently enhance humoral immunity and can promote isotype switching by stimulating dendritic cells in vivo. *Immunity,* 14**,** 461-70.
LEE-KIRSCH, M. A., GONG, M., CHOWDHURY, D., SENENKO, L., ENGEL, K., LEE, Y. A., DE SILVA, U., BAILEY, S. L., WITTE, T., VYSE, T. J., KERE, J., PFEIFFER, C., HARVEY, S., WONG, A., KOSKENMIES, S., HUMMEL, O., ROHDE, K., SCHMIDT, R. E., DOMINICZAK, A. F., GAHR, M., HOLLIS, T., PERRINO, F. W., LIEBERMAN, J. & HUBNER, N. 2007. Mutations in the gene encoding the 3'-5' DNA exonuclease TREX1 are associated with systemic lupus erythematosus. *Nat Genet,* 39**,** 1065-7.
LEE, Y. H. & SONG, G. G. 2009. Association between the rs2004640 functional polymorphism of interferon regulatory factor 5 and systemic lupus erythematosus: a meta-analysis. *Rheumatol Int,* 29**,** 1137-42.
LIU, C. C., KAO, A. H., MANZI, S. & AHEARN, J. M. 2013. Biomarkers in systemic lupus erythematosus: challenges and prospects for the future. *Ther Adv Musculoskelet Dis,* 5**,** 210-33.
LIU, C. C., MANZI, S. & AHEARN, J. M. 2005. Biomarkers for systemic lupus erythematosus: a review and perspective. *Curr Opin Rheumatol,* 17**,** 543-9.
LIU, Y. J. 2005. IPC: professional type 1 interferon-producing cells and plasmacytoid dendritic cell precursors. *Annu Rev Immunol,* 23**,** 275-306.
LOOD, C., GULLSTRAND, B., TRUEDSSON, L., OLIN, A. I., ALM, G. V., RONNBLOM, L., STURFELT, G., ELORANTA, M. L. & BENGTSSON, A. A. 2009. C1q inhibits immune complex-induced interferon-alpha production in plasmacytoid dendritic cells: a novel link between C1q deficiency and systemic lupus erythematosus pathogenesis. *Arthritis Rheum,* 60**,** 3081-90.
MAHAUAD-FERNANDEZ, W. D., BORCHERDING, N. C., ZHANG, W. & OKEOMA, C. M. 2015. Bone Marrow Stromal Antigen 2 (BST-2) DNA Is Demethylated in Breast Tumors and Breast Cancer Cells. *PLoS ONE,* 10**,** e0123931.
MAHAUAD-FERNANDEZ, W. D., DEMALI, K. A., OLIVIER, A. K. & OKEOMA, C. M. 2014a. Bone marrow stromal antigen 2 expressed in cancer cells promotes mammary tumor growth and metastasis. *Breast Cancer Research,* 16**,** 493.
MAHAUAD-FERNANDEZ, W. D., JONES, P. H. & OKEOMA, C. M. 2014b. Critical role for bone marrow stromal antigen 2 in acute Chikungunya virus infection. *J Gen Virol,* 95**,** 2450-61.
MAHAUAD-FERNANDEZ, W. D. & OKEOMA, C. M. 2016. The role of BST-2/Tetherin in host protection and disease manifestation. *Immun Inflamm Dis,* 4**,** 4-23.

MANDERSON, A. P., BOTTO, M. & WALPORT, M. J. 2004. The role of complement in the development of systemic lupus erythematosus. *Annu Rev Immunol,* 22**,** 431-56.

MANSOURI, M., VISWANATHAN, K., DOUGLAS, J. L., HINES, J., GUSTIN, J., MOSES, A. V. & FRUH, K. 2009. Molecular mechanism of BST2/tetherin downregulation by K5/MIR2 of Kaposi's sarcoma-associated herpesvirus. *J Virol,* 83**,** 9672-81.

MARITS, P., WIKSTROM, A. C., POPADIC, D., WINQVIST, O. & THUNBERG, S. 2014. Evaluation of T and B lymphocyte function in clinical practice using a flow cytometry based proliferation assay. *Clin Immunol,* 153**,** 332-42.

MARTIN-SERRANO, J. & NEIL, S. J. 2011. Host factors involved in retroviral budding and release. *Nat Rev Microbiol,* 9**,** 519-31.

MARTIN, D. A. & ELKON, K. B. 2005. Autoantibodies make a U-turn: the toll hypothesis for autoantibody specificity. *J Exp Med,* 202**,** 1465-9.

MD YUSOF, M. Y., SHAW, D., EL-SHERBINY, Y. M., DUNN, E., RAWSTRON, A. C., EMERY, P. & VITAL, E. M. 2017. Predicting and managing primary and secondary non-response to rituximab using B-cell biomarkers in systemic lupus erythematosus. *Ann Rheum Dis*.

MIN, D. J., KIM, S. J., PARK, S. H., SEO, Y. I., KANG, H. J., KIM, W. U., CHO, C. S. & KIM, H. Y. 2002. Anti-nucleosome antibody: significance in lupus patients lacking anti-double-stranded DNA antibody. *Clin Exp Rheumatol,* 20**,** 13-8.

MIYAGI, E., ANDREW, A. J., KAO, S. & STREBEL, K. 2009. Vpu enhances HIV-1 virus release in the absence of Bst-2 cell surface down-modulation and intracellular depletion. *Proc Natl Acad Sci U S A,* 106**,** 2868-73.

MOK, C. C. & LAU, C. S. 2003. Pathogenesis of systemic lupus erythematosus. *J Clin Pathol,* 56**,** 481-90.

MOSER, K. L., KELLY, J. A., LESSARD, C. J. & HARLEY, J. B. 2009. Recent insights into the genetic basis of systemic lupus erythematosus. *Genes Immun,* 10**,** 373-9.

MUNOZ, L. E., JANKO, C., GROSSMAYER, G. E., FREY, B., VOLL, R. E., KERN, P., KALDEN, J. R., SCHETT, G., FIETKAU, R., HERRMANN, M. & GAIPL, U. S. 2009. Remnants of secondarily necrotic cells fuel inflammation in systemic lupus erythematosus. *Arthritis Rheum,* 60**,** 1733-42.

MUNROE, M. E., VISTA, E. S., GUTHRIDGE, J. M., THOMPSON, L. F., MERRILL, J. T. & JAMES, J. A. 2014. Proinflammatory adaptive cytokine and shed tumor necrosis factor receptor levels are elevated preceding systemic lupus erythematosus disease flare. *Arthritis Rheumatol,* 66**,** 1888-99.

NAMJOU, B., KOTHARI, P. H., KELLY, J. A., GLENN, S. B., OJWANG, J. O., ADLER, A., ALARCON-RIQUELME, M. E., GALLANT, C. J., BOACKLE, S. A., CRISWELL, L. A., KIMBERLY, R. P., BROWN, E., EDBERG, J., STEVENS, A. M., JACOB, C. O., TSAO, B. P., GILKESON, G. S., KAMEN, D. L., MERRILL, J. T., PETRI, M., GOLDMAN, R. R., VILA, L. M., ANAYA, J. M., NIEWOLD, T. B., MARTIN, J., PONS-ESTEL, B. A., SABIO, J. M., CALLEJAS, J. L., VYSE, T. J., BAE, S. C., PERRINO, F. W., FREEDMAN, B. I., SCOFIELD, R. H., MOSER, K. L., GAFFNEY, P. M., JAMES, J. A., LANGEFELD, C. D., KAUFMAN, K. M., HARLEY, J.

B. & ATKINSON, J. P. 2011. Evaluation of the TREX1 gene in a large multi-ancestral lupus cohort. *Genes Immun,* 12**,** 270-9.
NASHI, E., WANG, Y. & DIAMOND, B. 2010. The role of B cells in lupus pathogenesis. *Int J Biochem Cell Biol,* 42**,** 543-50.
NATHAN, J. A. & LEHNER, P. J. 2009. The trafficking and regulation of membrane receptors by the RING-CH ubiquitin E3 ligases. *Exp Cell Res,* 315**,** 1593-600.
NEIL, S. J., ZANG, T. & BIENIASZ, P. D. 2008. Tetherin inhibits retrovirus release and is antagonized by HIV-1 Vpu. *Nature,* 451**,** 425-30.
NG, K. P., MANSON, J. J., RAHMAN, A. & ISENBERG, D. A. 2006. Association of antinucleosome antibodies with disease flare in serologically active clinically quiescent patients with systemic lupus erythematosus. *Arthritis Rheum,* 55**,** 900-4.
NIEWOLD, T. B., CLARK, D. N., SALLOUM, R. & POOLE, B. D. 2010. Interferon alpha in systemic lupus erythematosus. *J Biomed Biotechnol,* 2010**,** 948364.
NOPPERT, S. J., FITZGERALD, K. A. & HERTZOG, P. J. 2007. The role of type I interferons in TLR responses. *Immunol Cell Biol,* 85**,** 446-57.
OBERMOSER, G. & PASCUAL, V. 2010. The interferon-alpha signature of systemic lupus erythematosus. *Lupus,* 19**,** 1012-9.
OHTOMO, T., SUGAMATA, Y., OZAKI, Y., ONO, K., YOSHIMURA, Y., KAWAI, S., KOISHIHARA, Y., OZAKI, S., KOSAKA, M., HIRANO, T. & TSUCHIYA, M. 1999. Molecular cloning and characterization of a surface antigen preferentially overexpressed on multiple myeloma cells. *Biochem Biophys Res Commun,* 258**,** 583-91.
PARKIN, J. & COHEN, B. 2001. An overview of the immune system. *Lancet,* 357**,** 1777-89.
PENG, L., OGANESYAN, V., WU, H., DALL'ACQUA, W. F. & DAMSCHRODER, M. M. 2015. Molecular basis for antagonistic activity of anifrolumab, an anti-interferon-alpha receptor 1 antibody. *MAbs,* 7**,** 428-39.
PEREZ-CABALLERO, D., ZANG, T., EBRAHIMI, A., MCNATT, M. W., GREGORY, D. A., JOHNSON, M. C. & BIENIASZ, P. D. 2009. Tetherin inhibits HIV-1 release by directly tethering virions to cells. *Cell,* 139**,** 499-511.
PERL, A. 2010. Pathogenic mechanisms in systemic lupus erythematosus. *Autoimmunity,* 43**,** 1-6.
PERL, A., FERNANDEZ, D. R., TELARICO, T., DOHERTY, E., FRANCIS, L. & PHILLIPS, P. E. 2009. T-cell and B-cell signaling biomarkers and treatment targets in lupus. *Curr Opin Rheumatol,* 21**,** 454-64.
PERL, A., GERGELY, P., JR., NAGY, G., KONCZ, A. & BANKI, K. 2004. Mitochondrial hyperpolarization: a checkpoint of T-cell life, death and autoimmunity. *Trends Immunol,* 25**,** 360-7.
PETRI, M., ORBAI, A. M., ALARCON, G. S., GORDON, C., MERRILL, J. T., FORTIN, P. R., BRUCE, I. N., ISENBERG, D., WALLACE, D. J., NIVED, O., STURFELT, G., RAMSEY-GOLDMAN, R., BAE, S. C., HANLY, J. G., SANCHEZ-GUERRERO, J., CLARKE, A., ARANOW, C., MANZI, S., UROWITZ, M., GLADMAN, D., KALUNIAN, K., COSTNER, M., WERTH, V. P., ZOMA, A., BERNATSKY, S., RUIZ-IRASTORZA, G., KHAMASHTA, M. A., JACOBSEN, S., BUYON, J. P., MADDISON, P.,

DOOLEY, M. A., VAN VOLLENHOVEN, R. F., GINZLER, E., STOLL, T., PESCHKEN, C., JORIZZO, J. L., CALLEN, J. P., LIM, S. S., FESSLER, B. J., INANC, M., KAMEN, D. L., RAHMAN, A., STEINSSON, K., FRANKS, A. G., JR., SIGLER, L., HAMEED, S., FANG, H., PHAM, N., BREY, R., WEISMAN, M. H., MCGWIN, G., JR. & MAGDER, L. S. 2012. Derivation and validation of the Systemic Lupus International Collaborating Clinics classification criteria for systemic lupus erythematosus. *Arthritis Rheum,* 64**,** 2677-86.

PICKERING, M. C. & WALPORT, M. J. 2000. Links between complement abnormalities and systemic lupus erythematosus. *Rheumatology (Oxford),* 39**,** 133-41.

POWERS, A. D. & PALECEK, S. P. 2012. Protein analytical assays for diagnosing, monitoring, and choosing treatment for cancer patients. *J Healthc Eng,* 3**,** 503-534.

PREBLE, O. T., BLACK, R. J., FRIEDMAN, R. M., KLIPPEL, J. H. & VILCEK, J. 1982. Systemic lupus erythematosus: presence in human serum of an unusual acid-labile leukocyte interferon. *Science,* 216**,** 429-31.

PSARRAS, A., EMERY, P. & VITAL, E. M. 2017. Type I interferon-mediated autoimmune diseases: pathogenesis, diagnosis and targeted therapy. *Rheumatology (Oxford),* 56**,** 1662-1675.

RAHMAN, A. & ISENBERG, D. A. 2008. Systemic lupus erythematosus. *N Engl J Med,* 358**,** 929-39.

RIECK, M., ARECHIGA, A., ONENGUT-GUMUSCU, S., GREENBAUM, C., CONCANNON, P. & BUCKNER, J. H. 2007. Genetic variation in PTPN22 corresponds to altered function of T and B lymphocytes. *J Immunol,* 179**,** 4704-10.

RONNBLOM, L. E., ALM, G. V. & OBERG, K. 1991. Autoimmune phenomena in patients with malignant carcinoid tumors during interferon-alpha treatment. *Acta Oncol,* 30**,** 537-40.

SANTER, D. M., HALL, B. E., GEORGE, T. C., TANGSOMBATVISIT, S., LIU, C. L., ARKWRIGHT, P. D. & ELKON, K. B. 2010. C1q deficiency leads to the defective suppression of IFN-alpha in response to nucleoprotein containing immune complexes. *J Immunol,* 185**,** 4738-49.

SAUTER, D. 2014. Counteraction of the multifunctional restriction factor tetherin. *Front Microbiol,* 5**,** 163.

SESTAK, A. L., FURNROHR, B. G., HARLEY, J. B., MERRILL, J. T. & NAMJOU, B. 2011. The genetics of systemic lupus erythematosus and implications for targeted therapy. *Ann Rheum Dis,* 70 Suppl 1**,** i37-43.

SHLOMCHIK, M. J., CRAFT, J. E. & MAMULA, M. J. 2001. From T to B and back again: positive feedback in systemic autoimmune disease. *Nat Rev Immunol,* 1**,** 147-53.

SIEBER, J., DARIDON, C., FLEISCHER, S. J., FLEISCHER, V., HIEPE, F., ALEXANDER, T., HEINE, G., BURMESTER, G. R., FILLATREAU, S. & DORNER, T. 2014. Active systemic lupus erythematosus is associated with a reduced cytokine production by B cells in response to TLR9 stimulation. *Arthritis Res Ther,* 16**,** 477.

SIMON, J. A., CABIEDES, J., ORTIZ, E., ALCOCER-VARELA, J. & SANCHEZ-GUERRERO, J. 2004. Anti-nucleosome antibodies in patients with systemic lupus erythematosus of recent onset. Potential utility as a

diagnostic tool and disease activity marker. *Rheumatology (Oxford),* 43**,** 220-4.
STEINMAN, R. M., HAWIGER, D. & NUSSENZWEIG, M. C. 2003. Tolerogenic dendritic cells. *Annu Rev Immunol,* 21**,** 685-711.
TAKAOKA, A., YANAI, H., KONDO, S., DUNCAN, G., NEGISHI, H., MIZUTANI, T., KANO, S., HONDA, K., OHBA, Y., MAK, T. W. & TANIGUCHI, T. 2005. Integral role of IRF-5 in the gene induction programme activated by Toll-like receptors. *Nature,* 434**,** 243-9.
TAN, E. M. 1989. Antinuclear antibodies: diagnostic markers for autoimmune diseases and probes for cell biology. *Adv Immunol,* 44**,** 93-151.
THEOFILOPOULOS, A. N., BACCALA, R., BEUTLER, B. & KONO, D. H. 2005. Type I interferons (alpha/beta) in immunity and autoimmunity. *Annu Rev Immunol,* 23**,** 307-36.
TRUEDSSON, L., BENGTSSON, A. A. & STURFELT, G. 2007. Complement deficiencies and systemic lupus erythematosus. *Autoimmunity,* 40**,** 560-6.
TSOKOS, G. C. 2011. Systemic lupus erythematosus. *N Engl J Med,* 365**,** 2110-21.
VAN BOXEL-DEZAIRE, A. H., RANI, M. R. & STARK, G. R. 2006. Complex modulation of cell type-specific signaling in response to type I interferons. *Immunity,* 25**,** 361-72.
VAN DAMME, N., GOFF, D., KATSURA, C., JORGENSON, R. L., MITCHELL, R., JOHNSON, M. C., STEPHENS, E. B. & GUATELLI, J. 2008. The interferon-induced protein BST-2 restricts HIV-1 release and is downregulated from the cell surface by the viral Vpu protein. *Cell Host Microbe,* 3**,** 245-52.
VILCEK, J. 2006. Fifty years of interferon research: aiming at a moving target. *Immunity,* 25**,** 343-8.
VITAL, E. M., DASS, S., BUCH, M. H., HENSHAW, K., PEASE, C. T., MARTIN, M. F., PONCHEL, F., RAWSTRON, A. C. & EMERY, P. 2011. B cell biomarkers of rituximab responses in systemic lupus erythematosus. *Arthritis Rheum,* 63**,** 3038-47.
VITAL, E. M., DASS, S. & EMERY, P. 2012. Concomitant cyclophosphamide and oral immunosuppressants with rituximab for systemic lupus erythematosus. *Rheumatology (Oxford),* 51**,** 1131-2.
WALPORT, M. J. 2001. Complement. Second of two parts. *N Engl J Med,* 344**,** 1140-4.
WANDSTRAT, A. & WAKELAND, E. 2001. The genetics of complex autoimmune diseases: non-MHC susceptibility genes. *Nat Immunol,* 2**,** 802-9.
WANG, W., NISHIOKA, Y., OZAKI, S., JALILI, A., ABE, S., KAKIUCHI, S., KISHUKU, M., MINAKUCHI, K., MATSUMOTO, T. & SONE, S. 2009. HM1.24 (CD317) is a novel target against lung cancer for immunotherapy using anti-HM1.24 antibody. *Cancer Immunol Immunother,* 58**,** 967-76.
WANG, X., HERR, R. A. & HANSEN, T. 2008. Viral and cellular MARCH ubiquitin ligases and cancer. *Semin Cancer Biol,* 18**,** 441-50.
WILLIAMS, R. C., JR., MALONE, C., BLOOD, B. & SILVESTRIS, F. 1999. Anti-DNA and anti-nucleosome antibody affinity--a mirror image of lupus nephritis? *J Rheumatol,* 26**,** 331-46.

WILLIAMS, R. C., JR., MALONE, C. C., MEYERS, C., DECKER, P. & MULLER, S. 2001. Detection of nucleosome particles in serum and plasma from patients with systemic lupus erythematosus using monoclonal antibody 4H7. *J Rheumatol,* 28**,** 81-94.

WITTE, T. 2008. IgM antibodies against dsDNA in SLE. *Clin Rev Allergy Immunol,* 34**,** 345-7.

XIONG, Y. S., WU, A. L., MU, D., YU, J., ZENG, P., SUN, Y. & XIONG, J. 2017. Inhibition of siglec-1 by lentivirus mediated small interfering RNA attenuates atherogenesis in apoE-deficient mice. *Clin Immunol,* 174**,** 32-40.

YAO, Y., RICHMAN, L., HIGGS, B. W., MOREHOUSE, C. A., DE LOS REYES, M., BROHAWN, P., ZHANG, J., WHITE, B., COYLE, A. J., KIENER, P. A. & JALLAL, B. 2009. Neutralization of interferon-alpha/beta-inducible genes and downstream effect in a phase I trial of an anti-interferon-alpha monoclonal antibody in systemic lupus erythematosus. *Arthritis Rheum,* 60**,** 1785-96.

YURASOV, S., WARDEMANN, H., HAMMERSEN, J., TSUIJI, M., MEFFRE, E., PASCUAL, V. & NUSSENZWEIG, M. C. 2005. Defective B cell tolerance checkpoints in systemic lupus erythematosus. *J Exp Med,* 201**,** 703-11.

Appendices

Appendix 1

Reagents

FACSFlow solution (Becton Dickinson), FACS Shut-down solution (Becton Dickinson), FACS Cleaning solution (Becton Dickinson), monoclonal antibodies (Miltenyi Biotec), Isotype (Miltenyi Biotec), Fragment crystallisable receptor blocking (FC blocking) buffer (Miltenyi Biotec), lysing solution (Becton Dickinson), dilute to 1/10 with distilled water. 1% Phosphate-buffered saline / Foetal bovine serum (PBS / FBS) (wash buffer) - made with 500 mL PBS and 5 mL of foetal bovine serum (FBS Invitrogen). 0.5% Formaldehyde (Fix solution): made with 1.35 mL 37% formaldehyde (BDH) and 100 mL PBS.

Blocking Fc buffer: Add SIGMA 12411 - 10 MG IgG from human serum (reagent grade ≥ 95% (HPLC)) buffered aqueous solution into SIGMA m5905 - 10 mL mouse serum. Aliquot 300 µl into Eppendorf tubes and freeze, when in need of use defrost and add 1000 µl of 1% FACS buffer containing azide containing.

Wash buffer :1000 µL of 1% Phosphate-buffered saline / Foetal bovine serum (PBS / FBS) solution (wash buffer) and 49 mL of 1% PBS / FBS wash buffer was then added into the Falcon tube ((1% PBS / FBS - made with 500 mL PBS and 5 mL of foetal bovine serum (FBS Invitrogen)).

Cell Fix buffer: PBS + 0.5% formaldehyde. (Fix solution, made with 1.35 mL 37% formaldehyde (BDH) and 100 mL PBS).

TanMan

Required Reagents

• PreAmp Master Mix (Fluidigm, PN 100-5580, 100-5581)

• 20X TaqMan Gene Expression Assays (Applied Biosystems)

• 2X Assay Loading Reagent (Fluidigm, PN 85000736)

• 20X GE Sample Loading Reagent (Fluidigm, PN 85000735, 85000746)

Required Equipment

• Standard 96-well Thermal Cycler

• IFC Controller MX (for the 48.48 Dynamic Array IFC) or HX (for the 96.96 Dynamic Array IFC) or RX (for the 192.24 Gene Expression IFC)

• BioMark™ HD System

Required Software

Fluidigm® Real-Time PCR Analysis Software v.3.0.2 or higher and BioMark™ HD Data Collection Software v.3.0.2 or higher is required for this advanced development protocol.