Personalised Therapy Based on Immunopathogenesis to Improve Outcome of At-Risk and Established Systemic Lupus Erythematosus

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Chapter 2 is based on works from jointly authored publications by Md Yusof MY, Vital EM, Buch MH and Emery P. Dr Md Yusof performed the review of literature, critically appraised scientific evidences of the relevant topics and led the writing of the manuscripts. All other senior authors above revised the manuscripts for important intellectual content and final approval of the manuscript.

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Md Yusof MY, Vital EM and Emery P. Biologics in systemic lupus erythematosus: current options and future perspectives. Br J Hosp Med (Lond). 2014 Aug;75(8):440-7.

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b) the British Society of Rheumatology (BSR) Annual Meeting, Glasgow in April 2016.

Md Yusof MY et al. The effect of cyclophosphamide and rituximab for remission induction and maintenance in ANCA-associated vasculitis on immunoglobulin: Repeat cycles on clinical relapse with rituximab are associated with stable IgA and IgG. Presented at:

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Md Yusof MY et al. Identifying B cell Biomarkers for relapse in ANCA-associated Vasculitis. Presented at:

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Md Yusof MY et al. Validation of B Cell Depletion as A Predictor of Clinical Response and Efficacy of Retreatment of Non-Response in Rheumatoid Arthritis. *Presented at the EULAR Congress, London in June 2016.*

Md Yusof MY et al. Validation of Highly Sensitive Flow Cytometry as A Biomarker for Rituximab in SLE: A Rationale for More Intensive Treatment To Improve Clinical Outcomes. *Presented at the EULAR Congress, London in June 2016.*

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Abstract

Background: Pathogenesis of systemic lupus erythematosus (SLE) is thought to be closely related to B-cell dysfunction, and accordingly this is the usual target for therapies. However, non-B-cell mechanisms such as tumour necrosis factor (TNF) and interferons may also be important in the onset as well as established disease.

Objectives: (i) To assess biomarkers of progression from At-Risk (ANA-positive but limited symptoms) to connective tissue disease (AI-CTD); (ii) to identify predictors of non-response and serious infections with rituximab; and (iii) to assess new therapies to overcome rituximab deficiency with respect to anti-rituximab antibodies and B-cell-independent inflammation in discoid lupus erythematosus (DLE).

Methods: Prospective observational studies were conducted in (i) At-Risk of AI-CTD and (ii) SLE patients treated with rituximab. Patients with anti-rituximab antibodies were treated with alternative humanised anti-CD20 agents. (iii) A single arm, phase II open label trial of intra-dermal injection of etanercept for remission induction in DLE (TARGET-DLE) was undertaken.

Results: (i) Higher IFN-Score-B and a family history of autoimmune rheumatic diseases at baseline were predictive of progression from At-Risk to AI-CTD. (ii) B-cell depletion at 6 weeks post-rituximab was predictive of major response to rituximab and was not associated with increased serious infection post-therapy in SLE. During repeat rituximab cycles, 12% of SLE patients lost depletion, which was attributed to anti-rituximab antibodies. These patients were switched to humanised agents, and all depleted and responded. (iii) For TARGET-DLE, the primary and most of the key secondary endpoints were met. Therapy was tolerable without inducing systemic autoimmunity.

Conclusion: In this thesis, a personalised approach to treatment based on immunopathogenesis in At-Risk and established SLE led to better outcomes for patients. The predictive values of the biomarkers presented may allow stratification of patients for disease progression. While results from the use of novel therapies presented support further development in multi-centre trials.

Table of Contents

List of Figures

List of Tables

List of Abbreviations

2NDNR	Secondary non-depletion and non-response
AAV	ANCA-associated vasculitis
ACA	Anti-cardiolipin antibody
ACLE	Acute cutaneous lupus erythematosus
ACR	American College of Rheumatology
ADCC	Antibody-dependent cell-mediated cytotoxicity
AE	Adverse event
AI-CTD	Autoimmune connective tissue disease
ANA	Anti-nuclear antibody
ANCA	Anti-neutrophil cytoplasmic antibody
ANOVA	Analysis of variance
APRIL	A PRoliferation-Inducing Ligand
ARD	Autoimmune rheumatic disease
AS	Ankylosing spondylitis
AUROC	The area under the receiver operating characteristic
AZA	Azathioprine
BAFF	B-cell Activating Factor of the tumour ncrosis factor of the ligand Family
BCMA	B-cell maturation antigen
BCR	B-cell receptor
bDMARDs	Biological disease modifying anti-rheumatic drugs
BICLA	British Isles Assessment Group-based Combined Lupus Assessment
BILAG	British Isles Lupus Asessment Group
BILAG-BR	British Isles Lupus Assessment Assessment Group Biologics Registry

BLB	Belimumab
BlyS	B-lymphocyte stimulator
Bregs	Regulatory B-cells
BSR	British Society of Rheumatology
BVAS	Birmingham Vasculitis Activity Score
CAL	Calcineurin inhibitor
CCLE	Chronic cutaneous lupus erythematosus
CCP	Cyclic citrullinated peptide
CDC	Complement-dependent cytotoxicity
CI	Confidence interval
CLASI	Cutaneous Lupus Erythematosus Disease Area and Severity Index
CLL	Chronic lymphocytic anaemia
CNS	Central nervous system
CR	Complete response
CRP	C-reactive protein
csDMARDs	Conventional synthetic disease modifying anti-rheumatic drugs
CTLA4	Cytotoxic T-lymphocyte antigen 4
CyC	Cyclophosphamide
DAS28	Disease Activity Score in 28 joints
DIL	Drug-induced lupus
DLE	Discoid lupus erythematosus
DLQI	Dermatology Life Quality Index
DMEC	Data Monitoring Ethics Committee
dsDNA	Double stranded deoxyribonucleic acid
EBV	Epstein-Barr virus

eGFR	Estimated glomerular filtration rate
ELISA	Enzyme-linked immunosorbent assay
EMA	European Medicines Agency
ENA	Extract nuclear antigen
ESR	Erythrocyte sedimentation rate
EULAR	European League Against Rheumatism
FA	Factor Analysis
FcγR	Fc gamma receptor
FD	Fold difference
FDA	Food and Drug Administration
GCSF	Granulocyte-colony stimulating factor
GM-CSF	Granulocyte macrophage colony stimulating factor
GRADE	Grading of Recommendation, Assessment, Development and Evaluation
GS	Grey-scale
GWAS	Genome-wide association studies
HACA	Human anti-chimeric antibody
HC	Healthy control
HCQ	Hydroxychloroquine
HERV	Human endogenous retrovirus
HIV	Human immunodeficiency virus
HR	Hazard ratio
HSFC	Highly sensitive flow cytometry
HTLV-1	Human T-cell lymphotrphic virus type 1
IC	Immune complex
IFN	Interferon
IFNAR	Type 1 interferon receptor

IFN-I	Type 1 interferon
IFN-II	Type 2 interferon
IFN-III	Type 3 interferon
IL-6	Interleukin 6
ILD	Interstitial lung disease
IMP	Investigational medicine product
IQR	Interquartile range
ISG	Interferon-stimulated gene
JAK	Janus kinase
JIA	Juvenile idiopathic arthritis
LDI	Laser Doppler Imaging
LENS	Lupus erythematosus non-specific lesions
LFA	Lymphocyte function-associated antigen
LLN	Lower limit of normal
LN	Lupus nephritis
LRTI	Lower respiratory tract infection
LTE	Long-term extension
mAb	Monoclonal antibody
MCR	Major clinical response
MDRD	Modification of Diet in Renal Disease
MHC	Major histocompability
miRNA	Micro ribonucleic acid
ML-SADDLE	Modified limited Score of Activity and Damage in Discoid Lupus Erythematosus
MMF	Mycophenolate mofetil
MNC	Mononuclear cell
MPO	Myeloperoxidase

MSK-US	Musculoskeletal ultrasound
MTX	Methotrexate
NFκB	Nuclear factor kappa B
NHL	Non-Hodgkin's lymphoma
NHS	National Health Service
NICE	National Institute for healthcare and Clinical Excellence
NPV	Negative predictive value
NR	Non-response
NYHA	New York Heart Association
OCR	Ocrelizumab
OCT	Optical coherence tomography
OI	Opportunistic infection
OMERACT	Outcome Measures in rheumatoid arthritis clinical trials
OR	Odds ratio
PAS	Patient Access Centre
PBMC	Peripheral blood mononuclear cell
PCP	*Pneumocystis jiroveci pneumonia*
PCR	Partial clinical response
PD	Power Doppler
pDC	Plasmacytoid dendritic cell
PGA	Physician's Global Assessment
PK	Pharmacokinetic
PML	Progressive multifocal leucoencephalopathy
PPIA	Peptidylprolyl isomerase A
PPV	Positive predictive value
PR	Partial response

PR3	Proteinase-3
PsA	Psoriatic Arthritis
pSS	Primary Sjogren's Syndrome
PY	Patient-year
QALY	Quality Adjusted Life Years
RA	Rheumatoid arthritis
RCLASI	Revised Cutaneous Lupus Erythematosus Disease Area and Severity Index
RCT	Randomised controlled trial
REC	Research Ethics Committee
REF	Research Exercise Framework
RF	Rheumatoid factor
RNA	Ribonucleic acid
ROC	Receiver operator characteristic
RTX	Rituximab
SADDLE	Score of Activity and Damage in Discoid Lupus Erythematosus
SAE	Serious adverse event
SAR	Serious adverse reaction
SCLE	Subacute cutaneous lupus erythematosus
SD	Standard deviation
SELENA	Safety of Estrogens in Lupus National Assessment
SIE	Serious infection event
SLEDAI	Systemic Lupus Erythematosus Disease Activity Index
SLICC	Systemic Lupus International Collaborating Clinics
SNP	Single nucleotide polymorphism
SRI	Systemic Lupus Erythematosus Responder Index

STAT	Signalling transducer and activator of transcription
SUSAR	Suspected Unexpected Serious Adverse Reaction
TACI	Tumour necrosis factor receptor superfamily member 13b
TGF-β	Tissue growth factor beta
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TSH	Thyroid-stimulating hormone
UK	United Kingdom
UPCR	Urinary protein-to-creatinine ratio
US	United States
VAS	Visual analogue score
VAS	Vasculitis Damage Index
WP	Work-package

Chapter 1. Introduction

Systemic lupus erythematosus (SLE) is a multisystem autoimmune disease that causes significant morbidity and mortality (1). The management of SLE demands a personalised approach due to heterogeneity in clinical presentation, severity and response to therapy. If left untreated, patients with SLE have a 67% higher mortality rate than age- and gender-matched healthy individuals (2) and the annual direct medical cost is increased two-fold to over £4500 per patient in the UK for treatment of a severe flare (3).

Current treatment is mainly unlicensed and many therapies (especially corticosteroids) have significant long-term toxicity (4, 5). Therefore, the focus of management should ideally be directed towards intervention at the 'pre-clinical' stage; defined as a period of detectable autoimmunity and/or inflammation predating the onset of clinically apparent tissue inflammation and injury (6). In established disease, the challenge is to use the right therapy at the right time to prevent accumulation of damage and drug toxicity.

There is a significant unmet need for therapies that improve efficacy, for end-organ manifestations whilst improving quality of life. Nevertheless, many theoretically well-established agents did not meet their respective primary endpoints in clinical trials as a result of inefficacy, a problem with trial design and/or safety issues (7, 8). Belimumab, a human monoclonal antibody (mAb) that inhibits B-cell Activating Factor of the tumour necrosis factor ligand Family, (BAFF, also known as B-lymphocyte stimulator (BlyS)), is the only biologic approved by the United States Food and Drug Administration (US FDA) and the European Medicines Agency (EMA) since 2011 for use in moderate to severe autoantibody positive SLE (9). However, belimumab is expensive and the phase III trials excluded patients with severe lupus nephritis and neuropsychiatric manifestations (10, 11). Thus, it took 5 years from licensing as well as a substantial discounted price from the manufacturer before this drug was approved by the National Institute for Health Care and Excellence (NICE) in the UK in 2016 (12).

Rituximab, a chimeric anti-CD20 mAb is the mostly widely used biologic for SLE and is commissioned by NHS England for refractory SLE based on efficacy on a wide spectrum of SLE manifestations from strong open label evidence (13-15). However, the clinical response to therapy and the degree of B-cell depletion are highly variable; both of which can contribute to the poor response in some patients (16). Evidence from the use of rituximab in its licensed indications such as rheumatoid arthritis (RA) and B-cell malignancies have indicated several potential mechanisms that confer resistance of B-cells to rituximab along with modification of therapy that may improve clinical response. Data in SLE are more limited, thus will be a focus of my investigation in this thesis.

Certain manifestations of SLE appear to be B-cell-independent. Cutaneous lupus is particularly variable and we have previously shown that none of the patients with discoid lupus erythematosus (DLE) responded to rituximab. Additionally, new chronic cutaneous lupus erythematosus (CCLE) lesions were observed despite B-cell depletion (17). Therefore, alternative inflammation pathways should be targeted in DLE. Tumour necrosis factor (TNF) may be pathogenic in DLE and this may respond to TNF-blockade (18).

1.1 Thesis hypothesis

The unifying hypothesis of this thesis is:

A personalised approach to treatment based on immunopathogenesis in At-Risk and established SLE will lead to a better outcome for patients

1.2 Overview of planned investigations

Planned investigations to test the unifying and hypotheses for each chapter were divided into four Work-Packages (WPs). These are summarised in Figure 1.
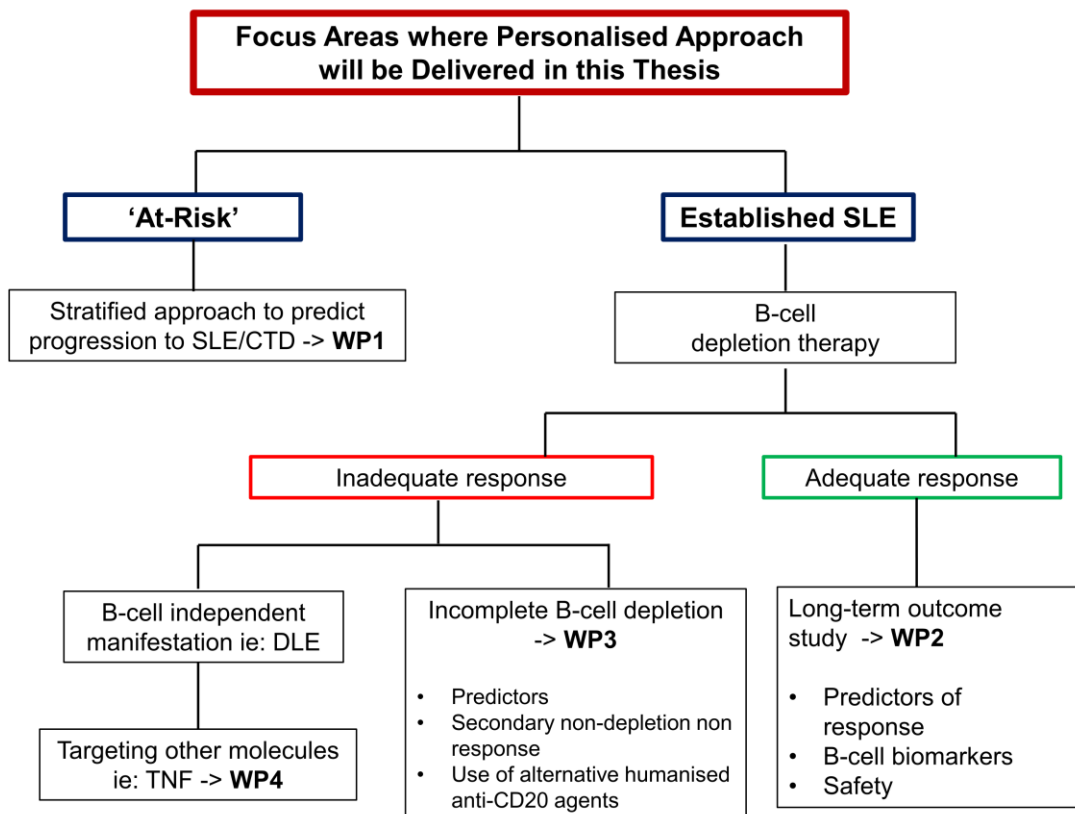
Figure 1-1 Planned investigations of this thesis

1.3 Thesis outline

The outline of this thesis is summarised below.

Chapter 2: Review of the literature

A review of literature was performed focusing on recent understanding of immunopathogenesis of SLE, the natural course of progression from benign autoreactivity to autoimmunity, outcome measures in assessing disease activity and management of moderate to severe SLE manifestations using B-cell and non B-cell targeted therapies including critical reviews of trial designs in lupus trials. This review concluded with the unmet needs in SLE, which were addressed in my thesis.

Chapter 3: PRediction to allow Early interVENTion in At-Risk of autoimmune connective tissue disease (PREVENT-CTD)

This chapter aimed to assess plausible predictors of progression from autoreactivity to autoimmune connective tissue disease (AI-CTD) including clinical, imaging, blood and skin interferon biomarkers with a view to formulate a strategy for disease prevention.

Chapter 4: Predicting and managing primary and secondary non-response to rituximab in systemic lupus erythematosus

This chapter sought to evaluate the long-term efficacy and safety of rituximab in SLE, identify predictors of primary and secondary non-response to rituximab including validation B-cell biomarkers for response prediction and management of secondary non-depletion and non-response by switching to alternative humanised anti-CD20 mAb agents.

Chapter 5: B-cell biomarkers in systemic lupus erythematosus and other B-cell mediated autoimmune rheumatic diseases

This chapter discussed the use of B-cell biomarkers, as measured using Highly Sensitive Flow Cytometry (HSFC) in predicting response and imminent relapse to rituximab in SLE including validation studies and factors contributing to incomplete B-cell depletion after rituximab. The use of these

biomarkers were compared to other B-cell mediated diseases including RA and anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV).

Chapter 6: Candidate predictors for safety and rationale for immunoglobulin monitoring during rituximab treatment in systemic lupus erythematosus and other autoimmune rheumatic diseases

This chapter aimed to assess predictors of serious infection within the first 12 months from starting rituximab and with repeat courses of therapy. In particular, the effect of B-cell depletion on the risk of serious infection as well as rationale for immunoglobulin monitoring were dissected, with a view to establish an algorithm for safe use of rituximab in SLE and other autoimmune rheumatic disease (ARDs).

Chapter 7: TARGeted therapy using intradermal injection of Etanercept for remission induction in Discoid Lupus Erythematosus (TARGET-DLE)

This chapter described the first results from a phase II open label trial of a novel route of administration using the existing drug, etanercept for remission induction in DLE.

Chapter 8: Discussion

This last chapter brought together the findings from all chapters of this thesis. Their contribution to the published literature, impact and future research direction were discussed.

Chapter 2. Review of the Literature

This review describes background of SLE including its classification criteria. This is important for early recognition of disease and treatment. Recent advance in the understanding of aetiology and immunopathogenesis of SLE will be discussed as well as the natural course of progression from benign autoreactivity to autoimmunity. Various outcome measures in assessing disease activity for lupus in general, organ-specific and objective outcome measures will also be reported. The use of B-cell and non B-cell targeted therapies in the management of moderate to severe SLE will be discussed, with a particular focus on critical review of trial designs in lupus trials. Finally, based on the current data, this review concludes by addressing the unmet needs in SLE.

2.1. Systemic lupus erythematosus

SLE is a complex inflammatory disorder, characterised by the presence of antinuclear antibodies (ANAs) and can affect virtually any organ or tissue. Its presentation is heterogeneous and encompasses a wide range of clinical and serological manifestations. SLE is a chronic autoimmune disease typically running a relapsing and remitting course. The severity of lupus symptoms can range from mild to severe and may vary tremendously between patients.

2.1.1. Epidemiology

In Europe, the prevalence of SLE ranges between 20 to 50 per 100 000 people, although much higher rates have been reported for Afro-Caribbean descent (19, 20). In the UK, the prevalence had increased from 65 per 100 000 in 1999 to 97 per 100 000 in 2012 (21). While in the US, prevalence ranges between 20 to 150 per 100 000 people (22, 23). African American and Hispanics are affected more than Caucasians, and have higher disease morbidity (24-26).

SLE has a female preponderance (10:1) with a peak incidence in the reproductive years between the age of 20 to 40 years old (20, 27). The

incidence of lupus has nearly tripled between 1950 to 1992, mainly due to improved recognition and diagnosis of mild disease (28). Incidence rates of SLE in North America, South America and Europe are estimated between 1 to 23 per 100 000 per year (23). In the UK, the age-standardised incidence is 8.3 per 100 000 per year for females and 1.4 per 100 000 per year for males, of which the highest incidence rates are seen in those of African-Caribbean descent, 31.4 per 100 000 per year compared with 6.7 per 100 000 per year for those of white European descent (21).

2.1.2. Classification criteria

Classification criteria for SLE were initially developed by the American College of Rheumatology (ACR) in 1971 (29), and subsequently revised in 1982 (30) and 1997 (31). However, there were notable deficiencies including i) the criteria were developed and validated in patients with longstanding disease and might exclude those with early or limited disease; ii) some systems were over-represented such as the mucocutaneous manifestations; iii) inability to classify patients with organ-threatening manifestation such as renal disease as SLE since they often presented with immunological abnormalities only and iv) classification of individuals with negative ANA as SLE should four criteria were met in other domains.

The Systemic Lupus International Collaborating Clinics (SLICC) group overcame these deficiencies and revised the classification criteria using real-case datasets and several validation steps. The advantages of the 2012 SLICC classification criteria over the ACR criteria include i) greater sensitivity but similar specificity for classifying SLE; ii) a reduction in items that are similar e.g. malar rash and photosensitivity are largely overlapping; iii) lupus nephritis in the presence of at least one of the immunologic variables as a "stand alone" criterion and iv) the requirement for at least ONE clinical and ONE immunologic criterion for SLE classification (32). A comparison between the two criteria is presented in Table 2-1.

At the time of this review, the 2018 revised ACR/European League Against Rheumatism (EULAR) Classification criteria for SLE is undergoing validation process and drafting. This proposed new criteria add weighting to the immunological and clinical items that are attributed to SLE. Within each

domain, only the highest weighted criterion is counted towards the total score. Patients with a score of at least 10 can be classified as SLE based on this revised criteria (33).

Table 2-1 The revised 1997 ACR and the 2012 SLICC classification criteria for SLE

Criteria	1997 Revised ACR (31)	2012 SLICC (32)
Mucocutaneous	1. Malar rash. Fixed erythema, flat or raised, over the malar eminences, tending to spare the nasolabial folds 2. Discoid rash. Erythematous raised patches with adherent keratotic scaling and follicular plugging; atrophic scarring occur in older lesions 3. Photosensitivity. Skin rash as a result of unusual reaction to sunlight either by patient history or physician observation 4. Oral or nasopharyngeal ulceration	1. Acute cutaneous lupus (ACLE) [lupus malar rash, bullous lupus, toxic epidermal necrolysis variant of SLE, maculopapular lupus rash and photosensitive lupus rash] OR subacute cutaneous lupus (SCLE) [non-indurated psoriasiform and/or annular polycyclic lesions that resolve without scarring] 2. Chronic cutaneous lupus (CCLE) [classic discoid rash: localised or generalised, hypertrophic verrucous lupus, lupus panniculitis (profundus), mucosal lupus, lupus erythematosus tumidus, chilblains lupus, discoid lupus/lichen planus overlap] 3. Non-scarring alopecia 4. Oral or nasal ulcers
Arthritis	5. Non-erosive arthritis involving ≥2 peripheral joints, characterised by tenderness, swelling or effusion	5. Inflammatory synovitis in ≥2 joints: a. Characterised by swelling or effusion, or b. Tenderness and ≥30 minutes of morning stiffness
Serositis	6. Any of: a. Pleuritis: convincing history of pleuritic pain or rub heard by a physician or evidence of pleural effusion	6. Any of a. Typical pleurisy lasting >1 day, or pleural effusions or pleural rub

Criteria	1997 Revised ACR (31)	2012 SLICC (32)
	b. Pericarditis: documented by ECG or rub or evidence of pericardial effusion	b. Typical pericardial pain (pain with recumbency, improved by sitting forward) for >1 day, or pericardial effusion, or pericardial rub or pericarditis by electrocardiography
Renal	7. Any of: a. Persistent proteinuria >0.5 g/day or >3+ on urine dipstick if measurement is not performed b. Cellular casts: red cell, haemoglobin or granular tubular	7. Any of: a. Urine protein/creatinine (or 24 h urine protein) representing ≥500 mg of protein/24 hour, or b. Red blood cell casts
Neurological	8. Any of: Seizures: in the absence of offending drugs or known metabolic derangements b. Psychosis: in the absence of offending drugs or known metabolic derangements	8. Any of: a. Seizures b. Psychosis c. Mononeuritis multiplex d. Myelitis e. Peripheral or cranial neuropathy f. Cerebritis (acute confusional state)
Haematological	9. Any of: a. Haemolytic anaemia with reticulocytosis b. Lymphopaenia: $<1500/mm^3$	9. Haemolytic anaemia 10. Leucopaenia ($<4000/mm^3$), or lymphopaenia ($<1000/mm^3$) of at least once

Criteria	1997 Revised ACR (31)	2012 SLICC (32)
	c. Thrombocytopaenia: <100 000/mm^3	11. Thrombocytopaenia (<100 000/mm^3) of at least once
Immunological	10. Any of: a. Anti-DNA: antibody to native DNA in abnormal titre b. Anti-Sm: presence of antibody to Sm nuclear antigen c. Positive finding of anti-phospholipid antibodies based on: (i) an abnormal serum concentration of IgG or IgM anti-cardiolipin antibodies, (ii) a positive test result for SLE anti-coagulant or (iii) a false-positive serological test for syphilis known to be positive for ≥6 months and confirmed by *Treponema pallidum* immobilisation or fluorescent Treponemal antibody absorption test	12. Anti-dsDNA above laboratory reference range (except enzyme-linked immunosorbent assay (ELISA): twice above reference range) 13. Anti-Sm 14. Anti-phospholipid antibody, SLE anti-coagulant, false-positive test for syphilis 15. Anti-cardiolipin (at least twice normal or medium–high titre), or anti-β2 glycoprotein 1 16. Low complement: low C3, or low C4, or low CH50 17. Direct Coombs test in the absence of haemolytic anaemia
Anti-nuclear antibody (ANA)	11. Abnormal titre of ANA by immunofluorescence or an equivalent assay at any time and in the absence of drugs known to be associated with 'drug-induced SLE' syndrome	18. ANA above laboratory reference range
Rules for Classification	At least 4 out of 11 criteria	Either biopsy-proven lupus nephritis in the presence of ANA OR anti-dsDNA as a 'stand-alone' criterion, OR four criteria with at least one of the clinical and one of the immunological/ANA criteria

2.2. Aetiology

The exact aetio-pathology of SLE remains elusive. Multifactorial interaction among various genetic, epigenetic, hormonal and environmental factors is probably involved (18). Recent understanding suggest that defective immune regulatory mechanisms such as the clearance of apoptotic cells and immune complexes are important contributors to the development of SLE (34, 35). Abnormalities of these factors may lead to an irreversible breakdown of immunological tolerance manifested by aberrant immune responses against endogenous nuclear and other self-antigens (36).

2.2.1. Genetic

The importance of genetics in the pathogenesis of SLE is supported by several observations including i) a high concordance rate of up to 57% of SLE in monozygotic twins (37, 38); ii) siblings of patients with SLE are 29 times more likely to develop SLE than those without an affected sibling (as reported in a large population-based study of over 23 million participants) (39) and iii) first-degree relatives of patients with SLE have a 17-fold increased risk to develop the disease compared with the general population (40).

The most common genetic predisposition is found at the major histocompatibility (MHC) locus which contains genes for antigen-presenting molecules i.e. class I human leukocyte antigens such as HLA-A, -B and –C and class II HLA molecules including HLA-DR, -DQ and DP. Predisposing loci which consists of HLA-DR2 and HLA-DR3 are associated with increased hazard ratio (HR) of approximately 2 (41, 42). Within the HLA-DRB1 loci, HLA-DRB1*0301 and HLA-DRB1*1501 predispose to SLE whereas HLADRB1*1401 reduces the risk of development (43).

Genetic factors that confer the highest HRs (between 5 to 25) for SLE are deficiencies in the complement components C1q (required to clear apoptotic cells), C2, C4A and B (44, 45) or the presence of a mutated TREX1 gene (46). The last encodes the 3-prime repair exonuclease1 enzyme that degrades deoxyribonucleic acid (DNA).

Genome-wide association studies (GWAS) have identified over 50 gene loci with polymorphisms (mutations or copy numbers) that predispose to SLE (47-49). These SLE susceptibility genes and their common variants have been associated with impaired apoptosis, autophagy and clearance defects resulting in increased exposure of nuclear autoantigens, pro-inflammation, nuclear factor-kappa B (NFκB) pathway and immune cell signalling or migration (50). They also highlight the importance of excessive activation of type I interferon (IFN-I) signalling via toll-like receptors (TLRs) or nucleic acid sensors pathways (51, 52). Additionally, several single-nucleotide polymorphisms (SNPs) have been associated with sub-phenotypes such as lupus nephritis (53). Nevertheless, this genetic information accounts for only about 20% of susceptibility to SLE (54), suggesting a large component of environmental or epigenetic influences.

2.2.2. Epigenetic effects

Epigenetic effects such as DNA methylation, post-translational histone modifications and micro ribonucleic acids (miRNAs), either genetically determined or environmentally induced may influence the risk of SLE (55). The role of epigenetics is supported (at least in part) by the discordance seen in identical twins who are discordant for SLE (56). The most well-understood type of epigenetic factor is DNA methylation, which affects specific genes and variation in acetylation of histones, thus influences transcription into protein. DNA methylation plays a role in a variety of human processes. Abnormal methylation has been associated with development of SLE (57).

miRNAs are essential in both adaptive and innate immunity by controlling the differentiation of various immune cell subsets such as B-cells, T-cells and their immunological functions (58, 59). Aberrantly expressed miRNAs have been observed in different cell types, tissues and play an important role in the progression of SLE (60).

2.2.3. Hormonal effects

The pathogenic role of hormones including oestrogen, testosterone, progesterone, prolactin and thyroid in SLE may be related to their effects on immune responsiveness.

Oestrogen stimulates thymocytes, CD8+ and CD4+ T-cells, B-cells, macrophages and the release of certain cytokines such as interleukin 1 (IL-1) (61). Oestrogen also reduces apoptotic activity in self-reactive B-cells, thus promoting selective maturation of autoreactive B-cells with high affinity for anti-double stranded DNA (dsDNA) (62). As a result, women are more predisposed than men to make autoantibodies that eventually lead to clinically apparent SLE. Indeed, data from the Nurse's Health study showed that women with early menarche or treated with oestrogen-containing regimens such as oral contraceptives or postmenopausal hormone replacement therapies had a significantly increased risk for SLE (HR of 1.5 to 2.1) (63, 64). In contrast, testosterone level is lower in male patients with SLE compared to those without the disease (65).

Progesterone contributes to the development of SLE by downregulating T-cell proliferation and increases the number of CD8+ cells (66). Both high progesterone and oestrogen levels promote a T-helper 2 (Th2) response, which favours autoantibody production (67). While hyperprolactinaemia has been associated with lupus flare (68). Lastly, there is an increased incidence of thyroid disease in patients with SLE compared to healthy controls (HCs) as well as elevation of both anti-thyroid antibodies and serum thyroid-stimulating hormone (TSH) levels (69).

2.2.4. Environmental factors

Candidate triggers of SLE include ultraviolet light, infectious, endogenous viruses or viral-like elements and certain drugs.

Exposure to ultraviolet causes aberrant apoptosis of keratinocytes and contributes to the accumulation of apoptotic cells in the skin of patients with SLE (70). Apoptotic cells that fail to be cleared by phagocytes undergo secondary necrosis and subsequently releasing inflammatory mediators including IL-1, IL-3, IL-6, granulocyte macrophage colony-stimulating factor

(GM-CSF), IFNs and TNF, thereby stimulating B-cells to make more antibodies (71). In addition to the local effects in skin, ultraviolet light may also increase systemic autoimmunity by interfering with antigen processing and activation of macrophage by decreasing T-cell DNA methylation, which in turn lead to overexpression of lymphocyte function-associated antigen (LFA)-1 (72). These T-cells may then become autoreactive, thus resulting in autoantibody formation.

A number of exogenous viruses particularly Epstein-Barr virus (EBV) have been linked to the pathogenesis of SLE. Patients with SLE have high antibody titres to EBV, increased circulating EBV viral loads and make antibodies to retroviruses including to protein regions that are homologous to nuclear antigens (73). Consequently, production of antibodies to these mimicry molecules and endogenous retroviruses may contribute to the development of autoimmunity. Moreover, human endogenous retroviruses (HERV) including HRES-1, ERV-3, HERV-E 4-1, HERV-K10 and HERV-K18 have also been implicated in SLE (74). HERVs can be inherited since they are incorporated into human DNA. Viral infection can also induce defective apoptosis, resulting in loss of immune tolerance.

More than 100 drugs have been reported to cause drug-induced lupus (DIL). For agents that are metabolised by acetylation such as procainamide and hydralazine, a genetic predisposition may play a role too since DIL cases are frequently reported in the slow acetylator phenotype (75).

2.3. Pathogenesis of SLE

As SLE is characterised by the generation of large amounts of autoantibodies directed against a variety of self-antigens, the loss of B-cell tolerance is believed to play a key role in pathogenesis of the disease (76). Evidence that the breakdown of B-cell tolerance occurs very early in SLE and may precede or trigger other immune abnormalities, is demonstrated by observation that SLE patients express ANAs several years before the onset of clinical disease (77, 78). Thus, in order to understand how tolerance is subverted in SLE, it is essential to review the normal B-cell development first.

In this section, B-cell biology and tolerance will be discussed. This comprises a carefully regulated process that involves the sequential differentiation of bone marrow precursors into immature and transitional B-cells, with further differentiation and maturation to mature naïve and memory subsets occurring in peripheral lymphoid organs (79).

Recent advances on pathogenesis of disease, particularly highlighting the role of innate immune mechanisms leading to the aberrant adaptive immune responses in SLE, will also be reviewed.

2.3.1. Overview of B-cell biology: Development, maturation and activation

B-cell development in bone marrow and B-cell receptor

B-cells arise from the bone marrow. They are continuously generated from pluripotent hematopoietic stem cells (pHSCs), multipotent myeloid progenitors (MPPs) and common lymphoid progenitors (CLPs) under the control of several key cytokines, chemokines and transcription factors including Fms-like tyrosine kinase 3 ligand (FLT3LG), Interleukin-6 (IL6), C-X-C motif chemokine 10 (CXCL10), CXCL12, transcription factor 3 (TF3) and paired box gene 5 (PAX5) (80, 81).

A critical step in B-cell development is the generation of B-cell receptors (BCRs). (82). BCRs for naïve mature B-cells are membrane-bound monomeric forms of IgD and IgM (83, 84). They have two identical heavy chains and two identical light chains, connected by disulfide bonds into a "Y" shape. Genetic rearrangement of hundreds of immunoglobulin (Ig) gene segments occur from the early stage of B-cell development; pre-pro B-cell to the late stage; pre B-cell, in order to provide the necessary diversity of BCR specificities (85, 86). The light and heavy chain loci comprise a series of V (variable) gene elements, followed by several D (diversity) segments (for the heavy chain gene only), some J (joining) segments, and C (constant region) exons. Genetic rearrangement of all possible combinations of V-D-J (heavy chain) and V-J (light chain) provides millions of unique antigen-binding sites for the BCR and for the antibodies secreted following B-cell activation (87, 88).

B-cell maturation

In bone marrow, the first step of B-cells maturation is an assessment of the functionality of their antigen-binding receptors. This is carried out through positive selection for B-cells with normal functional receptors (89). Next, a negative selection is used to eliminate autoreactive B-cells in order to minimise the risk of autoimmunity. This negative selection process includes elimination of autoreactive B-cells by clonal deletion (induction of apoptosis following IgM cross-linking), light chain editing (upregulation of recombination activation gene 1 (RAG-1) and RAG-2 following binding of IgM by self-antigen resulting in further light chain gene rearrangement), or induction of anergy in the B-cell (90-92). Subsequently, immature B-cells that pass these two selection processes in the bone marrow then travel to the spleen for their final stages of maturation. They then become naïve mature B-cells.

B-cell activation

Following maturation in the bone marrow and spleen, naïve B-cells remain in peripheral tissues until they encounter an antigen. Upon encounter with an antigen, naïve B-cells become activated and differentiate into antibody-producing plasma cells and memory B-cells. Some plasma cells migrate to the bone marrow, where they persist for several years. Moreover, they continue to produce antibodies even in the absence of antigen (93).

B-cell activation can occur through T-cell independent and T-cell dependent mechanisms. In T-cell independent activation of B-cells, BCRs interact with T-independent antigens (e.g., polysaccharide capsules, lipopolysaccharide). These T-independent antigens have repetitive epitope units within their structures, which allow for the cross-linkage of multiple BCRs, thus providing the first signal for activation (94, 95). Since T-cells are not involved in this activation of B-cells, the second signal has to come from other sources such as interactions of toll-like receptors (TLRs) with pathogen-associated molecular patterns (PAMPs) or interactions with factors from the complement system (96-98). However, this T-cell independent response is short-lived and does not result in the production of memory B-cells. Therefore, it will not result in a secondary response to subsequent exposures to T-independent antigens.

In contrast, T-cell dependent activation of B-cells is more complex than T-cell independent activation. Once a BCR binds a T-cell dependent antigen, the B-

cell internalises the antigen bound receptor through receptor-mediated endocytosis (99, 100). Following this internalisation, the peptide fragments of the antigen are presented to the cell surface of T-cells via MHC Class II molecules to cognate CD4+ T-cells. T-helper (T_h) cells, typically follicular T-helper cells (T_{FH}), that were activated with the same antigen, subsequently recognise and bind these MHC-II-peptide complexes through their T-cell receptor (TCR) (101). Following the binding of TCR-MHC-II-peptide, T-cells then express the surface protein CD40L as well as cytokines such as IL-4 and IL-21 (102, 103). CD40L is an important co-stimulatory factor for B-cell activation by binding the B-cell surface receptor CD40, which in turn promotes B-cell proliferation, immunoglobulin class switching, somatic hypermutation, sustains T-cell growth and differentiation (104, 105). B-cells are activated once these signals have been received.

Upon activation, B-cells participate in a two-step differentiation process that produces both short-lived plasmablasts for immediate protection, long-lived plasma cells and memory B-cells for persistent protection. The first step occurs outside lymphoid follicles but still in the secondary lymphoid organs. In this process, activated B-cells proliferate, may undergo immunoglobulin class switching and differentiate into plasmablasts that produce mostly IgM antibodies (106, 107). While in the second step, activated B-cells enter a lymphoid follicle and form a germinal center (GC), which is a specialised microenvironment where the B-cells can undergo extensive proliferation, immunoglobulin class switching and affinity maturation, directed by somatic hypermutation. As a result, both high-affinity memory B-cells and long-lived plasma cells are generated (108, 109).

Checkpoints that control B-cell development

Tolerance to autoreactive B-cells can occur through various mechanisms and at different B-cell differentiation stages. Although several stringent checkpoints are available, these autoreactive B-cells may evade them, leading to production of autoantibodies. These checkpoints are illustrated in Figure 2-1 and summarised below:

  i.   Central tolerance – Two checkpoints are available in the bone marrow. In human, a majority (55-75%) of immature B-cells and only a minority (6-20%) of mature B-cells are autoreactive (110), suggesting that a significant proportion of immature autoreactive B-cells are removed during maturation. During different phases of BCR expression, B-cells with high-affinity reactivity for self-antigens are eliminated by a process called clonal deletion (Checkpoint-1) (111) and/or receptor editing (Checkpoint-2). The latter can be a double-edge sword as it can potentially lead to immature B-cells that express both non-autoreactive i.e. receptor-edited and autoreactive i.e. primary autoreactive BCRs. These dual specificity B-cell expressing BCRs could contribute to autoantibody production (112, 113) since they may rearrange their receptors, express non-autoreactive BCRs and evade this negative selection (114, 115). Other mechanisms for central tolerance to be breached include 'ignorance' and 'anergy,' for which autoreactive B-cells evade removal by apoptotic cell death and emigrate from the bone marrow into peripheral circulation (110, 116).

  ii. Peripheral tolerance – Checkpoint-3 of tolerance occurs during B-cell maturation and/or differentiation in the periphery. Despite the mechanisms of central tolerance above, about 10% of the autoreactive B-cells bypass these checkpoints (117, 118). Here, the transitional B-cells in the periphery undergo peripheral tolerance based on their BCR specificity and signal strength, for which the signal strength must both be above the range of for positive selection and below the threshold signaling for negative selection (119, 120). This "transitional B-cell tolerance" is the first B-cell tolerance check point in the periphery,

which eliminates about two-thirds of the transitional B-cells (121, 122). Unlike the central tolerance mechanisms, transitional B-cell tolerance is not as stringent and depends on the interplay between BCR-mediated signals and B-cell Activating Factor of the TNF of the ligand Family (BAFF) signaling. Elevated BAFF levels lead to defect in transitional B-cell tolerance and a breach in the periphery (123, 124).
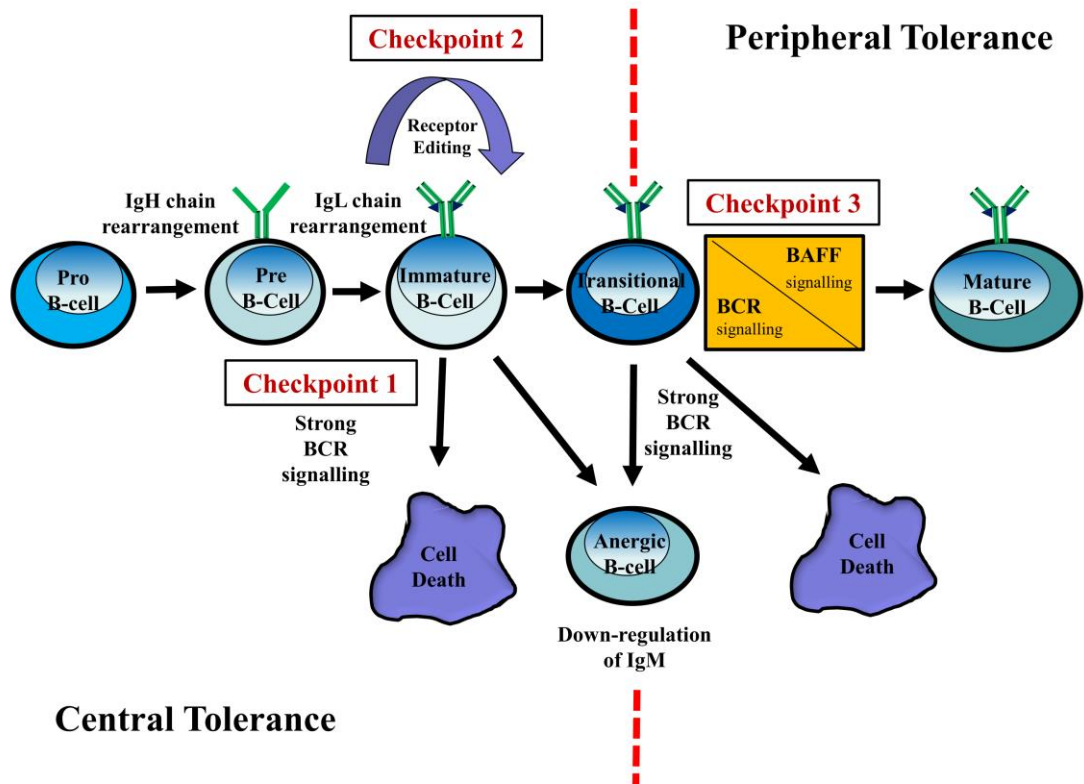
Figure 2-1 Checkpoints that control B-cell development

Development of B-cell begins in the bone marrow and completes in peripheral lymphoid tissues such as the spleen. In the bone marrow, development progresses sequentially through pro-B, pre-B, and immature B cell stages. B-cell receptors in the developing B-cells are generated by random V(D)J gene recombination, resulting in abundant B-cells that are autoreactive. Immature B-cells with strong reactivity to self-antigen undergo clonal deletion (Checkpoint 1) and/or rearrange their immunoglobulin gene segments; a process called receptor editing (Checkpoint 2), which eliminates self-reactivity and thus, allows entry to the transitional B-cell pool. Transitional B-cells depend on BAFF for survival and to differentiate into mature-B cells in the spleen (Checkpoint 3). The transitional 1 and 2 (T1/T2) B-cells with strong self-reactivity undergo clonal deletion or remain outside splenic follicles as hyporesponsive anergic B-cells that can be rescued upon receiving T-cell help to enter the mature B-cell pool. Overexpression of BAFF can lead to a breach in peripheral tolerance. BAFF: B-cell Activating Factor of the tumour necrosis factor of the ligand Family; BCR: B-cell receptor

2.3.2. The roles of innate immunity mechanisms in SLE pathogenesis

Pathogenesis of SLE is thought to be closely related to B-cell dysfunction, and accordingly this is the usual target for therapies. However, an intricate interplay between both innate and adaptive immune elements has been observed in protective anti-infective immunity (125) as well as in detrimental autoimmunity (126). Advances in the understanding of SLE pathogenesis also have shed light on innate immunity pathways in not only perpetuating inflammation cascades, leading to disease flares, but also continues to fuel adaptive immune responses throughout the course of the disease. Thus, this provides a rationale for targeting the innate immune system for the treatment of SLE.

Inducers and sensors of innate immunity

In contrast to adaptive immunity, which uses specific immune receptors for each antigen, the innate immune is equipped with receptors called pattern recognition receptors (PRRs) that are specialised in their recognition (127). They are expressed by antigen presenting cells such as dendritic cells (DCs) and macrophages as well as other immune and non-immune cells (128). At least three PRRs have been described including a) the toll-like receptors (TLRs), which recognise nucleic acids on the cell membranes or on endolysosomal compartments but not in the cytosol (129); b) the nucleotide binding and oligomerisation domain (NOD) receptors (NLRs), which monitor the cytosolic compartment closely and interacting with TLR signaling pathways (130); and c) the retinoid acid inducible gene (RIG)-I-like receptors that recognise RNA or DNA in the cytoplasm (RLRs) (131). Upon activation, they induce various cellular responses including the transcription of several genes that ultimately result in the elimination of the antigen. Moreover, some of these receptors (e.g. NLR) are also involved in sensing "danger" signals resulting from perturbations of normal cellular processes (132, 133).

Dendritic cells and innate immunity activation

DCs are a heterogenous population of professional antigen presenting cells, which link the innate and adaptive immunity. There are at least two distinct subsets of human DCs, arising from hematopoietic stem cell. Myeloid DCs

(mDCs) are thought to derive from a common myeloid progenitor and reside in tissues and lymphoid organs. They circulate as monocytic precursors (134). The other major subset of DCs, plasmacytoid (pDCs), are thought to derive from a common lymphoid precursor and circulate in blood (135). pDCs are considered the primary source of IFN-α (136, 137), which, makes them relevant to the immunopathology of SLE.

In the absence of exogenous triggers, DCs contribute to the clearance of dying or apoptotic cells and the maintenance of tolerance. Uptake of pathogenic antigens in the presence of a variety of accessory danger signals such as microbial-derived pathogen-associated molecular patterns (PAMPs), necrotic cells, heat-shock proteins and oxidation products induces DC maturation (138, 139), manifested by downregulation of phagocytic receptors, upregulation of antigen presentation machinery and costimulatory molecules (140, 141). Moreover, activated DCs play various roles including to secrete chemokines that attract innate and adaptive responders to the site of injury (142), stimulate naïve T-cells in order to generate immunological memory (143), as well as present antigen to antigen-specific T-cells (144), which subsequently can trigger both Th1 and Th2 responses (145). However, in the context of autoimmunity, a number of mechanisms by which aberrant DC function and regulation could have immunologic consequences have been described including imbalances in DC number, altered uptake and response to benign and harmful antigenic stimuli and aberrant interactions with other immune effector cells, triggering inappropriate downstream responses (146, 147).

Type I Interferon

Type I interferon (IFN-I) comprises a large family of cytokines, including multiple subtypes of IFN-α and the single IFN-β, which are rapidly produced in response to viral infections, and act as critical mediators of host antiviral responses. Although many cell types can produce IFN-I, the major producers are pDCs (136, 137). Many different immune complexes (ICs) can activate pDCs, but RNA containing ICs triggering endosomal TLR seems to be the best IFN-α inducer (148, 149). However, IFN-I production can occur without TLR activation (150).

IFN-I presents many immunological functions such as promoting B-cell differentiation, immunoglobulin switch, autoantibody production and survival of the activated B- and T-lymphocytes (151). Detection of circulating IFN is often challenging but many studies have shown a correlation between SLE activity and the expression of IFN-inducible genes; known as "IFN gene signature" in peripheral blood mononuclear cells (152, 153). IFN gene signature also has been reported in cutaneous lesions (154), glomerular (155) and synovial tissue (156), suggesting a key role of IFN-I in tissue damage. Further studies will be required for a better comprehension of the roles of IFN-I and DCs in SLE pathogenesis but at present, they appear to play a key role at the interface between innate and adaptive immunity.

2.3.3. Summary of the interplay between innate and adaptive immune systems in the pathogenesis of SLE

Key events in the immunopathogenesis of SLE are summarised into 5 steps below and are illustrated in Figure 2.2.

Step 1: Loss of self-tolerance

Deficiency in clearance of apoptotic cells leads to an abundance of nucleic acid remnants. These activate the TLR7 and TLR9 (157, 158), expressed by the pDCs, which then stimulate excessive production of various inflammatory cytokines including IFN-α. IFN-α activates variety components of the immune system including mDCs. Once activated, mDCs present self-antigens and other proteins (including BAFF and APRIL) to T- and B-cells leading to cell proliferation, maturation, differentiation and survival, and excess autoantibody and cytokine production (159, 160).

Step 2: Production of autoantibody

T-cell receptor interacts with MHC on antigen presenting cells and triggers the T-cell response. However, T-cell needs a second co-stimulatory signal. Co-stimulatory molecules such as CD28:B7 and CD40:CD40 ligands help activate B-cells (161). Thus, autoreactive T-cells provide help to B-cells which subsequently produce large quantities of autoantibodies. These autoantibodies form immune complexes (ICs).

Step 3: Deposition of immune complexes

Circulating ICs that are not adequately cleared will then be deposited in tissues or organs such as kidneys and skin.

Step 4: Immune complex-associated inflammation

In healthy individuals, immune complexes are cleared by Fc and complement receptors (162). However in SLE, genetic variations in FcR genes and the C3bi receptor gene (ITGAM) (47, 163) may impair the clearing of immune complexes, which then deposit and cause tissue injury at sites such as the skin and kidney. Immune complex-associated inflammation can also occur activation of macrophages and neutrophils via surface Fc-receptors which bind IgG (164).

Step 5: Tissue fibrosis and damage

Production of pro-inflammatory and pro-fibrotic cytokines including IL-4 and tissue growth factor-beta (TGF-β) lead to irreversible tissue damage or scarring (165, 166). Finally, when these immune elements remain dysregulated in SLE, these lead to further tissue damage and cell death, perpetuating the cycle of inflammation.
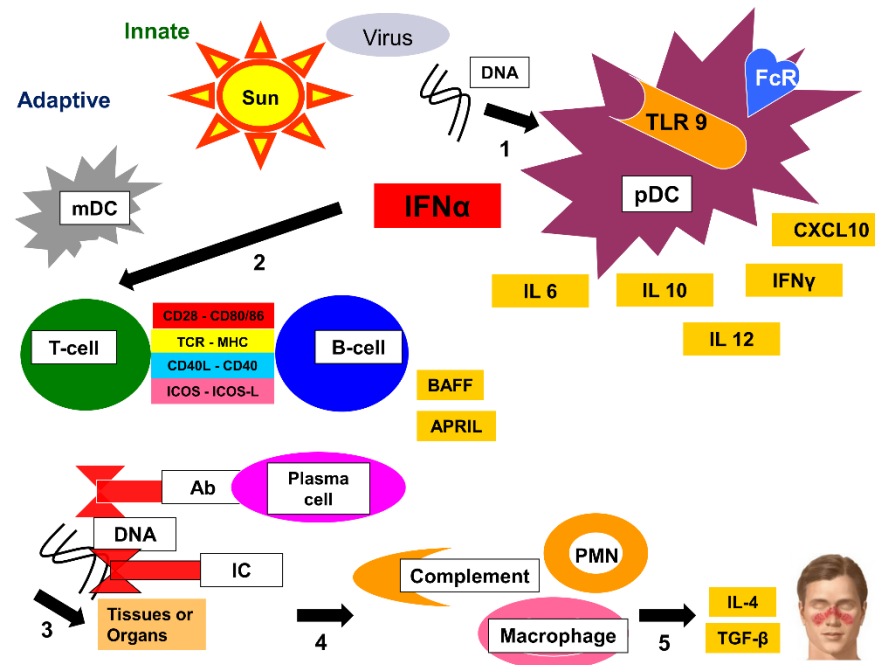
Figure 2-2 Immunopathogenesis of SLE

The interplay between innate and adaptive immune system in SLE pathogenesis. 1) Deficiency in clearance of apoptotic debris leads to abundance of nucleic acid remnants. These activate Toll-like receptors (TLR7 and 9) expressed by pDCs, which then stimulate excessive production of IFN-α. 2) IFN-α is a pluripotent cytokine that activates a variety components of the immune system including mDCs, T-cells and B-cells, leading to cell proliferation, maturation, differentiation and survival, as well as excess autoantibodies and cytokines production. 3) These autoantibodies then form ICs, which subsequently deposit in tissues or organs such as the kidneys and skin. 4) IC-associated inflammation can occur via complement activation classical pathway, activation of macrophages and neutrophils via surface Fc-receptors that bind IgG. 5) Finally, production of pro-inflammatory and pro-fibrotic cytokines including IL-4 and tissue growth factor-beta (TGF-β) can lead to irreversible tissue damage or scarring. APRIL: A PRoliferation-Inducing Ligand; Ab: antibody; BAFF: B-cell Activating Factor of the tumour ncrosis factor of the ligand Family; DNA: deoxyribonucleic acid; FcR: Fc receptor; IC: immune complex; mDC: myeloid dendritic cell; pDC: plasmacytoid dendritic cell; PMN: polymorphonuclear cell family; TGF-β: Tissue growth factor beta; TLR: Toll-like receptor

2.3.4. Autoreactivity and autoimmunity

The presence of ANAs with a range of specificities in apparently healthy individuals is common, with studies from geographically diverse locations reporting about one-quarter of individuals express at least low levels of autoantibodies, corresponding to immunofluorescence assay titres of at least 1:40 dilution (167, 168). The high prevalence of autoreactivity in the population suggests that autoantibodies may be expressed as part of a healthy immune response as well as exhibit important immune regulatory functions (169).

Nevertheless, in a small number of these individuals, presumably with additional pre-existing genetic and/or epigenetic susceptibility factors, these autoantibodies may promote activation of immune responses that result in progression from benign autoreactivity to autoimmunity. Therefore, individuals with ANA constitute an "At-Risk" population of whom a minority will progress to overt, clinically apparent disease. It remains unclear how this controlled state of autoimmunity develops into clinical disease. If this transition from pre-lupus to SLE could be predicted, early and potentially more effective intervention could be employed.

Over time, the risk of progression to systemic autoimmune disease may increase as autoimmune responses escalate and damage accumulates. Thus, non-specific intermittent symptoms that characterise the At-Risk individuals may develop into clinical SLE with subsequent progression of the disease, ultimately resulting in severe organ manifestations in a subset of patients. This natural course from benign autoreactivity to autoimmunity is illustrated in Figure 2-3.
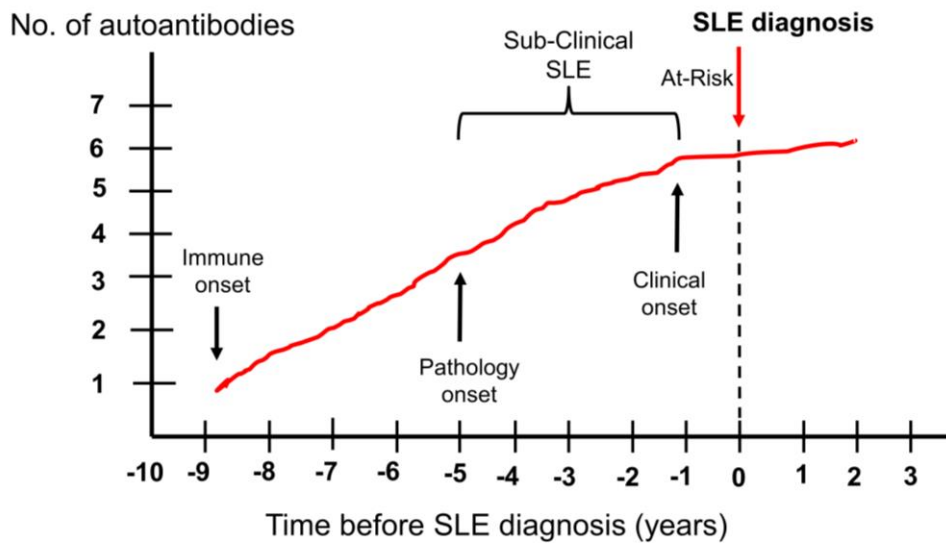
Figure 2-3 The natural course of progression from autoreactivity to clinical autoimmunity

> After a variable period of time from autoantibody production i.e. immune onset, circulating immune complexes can be formed and may be deposited in tissues and organs, where they can potentially initiate an inflammatory process. This process can be regarded as the pathology onset of SLE. Subsequently, immune complex-associated inflammation in the target tissues or organs may lead to formation of immune histopathological changes, albeit individuals are asymptomatic clinically. This is termed as sub-clinical SLE. Finally, all clinical and immunological abnormalities occurring between clinical onset and diagnosis or classification represents At-Risk of SLE. SLE: systemic lupus erythematosus

2.4. Outcome measures

Given the heterogeneity of disease presentation and clinical course among patients with SLE, the British Society of Rheumatology (BSR) 2017 Guidance recommends that all patients should be monitored on a regular basis for disease manifestations, clinical activity, drug toxicity and co-morbidities (170). Assessment and monitoring can be achieved by using several validated indices for clinical and clinical trial purposes. Only the widely used indices that are relevant to this thesis are described in details below. These indices or tools can be used to assess lupus in general or may be organ-specific. Several objective outcome measures that are relevant to this thesis are also described in this section. It is important to note that during these assessment, features attributable to active SLE must be distinguished from chronic damage, drug toxicities and other causes such as infection or malignancy.

2.4.1. British Isles Lupus Assessment Group (BILAG)-2004 Index

The classic British Isles Lupus Assessment Group (BILAG) was originally developed to match the physician's intention to change therapy (171). The revised BILAG Index (version 2004) measures disease activity (scored from grade A to E) in 9 body or organ systems affected by SLE based on clinical assessments and laboratory results (172). The BILAG-2004 index covers 97 items as opposed to 86 items in the classic BILAG and records disease activity occurring over the past 4 weeks. Each domain or system is then given an overall grade of: 0 = not present, 1 = improving, 2 = same, 3 = worse, or 4 = new. The grading is detailed Table 2-2. The numerical global BILAG-2004 score has also been introduced to facilitate comparison with other disease activity indices (173). Each grade is weighted as follows: grade A = 12 points, grade B = 8 points, grade C = 1 point and grades D/E = 0. The global BILAG-2004 score is then calculated by adding the total points from the nine BILAG grades.

The BILAG-2004 Index is recognised as a valid and comprehensive tool in measuring disease activity due to SLE (174). The strengths of this tool include highlighting the importance of assessing patients' individual bodily systems rather than a global or overall score, it incorporates the important element of

change in disease state with time, is sensitive to small changes (175) and distinguishes between disease activity and disease severity. Its disadvantages include high administrative burden and requirement of formal training as may be difficult to perform by inexperienced raters.

Table 2-2 Grade and definition of BILAG-2004 index

Category	Definition
A	Severely active disease (sufficient to require disease-modifying treatment ie: >20mg/day prednisolone, immunosuppressant and cytotoxics)
B	Moderately active disease (requires only symptomatic therapy, for example, prednisolone ≤20mg/day prednisolone, or anti-malarials
C	Mild stable disease (no indication for changes in treatment)
D	Inactive now but previously active
E	Never affected

2.4.2. SLE Disease Activity index (SLEDAI) and its versions

The SLE disease activity index (SLEDAI) was first developed and introduced in 1985. The SLEDAI-2K (version 2000) is a modified validated instrument that measures disease activity within the last 10 days. It is a global index and includes 24 clinical and laboratory variables that are weighted by the type of manifestation but not by severity (176). The total score falls between 0 and 105, with higher scores representing increased disease activity. The SLEDAI-2K has been shown to be a valid and reliable disease activity measure in multiple patient groups (177, 178).

Another modified version of SLEDAI was developed for use in the Safety of Estrogens in Lupus National Assessment (SELENA) study; SELENA-SLEDAI. A glossary was added and the modification allowed for documentation of persistent active disease in some descriptors such as rash, mucosal ulcers, and alopecia, which were previously not scored unless they were new or recurrent. Additionally, the SELENA-SLEDAI also accepts the presence of

either the objective or subjective findings for the descriptor to be scored as present (64).

The advantages of SLEDAI and its versions include low administrative burden, practicality and are the most commonly used global disease activity measures in longitudinal observational studies and clinical trials. The disadvantages include inability to capture improving or worsening of symptoms, do not account the severity within an organ system and some items that are clinically important and potentially life-threatening such as haemolytic anaemia and ascites, are not included as descriptors.

2.4.3. SLE Responder Index (SRI)

The SLE Responder Index (SRI) is a composite outcome that incorporates a modification of SELENA-SLEDAI, BILAG and a 3-cm visual analogue scale (VAS) of physician's global assessment (PGA) to determine patient improvement. The SRI was derived following *post-hoc* analysis of a phase II belimumab study in SLE in order identify subjects with a meaningful clinical improvement in disease activity and response to treatment (179). A responder was defined if fulfilling all of the following: (i) ≥ 4-point reduction in SELENA-SLEDAI score; (ii) no new BILAG A (severe disease activity) or two new BILAG B scores (moderate disease activity) and (iii) no deterioration from baseline in the PGA by at least 0.3 points (or 10 % of 3-point VAS).

The advantages of SRI include it ameliorates the limitations of each of the individual index that constitute this composite measure and SRI has been associated with global benefit for patients with SLE in clinical trials (180, 181). However, the SRI has some significant differences in outcome from the scores from which it was derived. For example, the BILAG component is only defined by no new worsening. Thus, a patient can qualify as a responder when a feature of SLEDAI resolves, while other features (if present at baseline) stayed the same or worsened slightly (one x BILAG B). The relevance of this index for clinical practice is therefore currently less clear.

2.4.4. British Isles Lupus Assessment Group-based Combined Lupus Assessment (BICLA)

The BILAG-Based Composite Lupus Assessment (BICLA) is a composite index that was derived by expert consensus of disease activity indices. The BICLA response was first used as the primary endpoint in the phase II randomised controlled trial (RCT) of Epratuzumab in autoantibody positive SLE (182). Requirement for the BICLA response were: (i) BILAG-2004 improvement (all A scores improved to B/C/D and all B scores improved to C or D); (ii) no worsening in disease activity (no new BILAG A or more than one new BILAG B score); (iii) no worsening of total SLEDAI-2K score from baseline; (iv) no significant deterioration (<10% worsening relative to baseline) in physician's global assessment and (v) no treatment failure (initiation of non-protocol treatment).

The strength of BICLA index is that it requires a stringent response in all body systems that are involved at baseline and require that there are no new flares in the remaining body system. Moreover, BICLA also incorporates treatment failure which is clinically meaningful particularly in terms disease burden and comorbidity perspectives, whether this may be cessation of therapy, increment in daily oral prednisolone dose or addition of alternative immunosuppressant. The disadvantage includes administrative burden as formal training is essential for commercial or academic optimal performance and further validation is needed for BICLA to be used widely in clinical practice or trials.

2.4.5. Cutaneous Lupus Erythematosus Disease Area and Severity Index (CLASI) and the revised version (RCLASI)

Several organ-specific disease activity indices have been developed in lupus. Since mucocutaneous and musculoskeletal complaints are two most common manifestations of SLE, indices and outcome measures related to these are described below.

Current methods for assessing cutaneous lupus erythematosus (CLE) include using the validated clinical indices such as the CLE Disease Area and Severity Index (CLASI) (183) and the revised CLASI (RCLASI) (184). CLASI is a comprehensive tool that comprises assessment for disease activity and damage in CLE. The activity components attempt to quantify the level of active inflammation in the skin, scalp, and oral mucosa. For example, lesions with greater visual intensity (dark purple is worse than faint erythema) and scaling are considered to be more active and would be scored appropriately higher than those without. While the components for damage are dyspigmentation, scarring or atrophy and scarring alopecia. CLASI has been shown to be valid, reliable and is sensitive to changes (185, 186). A four-point or 20% decrease in CLASI activity score has been shown to be the most specific criterion in classifying patients as responders or non-responders and represents the minimal clinically important change (187). Nonetheless, CLASI scoring is heavily influenced by the number of areas involved rather than the coverage of skin within each area, higher weighting of visible areas tends to cause greater patient impairment as well as subjective elements in assessing mucocutaneous ulcers and alopecia.

A study from Germany reported that the descriptors in CLASI did not reflect an accurate assessment for all CLE subtypes particularly the chronic CLE (CCLE) and lupus erythematous tumidus (188). Therefore, the RCLASI was derived by adding oedema or infiltration and subcutaneous nodules or plaques as descriptors for disease activity. RCLASI has been used in clinical trials however the limitations include high administrative burden and lack of use in clinical practice.

2.4.6. The Score of Activity and Damage in Discoid Lupus Erythematosus (SADDLE)

Discoid lupus erythematosus (DLE) is a disfiguring inflammatory skin disease and is a form of CCLE. Since DLE has distinct morphologies compared to other CLE subtypes, the Score of Activity and Damage in Discoid Lupus Erythematosus (SADDLE) was derived to account for items that were attributed to DLE (189). The items for activity include erythema, scaling and induration while for damage are scarring or atrophy and dyspigmentation. Each item is graded between 0 to 3 in 13 parts of the body with a total score ranges between 0 and 195. SADDLE index has been shown to be valid, correlates well with other global assessment scores and has been used in clinical studies (190, 191). Further studies are required to investigate its responsiveness to change with therapy.

2.4.7. Musculoskeletal Ultrasound

The most widely used disease activity indices for the assessment of musculoskeletal symptoms in SLE are the musculoskeletal items of the SLEDAI and BILAG-2004 indices. However, these tools were designed to assess multi-organ system disease and therefore might capture less details on an individual organ system compared to established composite index such as Disease Activity Score in 28 joints (DAS28) for RA.

The low frequency of clinical synovitis makes measurement of musculoskeletal disease activity difficult in SLE. Musculoskeletal ultrasound (MSK-US) can provide objective assessment of inflammation, detect subclinical synovitis and has been widely used in other rheumatologic conditions like RA for trial and clinical practice (192, 193). However, there are limited data in SLE. In a systematic review for which I am the co-author, we found that the rates of abnormality in terms of synovitis, tenosynovitis and erosions were highly variable among the studies included. Besides that, there were poor to moderate association between ultrasound abnormalities and disease activity indices and immunological findings. Notably, there was moderate to high risk of bias and there were concerns about applicability in most studies (194). Thus although MSK-US has potential value in the assessment of musculoskeletal symptoms in SLE, studies that address these methodological variation and promote consensus on ultrasound abnormalities are required before it can be used as a valid outcome measure in lupus.

2.4.8. Laser Doppler Imaging

Disease activity indices for CLE such as CLASI, RCLASI and SADDLE were developed to document activity in the whole parts of the body rather than localised lesions and were subjective to physicians and patient's report. Thus novel quantitative outcome measures that are valid and assess individual lesion accurately is needed.

One attractive approach is to measure the skin microcirculation. Microcirculatory abnormalities contribute to the pathophysiology of many autoimmune rheumatic diseases (ARDs) including CLE (195). Furthermore, various novel targeted therapies are currently under development or investigation in CLE with pharmacologic effect on skin microcirculation. Laser Doppler imaging (LDI) is a non-invasive imaging modality that monitors the total local microcirculatory blood perfusion including the perfusion in capillaries, arterioles, venules and shunting vessels and has been used to assess responsiveness after pharmacological stimuli (196). Alteration in peripheral blood flow (as measured by LDI) has been shown to correlate with the degree of inflammation in skin psoriasis (197). Our group for which I am the first author, is the first to report the use of LDI in CLE. We demonstrate that LDI is valid, reliable, has a better correlation with histology from skin biopsy compared to currently used clinical tools and is responsive to change in therapy (198).

2.5.   Overview of targeted therapies

A wide range of immunological abnormalities have been described in relation to pathogenesis of SLE. Figure 2-4 illustrates the range of target molecules and the corresponding therapeutic agents currently available or under investigations.

B-cells have traditionally been seen as central to this. The loss of B-cell tolerance is the key to the production of autoantibodies. In addition to functioning as sources of autoantibodies, B-cells are also efficient antigen-presenting cells (199) and secrete a wide range of cytokines which exhibit both autocrine and paracrine effects (200). For instance, once an appropriate stimulus is detected by both B-cell receptor (BCR) and CD40, B-cells secrete pro-inflammatory cytokines such as lymphotoxin-alpha, TNF and IL-6, which may act as growth and differentiation factors and assist in formation of germinal centre structures in inflamed tissues.

T-cells play a role in the pathogenesis via T-cell–antigen-presenting cell interaction and B-cell help (201), defects in various intracellular signal transductions in T-cell pathways (202) and inadequate suppression of autoreactive cells by regulatory T-cells (Tregs) (203).

Recent advances in SLE pathogenesis have focussed on abnormalities in clearance of apoptotic and secondary necrotic cells as well as increased innate sensing of nuclear antigen as demonstrated by overactive IFN-I and IFN-III production and TLR signalling in patients with SLE (151).

Despite heterogeneity in pathogenesis, translating targeted therapy from bench to bedside has been more problematic in SLE than in other ARDs, with many theoretically well-founded agents appearing to have failed in clinical trials as a result of inefficacy, problem with trial design and/or safety issues (7). Table 2-3 summarises biologics currently available and in late development for the treatment of SLE.
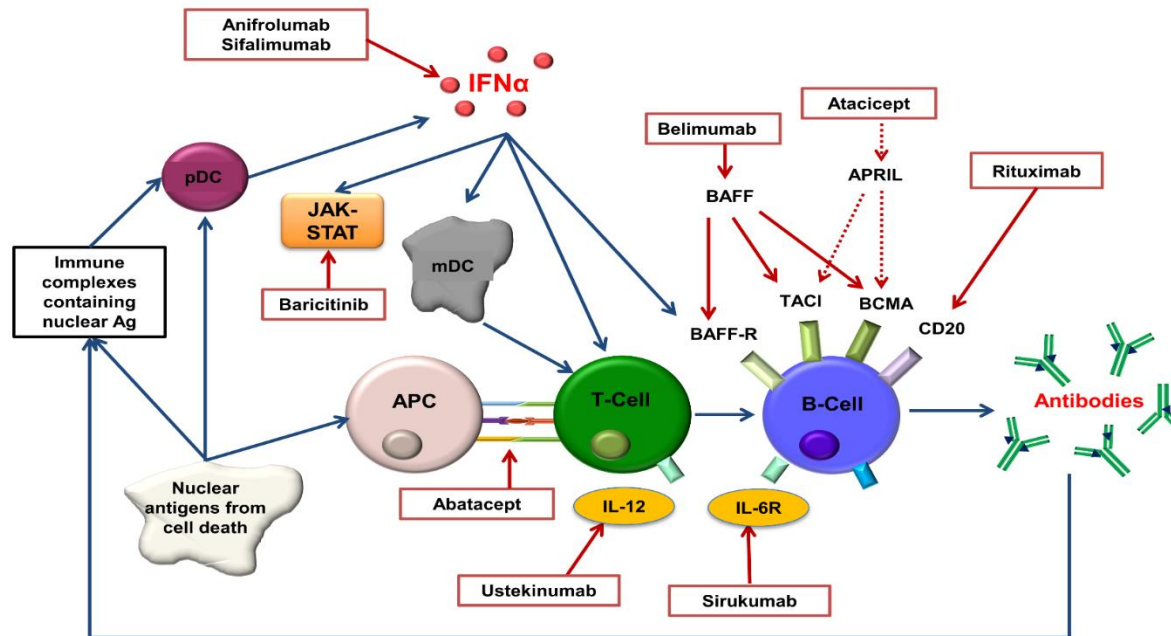
Figure 2-4 Range of target molecules based on immunopathogenesis of SLE and their corresponding therapeutic agents

Only therapeutic agents that are currently available and in late development in SLE are shown. IFN-α activates a variety of components of the immune system including mDCs, T-cells, B-cells and JAK-STAT signalling, leading to cell proliferation, maturation, differentiation, survival, and excess autoantibody and cytokine production. IFN and IFN receptor can be targeted using sifalimumab and anifrolumab respectively. While JAK-STAT can be targeted using baricitinib. T-cells need a second co-stimulatory signal such as CD28:B7 and CD40:CD40 ligands to activate B-cells. The latter co-stimulatory molecule can be targeted using abatacept. Activated B-cells can be targeted using anti-CD20 depleting agent such as rituximab and by inhibiting their B-cell activating factors either using belimumab or atacicept. Activated B-cells secrete a wide range of cytokines including interleukin-6 and interleukin-12. Both cytokines can be targeted using sirukumab and ustekinumab respectively. Ag, antigen; APC, antigen presenting cells; APRIL, a proliferation-inducing ligand; BAFF, B cell-activating factor of the tumour necrosis factor family receptor; BAFF-R, BAFF-receptor; BCMA, B cell maturation antigen; IFN-α, interferon-alpha; IL-6R, interleukin-6 receptor; JAK-STAT, janus kinase signal transducer and activator of transcription; mDC, myeloid dendritic cells; pDC, plasmacytoid dendritic cell; TACI, tumour necrosis factor receptor superfamily member 13

Table 2-3 Summary of biologics currently available and in late development for the treatment of SLE

Drug	Molecular Target	Phase	Comments	Ref
Belimumab	BAFF blockade	III	Both Phase III trials met their primary endpoints; improvement in the SRI-4 at week 52. The first biologic approved by the US FDA for use in SLE.	(10, 11)
Rituximab	CD20 depletion	III	Both non-renal and renal Phase III trials failed to meet their primary endpoints; achieving and maintaining BILAG overall clinical response and overall renal response respectively at week 52. *Post-hoc* analysis in non-renal trial showed that primary endpoint was achieved in Hispanic and African–American subgroups.	(204, 205)
Abatacept	Selective T-cell co-stimulation modulator	II/III	Phase II study in non-renal failed to achieve its primary endpoint; the proportion of patients with at least a new BILAG grade B flare at week 52 after the start of the steroid taper. Might be beneficial in arthritis manifestation. Phase III trial in renal lupus failed to meet its primary endpoint; complete renal response at week 52.	(206, 207)

Drug	Molecular Target	Phase	Comments	Ref
Atacicept	BAFF and APRIL blockade	II/III	Phase II/III trial in renal SLE was suspended due to severe infection. Phase II/III trial in non-renal SLE is ongoing.	(208)
Sifalimumab	Type I IFN-α blockade	II	Primary endpoint; SRI-4 at week 52 was met with improvement in skin and joint manifestations, although the treatment effects were modest.	(209)
Anifrolumab	Type I IFN-α receptor blockade	II	Primary endpoint; a composite of SRI-4 and sustained reduction of corticosteroid <10mg/day at week 24 was met in non-renal SLE indication with clinically important improvement in skin and joint manifestations.	(210)
Baricitinib	Selective janus kinase I (JAK1) and JAK2 blockade	II	Primary endpoint; the proportion of patients achieving resolution of SLEDAI-2K arthritis or rash at week 24 was met in non-renal SLE indication, with significantly greater resolution of arthritis or rash in baricitinib compared to placebo.	(211)
Ustekinumab	Interleukin 12/23 blockade	II	Primary endpoint, SRI-4 at week 26 was met including the largest SRI-4 response rate against placebo (29%) reported in any non-renal randomised controlled trial of SLE.	(212)

Drug	Molecular Target	Phase	Comments	Ref
Sirukumab	Interleukin-6 receptor blockade	II	Primary endpoint; change in proteinuria from baseline to week 24 was not met. About 15-20% of the sirukumab-treated group achieved meaningful reduction in proteinuria versus placebo, 0%.	(213)

APRIL, a proliferation-inducing ligand; BAFF, B cell-activating factor of the tumour necrosis factor family receptor; BILAG, British Isles Lupus Asessment Group; IFN-α, interferon-alpha; JAK, janus kinase; SRI-4, systemic lupus erythematosus responder index using a 4-point reduction in SLEDAI; SLE, systemic lupus erythematosus; SLEDAI-2K, systemic lupus erythematosus disease activity index

2.6. B-cell Targeted Therapy

As B-cells have multiple pathogenic roles in SLE, various strategies for B-cell blockade have been investigated including B-cell depletion, inhibition of the survival factors, inhibition of B-cell receptor signalling, development of B-cell tolerogens and targeting plasma cells with varying degree of success in clinical trials (214). In this literature review, only currently available biologics; belimumab, rituximab and abatacept are discussed in details, in line with the aim of my thesis.

2.6.1. Belimumab

Belimumab is a fully humanised mAb that specifically binds to and neutralises the soluble cytokine, BAFF, preventing it from binding to its receptors on the surface of B-cells. B-cell survival, maturation and differentiation are mediated by the two cytokines; BAFF and its homologue, A PRoliferation-Inducing Ligand (APRIL) (215, 216). They bind to three receptors that are expressed on B-cells at different developmental stages, thus activate their own signalling pathways: i) BAFF receptor (BAFF-R) binds BAFF strongly; ii) B-cell maturation antigen (BCMA) binds APRIL and iii) TNF receptor superfamily member 13b (TACI) binds both BAFF and APRIL (217).

Belimumab was the first therapy in over 50 years, which had gained approval from the US FDA and EMA for the treatment of active, autoantibody positive SLE. This approval was supported by the success of two RCTs; the Study of Belimumab in Subjects with SLE for 52 weeks (BLISS-52) (10) and the other was a parallel study that allowed the treatment to continue through 76 weeks (BLISS-76) (11). In these RCTs, both trials used a new composite index as the primary endpoint; improvement in the SRI-4 at week 52.

2.6.2. Critical review of trial design in belimumab studies

In BLISS-52 trial, higher SRI rates were achieved with belimumab 1 mg/kg (51%, p=0.0129) and 10 mg/kg (58%, p=0.0006) than placebo (44%) at week 52 (11). While significantly greater SRI response at week 52 was only achieved in the belimumab 10mg/kg dose compared with placebo (43.2% versus 33.5%; p=0.017) in BLISS-76 trial (10). Thus the higher dose was

approved due to consistency in efficacy in both trials. Over time, data from long-term extension studies (LTEs) of the phase II trials showed that SRI response and safety profile were maintained in autoantibody positive SLE patients taking belimumab plus standard therapy for up to 7 years (218).

The success of these trials is a prime example in modifying a clinical trial based on lessons learned from earlier trials. The results of a phase II trial of belimumab was negative. Co-primary endpoints; the change in the SELENA-SLEDAI score at week 24 and the time to first SLE flare were not met in the belimumab-treated group. This could be attributed to recruitment of around 30% patients without ANA (181). The *post-hoc* analysis showed that ANA positive patients maintained responses better in the extension studies (181). Thus, ANA positivity was set as the inclusion criteria for both BLISS trials. Moreover, the phase II investigators also reviewed the various components of clinical response criteria used in the phase II study and derived a new composite response index, the SRI as the primary endpoint. The SRI combined elements of the SLEDAI and BILAG to ensure that both clinical improvement and no simultaneous clinical worsening were documented. Lastly, large numbers of patients (over 800 patients in each trial) were recruited to increase the power of the study.

Despite meeting its primary endpoint; SRI-4 at week 52, the effect size of belimumab in autoantibody positive and active SLE appears small, prompting a search for subgroups with higher levels of response. Indeed, the magnitude of difference between rituximab and control groups in the phase III trial of Lupus Nephritis Assessment with Rituximab (LUNAR) was comparable with the differences observed in BLISS trials numerically but with ten times the numbers of patients in the latter (219).

In clinical practice, rheumatologists use biological agents in severe cases with end-organ involvement and refractory to conventional immunosuppressant whereas both BLISS trials recruited patients mainly with mucocutaneous and musculoskeletal manifestations (about 2/3 of cases). Patients with severe lupus nephritis and neuropsychiatric manifestation were excluded although 15% of the former had improvement in proteinuria in *post-hoc* analysis (32). These factors have contributed to the cost-effectiveness estimation being

problematic. In the UK, NICE estimated the cost of belimumab at £61,200 per quality adjusted life year (QALY), which was more than the normally acceptable cost of £20,000 to £30,000 per QALY. After several appeals as well as a substantial discounted price from the manufacturer, NICE finally recommended its use as an option or add-on treatment for active autoantibody-positive adults with SLE in 2016 (12).

Although a pooled analysis demonstrated that greater therapeutic benefit with belimumab group over standard of care, might be achieved in patients with higher disease activity as well as greater serologic activity (i.e. anti-dsDNA positivity and hypocomplementaemia) (220), a more accurate means to identify patients likely to respond well to belimumab is needed. Several studies are already in the pipeline to address this including a phase III trial in lupus nephritis.

2.6.3. Abatacept

Abatacept is a fusion protein of the extracellular domain of cytotoxic T-lymphocyte antigen 4 (CTLA4) and the constant region of IgG, that has been developed to block the co-stimulatory interactions between B- and T-cells. CTLA4-Ig acts as a competitive inhibitor for CD28 on the T-cell surface by binding with either CD80 (ligand B7-1) or CD86 (ligand B7-2), thus preventing T-cell activation (221). Consequently, this inhibits both T-cell dependent inflammatory pathways and T-cell dependent-B-cell responses.

Despite promising results in animal models, RCTs in extra-renal lupus and renal lupus failed to meet their primary endpoints; the proportion of patients with at least a new BILAG grade B flare after the start of the steroid taper and complete renal response respectively, at week 52. However in the trial of non-renal lupus, abatacept was associated with fewer major BILAG A flares compared to placebo and a subset of patients with polyarthritis showed significant response in secondary analyses (206).

2.6.4. Critical review of trial design in abatacept studies

In the trial of lupus nephritis, the problem with choosing an appropriate primary endpoint resurfaced. In a phase II/III trial of abatacept in lupus nephritis, complete response was defined if fulfilling all the following criteria: i)

Modification of Diet in Renal Disease (MDRD) estimated glomerular filtration rate (eGFR) ≥90% of screening level if normal at screening visit or eGFR ≥90% of 6-month pre-flare value if abnormal at screening); ii) urinary protein-to-creatinine ratio (UPCR) <0.26 gm/gm (30 mg/mmol); and iii) inactive urinary sediment. All complete response criteria had to be met once again, 4 weeks after they were initially achieved. These stringent endpoints used in defining renal response might have led to negative results, with only 8-11% achieving the complete renal response in both the treatment and placebo arms at 52 weeks (207). A comparative analysis was performed by applying the definition of renal response used in the LUNAR trial of rituximab (205), showed higher achievement of complete renal response rates in the abatacept versus placebo groups; 22% and 6% respectively (219). For this reason along with other findings from *post-hoc* analyses (i.e. significant reduction in proteinuria in patients with nephrotic-range proteinuria and improvement in anti-dsDNA levels), abatacept may still have role in SLE.

2.7. Rituximab

2.7.1. Therapeutic indications for rituximab

Rituximab was the first licensed, chimeric anti-CD20 mAb, initially approved in 1997 for the treatment of relapsed or refractory low grade or follicular CD20+ B-cell non-Hodgkin's lymphoma (NHL) (222). It has since been licensed for diffuse large B-cell lymphomas, chronic lymphocytic leukaemia (CLL), patients with severe RA who have had an inadequate response or intolerance to other conventional synthetic disease-modifying anti-rheumatic drugs (csDMARDs) including one or more TNF-inhibitors and remission induction of AAV (223).

In SLE, a high degree of efficacy in a wide spectrum of SLE manifestations including a systematic review of off-label use in 188 cases (13) and a pooled efficacy analysis of lupus nephritis (14) were reported in the initial open label case series of rituximab, generally in highly resistant SLE. Despite the success of these reports, two RCTs in non-renal lupus, The Exploratory Phase II/III SLE Evaluation of Rituximab (EXPLORER) and renal lupus, LUNAR failed to meet their primary endpoints; achieving and maintaining BILAG

overall response and overall renal response respectively at week 52. The key findings of both trials and trials of belimumab are summarised in Table 2-4.

Table 2-4 Results of phase III RCTs of rituximab and belimumab in SLE

Characteristic	EXPLORER (204)	LUNAR (205)	BLISS-52 (11)	BLISS-76 (10)
Number of patient	267	144	867	819
Follow-up (weeks)	52	52	52	52
Inclusion Criteria	ANA +ve, Active disease (>1 BILAG A or ≥2 BILAG B) and 1 stable DMARDs	ANA +ve history, ≥ Class 3 LN on biopsy and Proteinuria (UPCR ratio >1)	ANA/Anti-dsDNA +ve, Active disease (SELENA/SLEDAI ≥6) & stable DMARDs	ANA/Anti-dsDNA +ve, (SELENA/SLEDAI ≥6) & HCQ could be added up to week 16
Exclusion Criteria	Severe CNS or LN and ≤12 weeks recent use of CyC or CAL	> 50% glomerular sclerosis and eGFR <25 ml/minute/1.73 m^2	Severe LN or CNS, prior RTX & <6 months use of CyC	Severe LN or CNS, prior RTX & <1 year use of other biologics
Dosing Schedule	Pb + Pred + DMARDs vs RTX + Pred + DMARDs	Pb + Pred + MMF vs RTX + Pred + MMF	Pb + Pred vs BLB 1mg/kg + Pred vs BLB 10mg/kg + Pred	Pb + Pred vs BLB 1mg/kg + Pred vs BLB 10mg/kg + Pred
Primary Endpoint	Major or Partial or No response based on BILAG	Complete or Partial or No Renal Response Rate	SRI	SRI
Achieved? (Yes/No)	No	No	Yes	Yes
Comments	Secondary endpoints were not achieved. Primary endpoint was met in Hispanic & African-American. Anti-dsDNA ↓, Complement ↑	Secondary endpoints were not achieved. African-Americans showed more partial response than Caucasian although was not statistically significant	BLB 10 mg/kg was efficacious against the placebo in all three SRI components & reduction of >50% Pred dose from baseline	Only BLB 10 mg/kg statistically met primary endpoint and was still efficacious despite index threshold increased

ANA, anti-nuclear antibody; BILAG, British Isles Lupus Asessment Group; BLB, belimumab; CAL, calcineurin inhibitor; CNS, central nervous system; CYC, cyclophosphamide; DMARDs, disease modifying anti-rheumatic drugs; dsDNA, double-stranded deoxyribonucleic acid; HCQ, hydroxychloroquine; LN, lupus nephritis; MMF; mycophenolate mofetil; Pb, placebo; Pred, prednisolone; RTX, rituximab; SRI, systemic lupus erythematosus responder index; UPCR, urine protein creatinine ratio

2.7.2. Posology and pharmacokinetic of rituximab

Rituximab is formulated for intravenous administration and administered over several hours (based on the standard infusion schedule). For the treatment of RA and SLE, the licensed dose of a course of rituximab consists of two consecutive 1000 mg intravenous infusions, given on Days 1 and 15 (223).

Owing to the risk of infusion-related reactions, rituximab should be administered in clinical environments where full resuscitation facilities are available, although severe infusion reactions are uncommon in the treatment of ARDs. Prophylaxis with paracetamol (1 gram) and diphenhydramine hydrochloride (25 to 50mg, or equivalent dose of similar agent) should be given 30 to 60 minutes before infusion of rituximab (224).

Clinical pharmacokinetic data are available for two consecutive doses of the licensed dose; 1000mg (RTX1000) and half-dose regimen; 500mg (RTX500). Mean terminal elimination half-life ranged from 18 to 21 days (after the second infusion) for RTX1000 and 16 to 16.5 days (after the second infusion) for RTX500. With repeat courses of therapy, there was no difference in pharmacokinetics between first and second courses (223).

2.7.3. Pharmacology of rituximab

Rituximab is an anti-chimeric mAb. The chimeric structure of rituximab consists of human IgG 1 and kappa-chain constant regions and heavy- and light-chain variable regions, from a murine antibody to CD20. The murine variable regions selectively bind to the CD20 antigen, expressed on the surface of both normal B-lymphocytes and most autoreactive B-cells. While the Fc domain allows rituximab to bind to Fc receptors on human effector cells, to recruit antibodies and complements to mediate cell lysis (225).

The development of B-cell depletion targeted at the CD20 molecule as a therapeutic modality such as rituximab, represents a major advance in autoimmune rheumatic disease (ARD). CD20 is considered as a general B-cell marker but it is neither expressed on stem cells nor on plasma cells that have returned to the bone marrow (226). This selective expression on mature B-cells but not on precursors such as stem cells or antibody secreting plasma cells makes it an attractive therapeutic target, particularly from a safety point

of view. Depletion via CD20 permits B-cell regeneration and moderates reduction of immunoglobulin levels, at least with initial therapy (223, 227).

2.7.4. Mechanism of B-cell killing by rituximab

B-cells killing by rituximab can be achieved through a combination of (i) antibody-dependent cell-mediated cytotoxicity (ADCC) in the presence of effector cells; (ii) activation of complement resulting in complement-dependent cytotoxicity (CDC) and (iii) cross-linking of multiple CD20 molecules, resulting in cell death via induction of non-classical apoptosis (228). These mechanisms are illustrated in Figure 2-5**.**

ADCC is regarded to be the most important mechanism for cell killing (229). Evidence from use of rituximab in haematology patients has shown that targeted epitope expression may decline after infusion of mAbs. It has been demonstrated *in vitro* that rituximab/CD20 complexes may be removed from B-cells by acceptor cells via Fc gamma receptor (FcγR), preventing their killing via an endocytic process called CD20 shaving or trogocytosis (230).
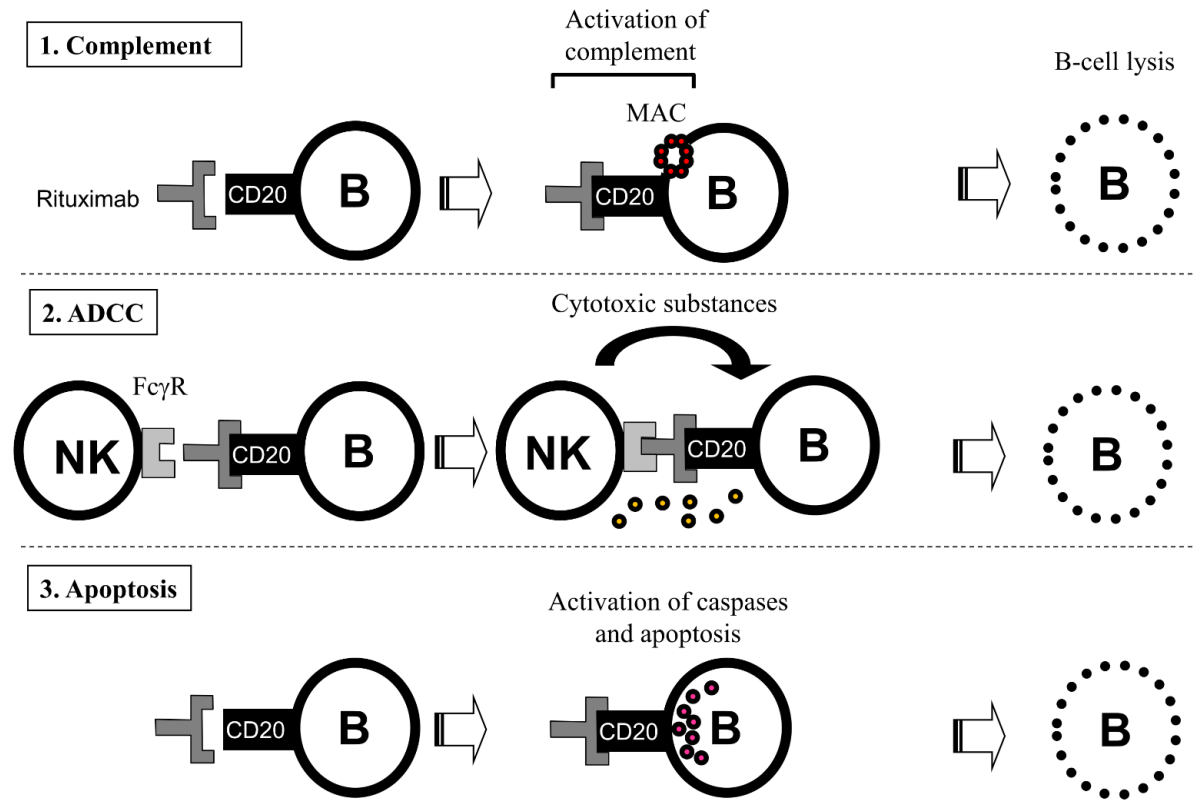
Figure 2-5 Mechanisms of B-cell killing by rituximab

There are three possible mechanisms of B-cell killing or lysis by rituximab; 1) antibody-dependent cell-mediated cytotoxicity (ADCC), 2) complement-dependent cytotoxicity (CDC) and 3) induction of apoptosis. ADCC is generally believed to be the predominant mechanism. In ADCC, cell killing depends on Fc-gamma receptor bearing natural killer (NK) cells or monocytes following the binding of rituximab to CD20. FcγR, Fc gamma receptor; MAC, the membrane attack complex

2.7.5. Critical review of trial design in rituximab studies

The EXPLORER trial was a placebo-controlled, double-blind, multicentre study which recruited 257 SLE patients with moderate to severe non-renal and non-central nervous system lupus from North America population (204). Patients received either a cycle of rituximab, 2 x 1000mg infusion given 2-week apart or a placebo with continuation of the background immunosuppressant. All patients also received 0.5–1.0 mg/kg of steroids which were tapered to 10mg daily by week 10. The primary endpoint was to achieve and maintain clinical response (major, partial or no clinical response) at week 52, assessed using BILAG criteria. No difference in both major clinical responses and partial clinical responses was observed between the placebo and rituximab groups with overall response rates of 28.4% and 29.6% respectively; p=0.973. *Post-hoc* analysis showed superiority of rituximab versus placebo in the African-American and Hispanic subgroups. The rates of BILAG A flares were also lower in the rituximab group (204).

To assess the efficacy and safety of rituximab in lupus nephritis, the LUNAR, a double-blind, placebo-controlled phase III trial, randomised 144 patients in a 1:1 ratio to receive either rituximab or placebo, both in combination with a background mycophenolate mofetil (MMF) and prednisolone (205). The primary endpoint at 52 weeks was not achieved. There was no statistically significant difference in the overall renal response (complete and partial) between the rituximab and placebo groups; 57% versus 46% respectively (p=0.180).

Despite the failure of both trials in meeting their primary endpoints, there were several lessons to be learned particularly the issues regarding to the trial design (214, 231). Firstly, both studies permitted aggressive background immunosuppressant and mandated concurrent high dose of oral prednisolone. Consequently, more than 50% of the patients in both rituximab and control groups had become steroid-dependent, while in the LUNAR study, patients were also co-prescribed a high dose of MMF up to 3g/day. Thus, the intensity of these "standard of care" therapy might have masked the therapeutic benefit of rituximab against placebo.

Secondly, the primary endpoint used in EXPLORER required patients to first meet a low disease activity landmark (BILAG C or better in all domains) at 6 months, and then to not flare in the second 6 months. However, the initial landmark was difficult to achieve since only 27% of patients achieved score C or better in all domains, resulting in lower power to detect a difference in flare rate subsequently. This difficulty might have been contributed by (i) most patients had considerably higher baseline scores than the 1 x BILAG A or 2 x BILAG B scores required in inclusion criteria, so even a substantial improvement in disease activity was not sufficient and (ii) because BILAG B scores were sometimes poor at differentiating partial responses. For instance, a mild transient malar rash scored the same as deep scarring discoid while the number of joints with synovitis (scored as B) were highly variable. Nonetheless, even in patients who did meet the 6-month endpoint, there was no evidence of a significant rise in BILAG total score again in the placebo arm since a high dose steroid regime might have been sufficient to restore stable disease when given with background immunosuppressant. To sum up, this endpoint relied on passing an initial endpoint in a first phase and then not flaring in a second. However, the first of these was rarely achieved and the second was probably unnecessary.

Lastly, the BILAG might have been scored inappropriately in EXPLORER, both as an inclusion and a response criterion. The BILAG index originally was developed to match the physician's intention to change therapy. Although only just over one third of patients achieved major clinical response (MCR) or partial clinical response (PCR), and post treatment mean global BILAG was approximately 8 (on the scale A=9, B=3, C=1), withdrawals due to rituximab inefficacy were relatively low. About 70% of patients continued the trial for one year as per trial protocol. This observation suggested that either residual disease activity was actually felt by treating physicians not to be as severe as the BILAG scores awarded, or that the disease activity present at baseline was not as severe as the BILAG scores suggested, or both.

It is worth noting that failure of RCTs of rituximab has been largely attributed to poor trial design including inappropriate endpoints, the use of an active comparator, inadequate inclusion criteria for a heterogeneous disease and underpowered sample size (214). For this reason as well as evidence of

efficacy from off-label use including the BILAG Biologics Registry (BILAG-BR) in the UK (232), rituximab is commissioned by the National Health Service (NHS) England for use in patients with refractory SLE (233). Nevertheless, further evidence pertaining to its efficacy is demanded. Better-designed trials of rituximab in renal and non-renal lupus are currently being planned.

2.7.6. B-cell depletion and association with clinical efficacy

Treatment with rituximab is followed by peripheral B-cell depletion, evident as early as two weeks after administration of the first infusion. Early studies indicated inconsistent relationship between peripheral B-cell numbers and clinical response to rituximab when they were measured using conventional flow cytometry. For example, in the EXPLORER trial, about 10% of the patients in the rituximab group did not achieve complete depletion after the second infusion. Removing patients with incomplete B-cell depletion did not change the primary outcome (204). While in the LUNAR trial, among rituximab-treated patients with high baseline anti-dsDNA titres (>123 IU/ml), those who were renal responders at week 52 had a greater depletion of CD19+ cells compared with renal non-responders (205).

A better means for enumerating peripheral B-cells is by using highly sensitive flow cytometry (HSFC), a protocol that is optimised for the detection of plasmablasts (234). Plasmablasts have a different morphology than mature naïve and memory B-cells. Naïve and memory B-cells are mostly resided within the lymphocyte regions while plasmablasts mostly outside lymphocyte region. Moreover, plasmablasts have lower and heterogenous CD19 expression. For this reason their numbers are underestimated should the gating only focus on the CD19 positive lymphocyte region as in conventional cytometry. Thus, enumeration of B-cells using HSFC results in a more stringent definition of depletion; peripheral CD19+ <1 cell/µl compared to the conventional cytometry; CD19+ between 50–100 cells/µl.

Our group previously reported that complete B-cell depletion (after the second infusion of rituximab) was associated with clinical response (MCR or PCR) compared to those with incomplete depletion; 100% versus 68%; p=0.012 respectively. This B-cell biomarker will require validation prior to its use in clinical practice.

In other ARD such as RA, persistence of plasmablasts after the first infusion of rituximab was associated with inferior clinical response at 6 months (234). Moreover, patients with lower numbers of plasmablasts pre-rituximab were associated with plasmablast depletion and good clinical response using half dose regimen (500mg x 2) (235) while doses of rituximab even higher than licensed might be employed in those with persistence of plasmablasts after therapy (236).

2.7.7. Factors associated with incomplete depletion during rituximab

Experience in B-cell malignancies has identified five different potential mechanisms that may explain the reasons for incomplete B-cell depletion with rituximab. However to date, there are no data on which of these mechanisms predominates in SLE. This knowledge will be fundamental in order modify the use of rituximab for a more efficient depletion and outcome.

Insufficient rituximab dose

There are two dosing regimens to administer rituximab in ARDs: (i) 1g given 2-week apart (standard RA dose) and (ii) 375 mg/m^2 of body-surface area, once weekly for 4 weeks (full lymphoma dose). However, there is no head-to-head study that compares these regimens. Our group previously showed that adding an extra 1g infusion of rituximab at 4 weeks in RA patients with incomplete B-cell depletion, resulted in better depletion and clinical response than the standard RA dose (236).

In SLE, a group in London reported that patients with SLE had markedly lower serum rituximab levels than patients with RA at both 1 and 3 months, regardless of the level of depletion (237). This could be explained by internalisation and destruction of rituximab by target B-cells regulated by FcλRIIb (238). Other plausible reason could be due to trogocytosis (CD20 shaving) as reported in CLL (239). Nevertheless, the absence of an inverse correlation between serum rituximab levels and B-cell counts at 1 month might suggest other intrinsic resistance of B-cells to rituximab-induced depletion.

In our previously reported SLE cohort, the time-to-relapse was highly variable. The median time to re-treatment with rituximab was 18 months, by which time substantial B-cell repopulation and clinical relapse had occurred (16). Data in

RA indicated that giving a second cycle of rituximab at 6 months, if there was active disease, enhanced depletion and clinical response (240).

Ineffective complement function

Complement is a group of over 30 proteins that act in concert in the recognition and elimination of pathogens via direct killing and/or stimulation of phagocytosis (241). Type 1 mAbs, such as rituximab localises CD20 into lipid rafts, thus enhances C1q recruitment and activation of CDC. Induction of CDC is critically dependent on the distance between the mAb binding site and the plasma membrane, with closer binding associated with more efficient coating of active complement components onto the target cell (229).

To maximise the clinical effect of rituximab, it is important to consider whether patients with SLE will be able to elicit CDC and ADCC responses to the drug. Some patients harbour deficiencies in classical complement components, C1q, C2 and C4, resulting in defective immune complex clearance. Studies in CLL showed that over a third of patients were deficient in one or more complement components, correlating with reduced CDC responses (242). However, there is limited data in SLE. Deficiencies in classical complement components have also been linked to patients being more susceptible to infections with organisms such as *Streptococcus pneumonia* and *Nisseria meningitides* (243)*.* These findings raise an important questions: first, do complement deficiencies correlate with ineffective complement function; and second, how do these affect the efficiency of mAb treatments?

Fc-gamma receptor (FCGR) genetics variants

ADCC is an important effector mechanism in the eradication of the autoreactive cells particularly *in vivo.* Most biological agents including rituximab have IgG1 Fc regions, which bind to the FCGR on immune effector cells. SNPs in the coding regions of the FCGR2A and FCGR3A genes appear to have clinical significance as they have been reported to correlate with responses to therapeutic mAbs in B-cell malignancies (244). Data in SLE are limited. A small study (n=12) suggested that low-affinity (FF) alleles of the FCGR3A gene required about a 10-fold increase in rituximab levels to achieve

the same degree of B-cell depletion in patients with high-affinity genotypes (VV or VF) (245).

Development of human anti-chimeric antibody (HACA)

In the EXPLORER and LUNAR trials, 26.0% and 15% of patients respectively in the rituximab group were tested positive for human anti-chimeric antibody (HACA) at any time during the 52-week follow-up. The results of HACA analyses did not affect the primary and secondary efficacy outcomes (204, 205).

Interestingly, HACAs were also detected in less than 10% of patients who were in the placebo arm and did not receive treatment with rituximab. Their titres were lower than those who were treated with rituximab. These observations highlighted the need to interpret a positive test for HACA with caution during rituximab treatment and raised a question on their applicability in clinical practice. Nevertheless, in LUNAR trial, the rituximab-treated patient with the highest HACA titre experienced a severe infusion reaction while infusion-related adverse events (AEs) were less frequent in HACA-negative patients (205).

Another means in assessing the impact of immunogenicity (clinically) to rituximab is by combining clinical characteristics such as severe infusion reaction after the first infusion of rituximab, measuring one's ability to deplete the CD20+ B-cells and the degree of clinical response (246). Risk factors for developing HACA with rituximab have not been reported. In patients with high HACA titres and with incomplete B-cell depletion, treatment with rituximab should be withdrawn as these patients will be at risk of severe infusion reactions if re-treated with the therapy.

B-cell killing inhibited by BAFF and APRIL

BAFF and its homologue, APRIL, mediate B-cell survival, maturation and differentiation (247). A recent study showed following an initial treatment with rituximab in SLE, BAFF levels were elevated and these were associated with rising anti-dsDNA antibody levels and subsequent disease flare (248). Thus, several studies are in progress to investigate the efficacy of sequential therapy of a BAFF-inhibitor, belimumab as maintenance following remission induction with rituximab in terms of flare reduction.

2.7.8. Rituximab biosmilars

Roche's patent on the reference rituximab, MabThera for its licenced indication had expired in the European in November 2013 and the US in September 2016 (223). As a result, several manufacturers have developed biosimilars. A biosimilar is defined as a biological product that is highly similar to the reference product notwithstanding minor differences in clinically inactive components, with no clinically meaningful differences between the biological product and the reference product in terms of the safety, purity, and potency of the product. Because of the potential differences in efficacy and immunogenicity from a new manufacturing process, *in vitro* analytical studies and *in vivo* clinical trials are required to confirm clinical equivalence of the biosimilar with the reference rituximab prior to approvals (249). At the time of writing this chapter, two rituximab biosimilars; Truxima and Rixathon have gained marketing approvals in Europe and the US following RCTs that showed bio- and clinical equivalences to MabThera in RA (250, 251). The licensing of these biosimilars will further enhance rituximab cost-effectiveness profile since they are often cheaper than the originator agent.

2.7.9. Other type 1 and 2 anti-CD20 molecules

Anti-CD20 mAbs can be classified into type 1 or type 2 based on their ability to induce the reorganisation of CD20 molecules into lipid rafts upon binding (228). Type I mAbs induce a translocation of CD20 into lipid rafts and efficiently activate the classical pathway of the complement system. Examples of type 1 mAbs include rituximab, ofatumumab and ocrelizumab. In contrast, type 2 mAb such as obinutuzumab poorly activates complement but directly

induces cell death upon binding to CD20 without cross-linking by secondary antibodies. Both types of mAbs are capable of inducing ADCC in the presence of effector cells.

Ocrelizumab (OCR) is a humanised anti-CD20 mAb binding to an overlapping CD20 epitope to rituximab with increased ADCC and reduced CDC. It met its endpoints; American College of Rheumatology 20% improvement criteria (ACR20) in clinical trials in RA (252) and was investigated in SLE (253). Development in these indications was halted due to major safety concerns (254). In *post-hoc* analysis of the RA trial, OCR500 + methotrexate group demonstrated improvement in clinical response included radiographic outcome but this dose was associated with an increased incidence of serious infection events (SIEs). In contrast, OCR200 + methotrexate group did not show superior efficacy compared with existing therapies, but there were no major safety signals and therapy was well-tolerated (254). In the RCT of OCR in renal lupus, two doses of OCR were used; 400mg and 1000mg. Despite enhanced B-cell depletion being achieved in the OCR groups, the trial was halted due to increased SIE and opportunistic infections; some of which were fatal in the OCR + standard of care groups (253). The drug is still being developed in multiple sclerosis.

Ofatumumab is a fully human anti-CD20 mAb binding to a different epitope than rituximab. It is licensed for resistant CLL and with evidence for efficacy in RA (255). No study has been done in SLE. Obinutuzumab is a humanised anti-CD20, also binding a different epitope to rituximab as well as an Fc region of the molecule that is glycoengineered to enhance ADCC. It has been licensed in combination with chlorambucil as a first-line treatment for CLL and is currently investigated in an RCT of lupus nephritis in SLE.

2.8.   Safety of rituximab in SLE and other B-cell mediated diseases

The immunomodulatory properties of biological therapies including rituximab have naturally raised safety concerns prompting careful evaluation in clinical trials and intensive post-marketing surveillance. Observations from these data also provide insight into pathogenic basis of infectious diseases (8).

There are no new safety signals including the risk of infection and malignancies from the long-term data (RCTs + LTE studies up to 11 years) on use of rituximab in RA (256). With regards to SLE, although the rates of any infection event were similar between placebo and rituximab groups, interestingly, higher SIEs were observed in the placebo groups; 17% and 9.5% respectively in the EXPLORER trial (204) and 21% versus 16% respectively in the LUNAR trial (205). Data regarding SIEs from RCTs and LTE in RA, SLE and AAV are summarised in Table 2-5.

Table 2-5 Summary of safety data from RCTs and LTE studies on the use of rituximab in autoimmune rheumatic diseases

Source of Data	Disease group (No of Patients Treated with RTX)	Duration of Follow-up	Treatment Schedule	Serious Infection Event (SIE)	Opportunistic Infections	Risk Factors for Infection
Pooled data (RCT + LTE)						
8 RCTs + 2 LTE studies (257)	RA (n=3194)	11 962 PY	RTX (Either 2×1000 mg or 2×500 mg dose) 2428 (76%) were retreated with RTX	3.94/100 PY (3.26/100 PY in patients observed >5 years in RTX Group versus 3.79/100 PY in Placebo + MTX group)	Lung TB reactivation (n=2) Rate of other OIs: 0.06/100 PY (Atypical pneumonia; n=2, Candida; n=1, pharyngeal abscess; n=1, *Scedosporium* lung infection; n=1, *Pneumocystis jirovecii* pneumonia; n=1 and JC Virus; n=1) Rate of herpes zoster in RTX group (9/100 PY) versus (11.7/100 PY in Placebo + MTX Group	SIE rates were similar before and during/after low IgG, but both rates were significantly higher than in patients who never developed low IgG Baseline risk factors for development of low IgG: older, longer disease duration, lower mean CD19+count, lower mean IgG levels and had received more csDMARDs

Source of Data	Disease group (No of Patients Treated with RTX)	Duration of Follow-up	Treatment Schedule	Serious Infection Event (SIE)	Opportunistic Infections	Risk Factors for Infection
RCT						
RAVE (258)	AAV (n=99)	18 months	Single Course RTX (375mg/m^2 body surface area weekly for 4 weeks) for remission induction	Rate of SIE was similar: 7% and 12% at 6 and 18 months in RTX monotherapy Group versus 7% and 11% in CyC + AZA maintenance Group	None in RTX group but 3 x fatal infections in CyC + AZA maintenance Group (i) *Enterococcus/Escherichia coli* sepsis (ii) *Pseudomonas aeruginosa* sepsis and (iii) *Pneumocystis jirovecii* pneumonia with secondary *Staphylococcus aureus/ Escherichia coli* sepsis in a patient who was not compliant with prophylaxis	No risk factors were identified
RITUXVAS (259)	AAV (n=33)	2 years	Single Course RTX (375mg/m^2 body surface area weekly for 4 weeks) for remission induction	Rate of SIE similar: 0.25/PY in RTX monotherapy Group versus CyC + AZA maintenance Group	2 cytomegalovirus infections reported in RTX Group and none in the CyC + AZA maintenance Group	RTX-treated patients had higher VDI at baseline (median, IQR: 2 (0-3)) compared to CyC + AZA maintenance (1 (0-2))

Source of Data	Disease group (No of Patients Treated with RTX)	Duration of Follow-up	Treatment Schedule	Serious Infection Event (SIE)	Opportunistic Infections	Risk Factors for Infection
EXPLORER (204)	SLE (n=169)	78 weeks	Single course RTX (2 x 1000mg)	Higher rate of SIE in the Placebo + csDMARDs group versus RTX + csDMARDs group; 17% and 9.5% respectively	More herpes zoster infection in 16 RTX + Group (9.5%) versus 3 in Placebo + csDMARDs Group (3.4%)	None reported
LUNAR (205)	SLE (n=72)	78 weeks	Single course RTX (2 x 1000mg)	4 SIEs reported in each RTX + csDMARDs (16.6/100 PY) and Placebo +csDMARDs (19.9/100PY)	3 OIs in RTX + csDMARDs Group: colitis, histoplasmosis, and *cryptococcal* pneumonia plus fungal sepsis, respectively versus 1 OI in Placebo + csDMARDs Group: cytomegaloviral pneumonitis	None reported

AZA: azathioprine, OI: opportunistic infection, PY: patient-year, RTX: rituximab, SIE: serious infection event, TB: tuberculosis and VDI: Vasculitis Damage Index

2.8.1. Risk factors for serious infection

Incomplete and transient depletion of B-cells as well as sparing of plasma cells may be one reason for the relative safety of rituximab. However, excessive repeat cycles of rituximab may lead to progressive reduction of immunoglobulin levels and increase the risk of infection (260, 261). There is a notable absence of data pertaining to the effect of B-cell depletion and B-cell numbers with infection. Other risk factors associated with serious infection during rituximab are described below.

Low immunoglobulin levels prior to and after therapy

Data from a French registry in RA showed that low IgG level (<6 g/L) before a cycle of rituximab was associated with SIE, particularly in the 3 months following rituximab infusion; 16.2% versus 3.9% without low IgG (262). In RCTs and LTE studies in RA, low IgM and IgG levels were exclusion criteria for trial entry. In these studies, a similar increase in SIE rate was seen following rituximab both before and after the development of low IgG compared with those who never developed low IgG (257). This might attribute the SIE to other pre-existing demographic or clinical risk factors rather than the low IgG itself. Careful interpretation is needed, as the number of patients with low IgG level post-therapy was low, 3.5%. More data are needed to assess the effect of secondary low IgG with the risk of SIE after repeat cycles of rituximab.

IgM level tends to decline following therapy. In particular, IgM levels were lower in patients receiving 2x1000 mg rituximab versus those receiving 2x500 mg doses (263). In the RCTs + LTE studies in RA, 22.4% of patients developed low IgM (257). Low IgM level prior to and post-rituximab was not associated with SIE (257, 262) although this analysis might have been limited by the low incidence of SIE. The development of low IgA level post-rituximab was uncommon (1.1%) and thus, did not appear to be associated with SIE (257).

In AAV, remission induction with cyclophosphamide was associated with a decline in all immunoglobulin classes at 6 months post-therapy and remained below the baseline levels up to 36 months. Following a clinical relapse, a

single cycle of rituximab further aggravated the decline in IgM and IgG levels (264).

Concomitant glucocorticoid and csDMARDs

Glucocorticoids impair phagocyte function and suppress cell-mediated immunity, thus may increase the risk of infection. Data from registries in France and Spain showed an increased risk of SIE with concomitant glucocorticoids (262, 265).

Interestingly, the co-prescription of csDMARD appears to be safe. The initial phase II RCT in RA showed no increase in SIE rates using combination methotrexate or cyclophosphamide with rituximab (266). Data from a registry in Spain showed no significant difference in infection rate when rituximab was used either in combination with leflunomide, methotrexate or as monotherapy; rates were 6.2%, 6.6% and 7.4% respectively (267).

Age and Comorbidity

An increasing number of elderly patients with multiple comorbidities are diagnosed with ARDs in current practice. These patients are at increased risk of SIE post-rituximab (262, 265). Comorbidities investigated including chronic obstructive pulmonary disease, interstitial lung disease (ILD), renal failure, hypertension and cardiac insufficiency (262).

Rituximab-associated neutropaenia

Neutropaenia is recognised as a complication of rituximab when used in B-cell malignancies with an incidence of 3-27% (268). Data in ARDs are more limited and the optimal management of these patients has not been defined. Data from large cohort studies suggested an incidence of 2.5-3.0% in RA and increased to 20% in AAV (269-271). In most cases, neutropaenia recovered promptly but counts <0.5 x10^9/L were associated with severe infection requiring treatment with intravenous antibiotics and granulocyte-colony stimulating factor (GCSF). Current data therefore suggests retreatment with monitoring is appropriate, with additional caution needed only in severely neutropaenic patients (270). The mechanism of rituximab-associated neutropaenia remains elusive.

2.9. B-cell independent SLE manifestations

It is important to acknowledge that SLE is not exclusively a B-cell-dependent disease as evidenced by various molecules or immune pathways that are responsible for the pathogenesis and variability in the success of various B-cell targeted therapies in SLE as described above.

In a paper of which I am the co-author, we previously showed that the clinical response to rituximab in cutaneous manifestations of SLE relied on the subtype. Of 8 patients with CCLE, none of them responded. Additionally, new CCLE lesions were observed during B-cell depletion, suggesting that the initiation and activity of these lesions were not B-cell dependent (17).

2.9.1. Discoid lupus erythematosus and the unmet needs

DLE is a form of CCLE. It usually occurs on sun-exposed skin such as the face, ears and scalp. The prevalence has been reported ranging from 12.5 per 100 000 in England to 50.8 per 100 000 in certain groups in the US (272). DLE can occur as an independent entity or form a part of SLE.

Without effective treatment, DLE leads to permanent destructive scarring on the face and scalp and irreversible, severe hair loss. DLE can severely impair quality of life through impact on body image, leading to physical and psychological disability (273). Thus, there is a significant unmet need for effective therapy to improve the quality of life of patients with DLE.

2.9.2. Challenges and opportunities in the management of DLE

A significant proportion of patients with DLE are resistant to conventional therapies. There is no guideline pertaining to management of cases that are refractory to steroid as well as after the first line agent, anti-malarials. The latter are only effective in 50% of the patients at 6 months (190). For patients who have exhausted conventional therapies, most clinicians would use high dose systemic corticosteroids, experimental therapies or enrolment in clinical trials as available.

One attractive opportunity is to develop a targeted approach based on the immunopathogenesis of DLE. Skin disease is particularly heterogeneous. Although immune complex deposition is a common feature, non B-cell

mechanisms are also responsible through activation and apoptosis of keratinocytes and production of inflammatory cytokines and chemokines such as TNF (70, 274).

2.10. Non B-cell targeted therapies

Given the role of pro-inflammatory cytokines in both the innate and adaptive immunopathogenesis of lupus, a number of anti-cytokine therapies have been the focus of research to more selectively and efficaciously manage patients with SLE.

2.10.1. Interferon-blocking therapy

IFN-I is a key mediator that links the sensing of classic lupus-associated nuclear antigens with an adaptive immune response. Its importance is indicated by numerous genetic associations as well as the high spontaneous expression of type I IFN-induced genes in circulating mononuclear cells (MNCs) and peripheral tissues in patients with SLE. High expression levels of these genes are associated with high disease activity in SLE (275).

IFN-I is therefore a logical therapeutic target that may have certain advantages over B- and T-cell targets. Rontalizumab is an anti-IFN-α mAb. Although it did not demonstrate efficacy in a phase II RCT (276), there was evidence that baseline IFN activity might help to identify patients who would likely to respond. In this study, patients were screened at baseline to characterise IFN signature-positive vs -negative patients using gene expression in a 3:1 ratio. Patients with a positive signature had higher biological indices of disease activity compared with signature-negative patients, although clinical indices of disease activity were similar in both groups. Surprisingly, higher SRI response rates as well as reduced number of flares were observed in the rontalizumab group in the IFN signature-negative patients (versus placebo). None of these differences were observed in patients with a positive IFN signature at baseline. This intriguing evidence might suggest that either the dose of rontalizumab was too low to neutralise the IFN in patients with high IFN scores or a problem with variability in

detection of type 1 IFN-inducible genes with the simple 3 gene whole blood signature that was used.

Results for Sifalimumab, another anti-IFN-α mAb was positive according to the phase II RCT of moderately to severe SLE (excluding severe lupus nephritis and neuropsychiatric). The primary endpoint, the SRI-4 at week 52 was met with clinically important improvement in skin and joint manifestations, although the treatment effects were modest. The Cochran-Armitage trend test of all treatment groups showed that the number of patients achieving the primary endpoint was greater for sifalimumab versus placebo (p=0.053). Pairwise comparisons demonstrated that these effects were consistent for each sifalimumab dosage (200 mg monthly: 58.3%, p=0.057; 600 mg monthly: 56.5%, p=0.094; 1200 mg monthly: 59.8%, p=0.031) compared with placebo (45.4%) with improvement reaching a peak at week 24, after which there was a plateau in the effect. Although the 1200 mg dosage provided the most consistent results pertaining to primary and secondary endpoints, no clear sifalimumab dosage effect was observed in this study (209).

Of numerous anti-IFN therapies currently in development, the most promising agent is anifrolumab, an anti-IFN receptor mAb. Cell signalling by all type I IFNs including IFN-α, IFN-beta (IFN-β), IFN-epsilon (IFN-ε), IFN-kappa (IFN-κ) and IFN-omega (IFN-ω) is mediated by the IFN-I receptor (IFNAR). In a phase II RCT of adults with moderate-to-severe SLE (severe lupus nephritis and neuropsychiatric excluded), patients were randomised to receive intravenous anifrolumab (300 mg or 1000 mg) or placebo, in addition to standard therapy for 48 weeks. Randomisation was stratified by SLEDAI-2K (<10 or ≥10), oral corticosteroid dosage (<10 or ≥10 mg/day) and IFN-I gene signature test status (high or low) based on a 4-gene expression assay. The primary endpoint was the SRI-4 rate at week 24 as well as sustained reduction of oral corticosteroids (<10 mg/day) from week 12 through 24. In this study, the primary endpoint was met in the anifrolumab (34.3% of 99 patients for 300 mg and 28.8% of 104 patients for 1000 mg) than the placebo groups (17.6% of 102 patients); p=0.014 for 300 mg and p=0.063 for 1000 mg versus placebo respectively. Greater effect sizes were reported in patients with a high IFN signature at baseline (13.2% in placebo-treated patients versus 36.0% [p= 0.004] and 28.2% [p = 0.029]) in patients treated with anifrolumab 300 mg

and 1000 mg respectively. In terms of safety, herpes zoster was more frequent in the anifrolumab-treated patients (5.1% and 9.5% with anifrolumab 300 mg and 1000 mg respectively versus 2% in placebo), as were cases reported for influenza, consistent with the physiological role of IFN in viral immunity (210). The greater efficacy and broader impact demonstrated by anifrolumab were likely due to the result of achieving greater suppression of the IFN-I pathway by blocking the IFNAR. Phase III RCTs of anifrolumab are currently undergoing and their results are expected by the end of 2018.

2.10.2. Interleukin-12/23 blocking therapy

The interleukin 12/23 (IL-12/23) pathway has been implicated in the pathogenesis of SLE (277). In a phase 2 RCT of ustekinumab, a fully human IgG1κ mAb to interleukin IL-12/23 in autoantibody positive SLE, the primary endpoint, the SRI-4 at 26 weeks was met in the ustekinumab versus placebo groups; 60% and 31% respectively; p=0.0056, with a treatment effect favouring ustekinumab from the beginning of week 12. Most secondary endpoints were met apart from the BICLA response, although among BICLA non-responders, a greater proportion of ustekinumab group had no BILAG worsening versus placebo. Of particular interest, more patients with improvement of >50% in CLASI in the ustekinumab group versus placebo; 59% vs 25% was reported (212). Safety events were consistent with the ustekinumab safety profile in other studied indications such as psoriasis and psoriatic arthritis.

2.10.3. Janus kinase-signal transducer and activator of transcription (JAK-STAT) blockade therapy

Recent advance in the JAK-signal transducer and activator of transcription (STAT) signalling pathway reveals aberrant STAT signalling in SLE. There are four JAKs; JAK1, JAK2, JAK3 and TYK2. Each involves in the signalling cascade of various cytokines. A theoretical advantage of modulating the JAK-STAT pathway in SLE is the inhibition of pathogenic cytokines, hormones and growth factors regulating the key cellular processes such as survival, proliferation, and differentiation.

Baricitinib, an oral selective JAK1 and JAK2 inhibitor was investigated in a phase II RCT of non-renal SLE. Patients had to have positive ANA and clinical SLEDAI-2K≥4 including arthritis or rash. The primary endpoint was resolution of SLEDAI-defined arthritis or rash. At week 24, a significantly greater proportion of patients in the baricitinib 4mg group achieved resolution of SLEDAI-2K arthritis or rash compared to placebo; 67% versus 53%; as well as SRI-4 response rate; 64% versus 48% respectively; all $p<0.05$. No significant difference was observed in the baricitinib 2mg dose. The rates of discontinuation due to AE were higher in both baricitinib groups versus placebo. No other major safety signals apart from one case of deep venous thrombosis (in a patient with risk factors) reported in baricitinib 4mg group (211).

2.10.4. Interleukin-6 blocking therapy

Interleukin-6 (IL-6) is a highly pleiotropic cytokine that is overexpressed in SLE. Targeting IL-6 signalling may offer a novel therapeutic approach for SLE as supported by promising clinical and serological responses observed with the soluble IL-6 receptor inhibitor, tocilizumab in a small, open-label phase I study (278).

Sirukumab, anti-IL-6 mAb was evaluated in a phase II RCT of patients with either class III or IV lupus nephritis, refractory to azathioprine or MMF. The primary endpoint, the change in proteinuria at week 24 was not met in the sirukumab-treated group (279). However, 20% of the sirukumab-treated group achieved meaningful reduction in proteinuria i.e. >50% from the baseline versus 0% in the placebo group. Nearly half of the sirukumab-treated group had ≥ 1 serious adverse events.

2.11. Tumour necrosis factor-blockade therapy

2.11.1. Therapeutic indications for etanercept

Etanercept in combination with methotrexate is indicated for the treatment of moderate to severe active RA, psoriatic arthritis (PsA). psoriasis and juvenile idiopathic arthritis (JIA), in patients with inadequate response to csDMARDs including methotrexate. It is also licensed for ankylosing spondylitis (AS) and non-radiographic axial spondyloarthropathy, in those with inadequate response to non-steroidal anti-inflammatory drugs (NSAIDs) (280).

Despite the remarkable success of TNF-blockade therapies in the treatment of various ARDs above, there has been no completed RCT assessing efficacy of these agents in SLE. Two RCTs involving TNF- blockers in patients with lupus nephritis (one study each using infliximab and etanercept) were both aborted prior to study completion (281). The reason for this was because of a potential concern in the induction of pathogenic autoantibodies that might render SLE worse, thus both trials found difficulty to recruit patient. Approximately 0.5-1.0% of patients treated with systemic TNF-blockers developed high affinity IgG autoantibodies to anti-dsDNA, which were associated with mild lupus-like syndromes (282, 283). Nevertheless, data from open label use have demonstrated the efficacy and safety in both renal and non-renal manifestations (284, 285).

2.11.2. Posology and Pharmacokinetic

Treatment with etanercept should be initiated and supervised by specialist physicians experienced in the diagnosis and treatment of its licensed indications above. Patients should be given the Etanercept Patient Alert Card (280).

Pfizer's patent on the reference etanercept, Enbrel for its licensed indication had expired in the Europe in October 2012 but, in the US, a second patent, granting exclusivity for another 16 years i.e. 2028, has been granted (280). At the time of writing this chapter, two etanercept biosimilars; Benepali and Erelzi have gained marketing approvals in Europe.

Enbrel is available in strengths of 10, 25 and 50 mg.

The recommended dose for the indications above (apart from JIA) are either 25 mg etanercept, administered twice weekly or 50 mg administered once weekly. For JIA, the recommended dose is 0.4 mg/kg (up to a maximum of 25 mg per dose), given twice weekly as a subcutaneous injection with an interval of 3-4 days between doses or 0.8 mg/kg (up to a maximum of 50 mg per dose) given once weekly (280).

In terms of drug elimination, etanercept is cleared slowly from the body. The half-life is long, approximately 70 hours as well as slower clearance rate in patients RA compared to healthy volunteers (286, 287). The pharmacokinetics of etanercept are comparable between the different diseases as well as gender (288).

2.11.3.   Pharmacology of etanercept

TNF is a dominant cytokine produced by lymphocytes and macrophages. It mediates the immune response by attracting additional white blood cells to the sites of inflammation, as well as through additional molecular mechanisms which initiate and amplify inflammation (289).

Etanercept is a competitive inhibitor of TNF, binding to its cell surface receptors. TNF-α and TNF-β (lymphotoxin) are pro-inflammatory cytokines that bind to two distinct cell surface receptors; the 55-kilodalton (p55) and 75-kilodalton (p75) TNF receptors. These receptors exist naturally in membrane-bound and soluble forms; with the latter form is thought to regulate TNF biological activity. TNF and lymphotoxin exist as homotrimers. Their biological activity dependent on cross-linking of cell surface TNF receptors (280).

Etanercept is developed by recombinant DNA technology in Chinese hamster ovary mammalian cell expression system. Etanercept is made from the combination of two naturally occurring soluble human 75-kilodalton TNF receptors linked to an Fc portion of an IgG1 (280). Etanercept is a dimeric molecule, and this dimeric structure exerts a higher affinity for TNF and are considerably more potent competitive inhibitors of TNF binding to its cellular receptors than other monomeric receptors. Moreover, its composition of an immunoglobulin Fc region as a fusion element in the construction of a dimeric receptor imparts a longer serum half-life (290).

2.11.4. Mode of action of etanercept

Etanercept competitively inhibits the binding of both TNF and lymphotoxin to cell surface TNF receptors, rendering TNF biologically inactive (291).

Etanercept also indirectly modulates several downstream molecules that are regulated by TNF, such as the expression of adhesion molecules E-selectin and to a lesser extent intercellular adhesion molecule 1 (ICAM-1), the production of interleukin-6 (IL-6), matrix metalloproteinase 3 (MMP-3) and IL-1 (292).

2.11.5. Immunogenecity of etanercept

The currently available TNF-inhibitors have different molecular structures, dosing regimens, routes of administration, pharmacokinetic properties and immunogenicity. Etanercept is a TNF receptor–Fcγ1 fusion protein (as described in section 2.11.3). Infliximab is a chimeric mAb with a murine variable region fused to a human Fcγ1 Ig. Adalimumab and golimumab are fully human mAbs while certolizumab is a humanized Fab' fragment bound to polyethylene glycol molecules (293). The quantification and comparison of the immunogenicity of these TNF-inhibitors are largely dependent on the assay used to detect the anti-drug antibodies (ADAs). However, some general inferences can be drawn concerning their immunogenicity.

Data from RCTs showed that etanercept had the lowest rate of ADA (range 0-3%) (294, 295) compared to infliximab (range 7-53%) (296-298) and adalimumab (range 1-31%) (299, 300). Real-world data also concurred with the rates of ADA in etanercept, adalimumab and infliximab-treated patients were 0%, 17% and 31% respectively (301). Infliximab is the most immunogenic TNF-inhibitor, particularly when it is used without concomitant methotrexate (302, 303). ADAs can bind to the idiotope (e.g. the antigen-binding region) of the fully human mAb such as adalimumab and golimumab and the chimeric mAb like infliximab. As a receptor construct, etanercept does not have an idiotype, which may explain the reason for reduced immunogenicity with therapy. The clinical implication of ADAs remains unclear, although ADAs are known to contribute to secondary drug failure (293).

2.12. The unmet needs in SLE

The management of SLE is challenging due to the heterogeneity in pathogenesis, clinical features and response to therapy. Despite treatment, SLE results in work disability and job loss (304, 305), reduced quality of life (306) and increased mortality with a Standardised Mortality Ratio of 2-3 (221, 307). In addition, severe flare as a result of uncontrolled inflammation can increase the annual direct medical cost by two-folds to over £4500 per patient in the UK (3).

Thus, there is a significant unmet need for therapies of proven efficacy and safety. Given the heterogeneity in immunopathogenesis, it may be that there is no one-size-fits-all therapy for SLE. Mechanistic studies concerning stratification and personalisation of therapy to individual patient and disease manifestations are currently limited. Lastly, in line with the principle of treat-to-target in SLE (308), prevention of damage accrual should be a major therapeutic goal, which can be achieved through early recognition and intervention (309-311).

2.13. Summary

This review has described the importance of the interplay between innate and adaptive immunity in the pathogenesis of SLE. Immune dysregulation can occur several years before clinical onset. It appears that a 'second hit' is needed whether this may be environmental, hormonal or epigenetics effect for progression from benign autoreactivity to autoimmunity to occur. Currently, this factor is unknown. Should this and other potential biomarkers be identified, then early intervention can be employed to prevent from disease progression and reversible damage.

Assessment of disease activity in SLE can be difficult owing to concurrent infection and multiple comorbidities often present in SLE patients. Of the currently available disease activity indexes, the BILAG requires adequate formal training and may be complex for the inexperienced clinicians while the SLEDAI may fail to capture partial response to therapy. Thus, the development of composite indices such as SRI and BICLA is welcomed with

interest. However, further work is needed to define a clinically meaningful response.

This review also showed that results from RCTs in SLE demonstrated variable response rates ranging from 30-60%. Translating findings from bench to bedside has been problematic with many of the promising targeted therapies failing in clinical trials due to inefficacy, problem with trial design and/or safety issues. Belimumab is the only biologic licensed for autoantibody positive SLE but choosing the right patient for the therapy remains problematic. Better indication for use including the clinical phenotype that will respond to therapy may be identified from studies currently planned.

Although RCTs of rituximab in SLE were negative, their methodology has been disputed and NHS England has agreed to commission rituximab based on strong open label evidence. Mechanistic studies to delineate predictors of B-cell depletion, rituximab resistance and stratification of therapy to lupus manifestations are needed but currently limited. With regards to safety, it is worth noting that another anti-CD20 mAb agent, ocrelizumab has failed in the trials due to safety issues. The effect of B-cell depletion and repeat cycles of treatment on immunoglobulin levels and infection risk is therefore of paramount importance.

2.14. Key messages

i. A period of ANA positivity and other immune dysregulation precedes clinically overt disease, thus provides a window of opportunity for early intervention.

ii. The development of biologics targeting B-cells and non B-cell blockade represents a major advancement in SLE therapy, however many of these agents have failed in clinical trials due to inefficacy, problem with trial design and/or safety issues.

iii. Although trials in rituximab have failed to meet their primary endpoints, their methodology has been disputed and rituximab is still widely used based on strong open label evidence and is commissioned by NHS England.

iv.   Further studies to identify predictors of response to rituximab, mechanism of resistance and stratification of therapy to lupus manifestations are needed and may help to optimise its use in clinical practice.

Chapter 3. PRediction to allow Early interVENTion in At-Risk of autoimmune connective tissue disease (PREVENT-CTD)

3.1. Introduction

AI-CTD include SLE, primary Sjogren's Syndrome (pSS), systemic sclerosis, inflammatory myopathies, mixed and undifferentiated CTDs. A hallmark of their pathogenesis is the loss of self-tolerance leading to autoreactivity and production of antibodies against numerous self-nuclear antigens. ANA can be detected in bloods up to 10 years before clinical features, representing a phase of subclinical autoimmunity (78). However, detecting ANA alone will not be sufficient to predict progression to disease. ANA is present in up to 25% of general population, of whom less than 1% develop clinical autoimmunity (167, 312). Individuals with positive ANA therefore constitute At-Risk population of whom a minority will progress to AI-CTD (169, 313). The factors that dictate whether this autoreactivity develops into autoimmune disease are unknown. But if these were understood and predictable, then early and effective intervention could be employed, preventing the severe disease and heavy glucocorticoid use for remission induction of a newly diagnosed AI-CTD. Indeed, improved clinical outcome of patients with lupus nephritis, as diagnosed in the 1990s compared to 1980s, was attributed to early diagnosis and treatment (311) while failure to achieve lupus low disease activity state (LLDAS) six months after diagnosis was associated with early damage accrual in patients with SLE (309).

Plausible biomarkers have not been investigated prospectively in At-Risk individuals. Variants in IFN-I pathway are prominent in the genetic susceptibility to AI-CTDs (as described in section 2.2.1) and therefore a focus for investigation (151, 314, 315). However, their role in disease initiation is unclear at present. IFN activity is difficult to measure but usually quantified using expression of interferon-stimulated genes (ISGs). Interpretation of ISG expression is complex with multiple IFN subtypes produced by different

tissues and cell types, as well as a transcriptional response in all nucleated cells with variation between cell types. Methods of measuring IFN-I activity are through indirect measurement of the expression of ISGs; either by assigning the patients into "High versus Low IFN signature" group (276, 316) or by combining level of protein expression of a set of ISGs of interest (317-319). However, the former poorly differentiates IFN-I activity between individuals as the outcome is categorical while the latter may be affected by the weighting and selection of the genes, as some of them may have purely anti-viral properties and not necessarily pathogenic. In a paper for which I am the co-author, we recently described two continuous ISG expression scores (IFN-Score-A and IFN-Score-B) that in combination better identified clinically meaningful differences in IFN status between, and within ARDs and these scores were associated with certain features of SLE manifestations (320).

In other ARDs such as RA, early evidence of progression to disease may be found at a target tissue level (321, 322). The tissues most commonly affected in AI-CTDs are skin and the joints. In a systematic literature review of which I am a co-author, we show that musculoskeletal ultrasound (MSK-US) can detect sub-clinical synovitis in SLE (194). However, this has not been assessed in At-Risk individuals. In skin, locally produced cytokines, which contribute to affected tissue injury and inflammation have been described in SLE. Previous studies comparing keratinocytes or skin biopsies that were isolated from cutaneous lupus patients and HCs found marked differences in interleukin-18 receptor (IL-18R) responsiveness (323), IFN-λ expression (324), as well as a role of IFN-κ in initiating a feed-forward loop which promoted exaggerated ISG activation in cutaneous lupus (325). IFN-I status in the skin has not been reported in At-Risk individuals.

A wide range of targets for therapy are currently under investigation in established SLE (see section 2.4). However, there are no data on which of these are relevant in early disease.

3.1.1. Hypotheses

i. Risk of progression from At-Risk to SLE can be predicted using clinical, serological, histological or imaging parameters at presentation.

ii. Immunological assessment of At-Risk individuals will provide a basis on the appropriate targeted therapy for disease prevention in a high risk population.

3.1.2. Objectives

i. To evaluate clinical, blood and tissue interferon and imaging biomarkers of progression from At-Risk to AI-CTD

ii. To assess the relationships between blood interferon biomarkers and autoantibodies and routine immunological markers

iii. To define biomarkers thresholds of progression to AI-CTD in At-Risk individuals

3.2. Methods

3.2.1. Candidate's roles in this project

In this work, the initial concept and design of the study were set by myself, Dr Psarras, Dr El-Sherbiny, Prof Emery and Dr Vital. I set-up and carried out the weekly pre-CTD clinic in Leeds under the supervision of Dr Vital, recruited and consented all 135 participants to the study, designed the proforma for data collection, performed a significant proportion of the clinical and 50% of the imaging assessments (the other half was done by Dr Zayat while I underwent training to perform MSK-US), undertaken all skin biopsy from consenting participants and was responsible to answer the enquiry line when contacted by the participants. The research blood and skin samples were processed and stored by members of my group; namely Dr Antonios Psarras, Dr Alase, Dr Shalbaf, Mrs Corscadden, Mrs Mbara and Ms Wigston. I organised and entered data onto the study database. In terms of statistical analysis, Dr Hensor initially performed the single imputation of the missing ISG data as well as the factor analysis. I then performed the descriptive statistics analyses, multiple imputation of missing data and multivariable analyses of prognostic predictors of progression. My results were then checked by Dr Hensor. Finally,

I led the writing of the manuscript while other co-authors revised the draft critically for important intellectual content and final approval of the manuscript prior to submission to journal for publication.

3.2.2. Design and Patient

A prospective observational study was undertaken in consecutive individuals who were referred from primary care to Leeds Teaching Hospitals NHS Trust for a suspected AI-CTD between November 2014 and May 2017.

Inclusion criteria were: (i) ANA-positive of at least 1:80 titre on indirect immunofluorescence and using multiplex immunoassays (excluding those with scleroderma [Centromere, Scl-70] or myositis-specific [PL-12, OJ, PL-7, Mi-2, Ku, Jo-1, PM-Scl75, PM-Scl100, SRP and EJ] antibodies only); (ii) ≤1 clinical criterion based on 2012 SLICC (32) and not meeting classification criteria for other AI-CTDs (326-328) or RA (329); (iii) symptom duration <12 months; (iv) glucocorticoid, antimalarial and immunosuppressive treatment-naïve. Forty-nine HCs and 114 patients with SLE who met the SLICC criteria were used as negative and positive controls.

3.2.3. Ethical approval

All individuals provided informed written consent and this research was undertaken in compliance with the Declaration of Helsinki. The patients' blood samples used for this study were collected under ethical approval, REC 10/H1306/88, National Research Ethics Committee Yorkshire and Humber–Leeds East, and HCs' blood samples were collected under the study number 04/Q1206/107. All experiments were performed in accordance with the relevant guidelines and regulations. The University of Leeds was contracted with administrative sponsorship.

3.2.4. Assessment schedule and outcome

Comprehensive assessments including clinical, laboratory, imaging, bloods and skin biomarkers were performed at baseline, 12 months, annually for 3 years. Participants were given a helpline number to contact for an additional flare visit to be organised if they had new or worsening inflammatory symptoms.

Progression was defined by meeting the 2012 SLICC classification criteria for SLE (32), 2016 ACR/EULAR classification criteria for pSS (326) or other relevant classification criteria for AI-CTD (327, 328) at 12 months as assessed by rheumatologists.

3.2.5. Clinical and laboratory assessment

Age, gender, ethnicity, history of first or second degree relative(s) with ARDs, smoking history, SLICC criteria for SLE (32), signs or glandular symptoms criteria for pSS (326), patient and physician global health assessment using 100mm VAS were recorded.

ANA was tested using indirect immunofluorescence as well as a panel of nuclear autoantibodies including anti-dsDNA, extractable nuclear antigens (ENA, including Ro52, Ro60, La, Sm, Chromatin, RNP, Sm/RNP and Ribosomal P) and anti-phospholipid antibodies (Cardiolipin and B2-Glycoprotein IgGs) using Bioplex 2200 Immunoassay. Lupus anti-coagulant tests including activated prolonged thromboplastin time (APTT), APTT-synthetic peptide (APTT-SP) (with correction) and dilute Russell's viper venom test (dRVVT) (with correction) were deemed positive if persistent when repeated at 12 weeks. Full blood count was processed at a single accredited diagnostic laboratory. Complement levels (C3 and C4) were measured by nephelometry.

3.2.6. Musculoskeletal ultrasound

MSK-US examination of wrists, metacarpophalangeal and proximal interphalangeal joints were performed by two rheumatologists, using General Electric S7 machine with a 6–15 MHz transducer. Outcome Measures in RA Clinical Trials (OMERACT) criteria (330) were used to define synovitis i.e. the presence of grey-scale (GS) ≥grade 2 and/or power Doppler (PD) ≥grade 1.

3.2.7. Blood IFN Scores

A two-score system of ISGs as previously described (320), was calculated without the knowledge of participants' clinical status.

Gene probe selection and gene expression

Ten genes were selected from each IFN-annotated module (M1.2, M3.4, M5.12) of a previous microarray study as reported by Chiche L et al. 2014 (314), with addition of other common ISGs i.e. IFI27 and IFI6. Thus a total of 31 ISGs were evaluated. Peptidylprolyl isomerase A (cyclophilin A) (PPIA) was used as a reference gene (confirmed not responsive to IFN-I).

Peripheral blood mononuclear cells (PBMCs) were separated using density gradient method (LymphoprepTM, Alere Technologies, Norway) from ethylenediaminetetraacetic acid (EDTA)-anticoagulated blood. Total ribonucleic acid (RNA) purification kit (Norgen Biotek, Canada) was used to extract RNA from PBMCs and sorted cell subsets. To obtain the complementary DNA (cDNA) synthesis from total RNA acquired, Fluidigm® Reverse Transcription Master Mix buffer was used including a mixture of random primers and oligo dT for priming. TaqMan assays (Applied Biosystems, Invitrogen) were used to perform the quantitative real-time reverse transcriptase-polymerase chain reaction (qRT-PCR) for the selected 31 ISGs. These assays were performed using the BioMark™ HD System with appropriate cycling protocols for the 96.96 chip. Data were normalised using PPIA as the reference gene to calculate ΔCt.

Factor analysis

Factor analysis (FA) was performed to reduce the 31 ISGs into a smaller number of factors (331). Prior to FA, undetected ΔCt values were singly imputed using the R package nondetects. The Kaiser-Meyer-Olkin measure was used to verify the sampling adequacy of the analysis. Principal factor extraction (without rotation) was used to identify the optimum number of factors, which was initially determined according to a parallel analysis (Monte Carlo simulation using 1000 replications). This indicated the maximum number of factors present. However, if a smaller number of factors were required to explain 80% of the variance and resulted in lower levels of cross-loading (genes loaded by 2 or more factors at >0.4), a simpler structure was selected. Having identified the number of factors present, oblique (promax; kappa=4) rotation was used to obtain the final factor solution.

In this present study, to calculate factor scores for each patient, median gene expression was calculated for genes loaded at ≥0.4 by each factor, provided

they did not cross-load onto more than one factor. The advantage of this approach was that it reflected the variability of the data and respected the within-patient ordinal scaling of ΔCt values.

As previously described (320), two factors explained 84% of the variance with limited cross-loading among the ISGs. Table 3-1 shows the ISGs that contributed to each factor; we called these IFN-Score-A (comprises 12 co-clustered genes) and IFN-Score-B (comprises 14 co-clustered genes).

Table 3-1 ISGs that constitute IFN-Score-A and IFN-Score-B

Genes	Modules from previous study using microarray	Rotated Factor Loading	
		Factor 1: IFN-Score-A	Factor 2: IFN-Score-B
ISG15	1.2	**0.96***	
IFI44	1.2	**0.80***	
IFI27	n/a	**0.77***	
CXCL10	1.2	**0.71***	
RSAD2	1.2	**0.70***	
IFIT1	1.2	**0.67***	
IFI44L	1.2	**0.66***	
CCL8	3.4	**0.58***	
XAF1	1.2	**0.54***	
IFI6	n/a	0.51	0.45
GBP1	3.4	**0.46***	
IRF7	3.4	**0.46***	
CEACAM1	3.4	**0.45***	
HERC5	1.2	0.43	0.59
EIF2AK2	3.4	0.42	0.64
MX1	1.2	0.40	0.56
LAMP3	1.2		**0.40***
IFIH1	3.4		**0.45***
PHF11	5.12		**0.58***
SERPING1	1.2		**0.60***
IFI16	5.12		**0.64***
BST2	5.12		**0.74***
SP100	5.12		**0.74***
NT5C3B	5.12		**0.80***
SOCS1	3.4		**0.84***
TRIM38	5.12		**0.87***
UNC93B1	5.12		**0.88***
UBE2L6	3.4		**0.89***
STAT1	3.4		**0.94***
TAP1	5.12		**0.98***
CASP1	5.12	<0.40	<0.40

* Indicates genes that were included in the factor scores

3.2.8. Skin biopsy

One 4 mm biopsy was obtained from non-lesional, non-sun-exposed areas (upper back or upper arms) of At-Risk individuals (n=10) and HCs (n=6), and from active lesions of SLE patients (n=10). Biopsies were snap frozen in optimum cutting temperature compound and sectioned at a thickness of 5 μm ensuring no remaining material contaminating subsequent RNA extraction/RT procedures. Total RNA were extracted using RNeasy mini kit (Qiagen, Manchester, UK). The RNA quantity was measured and assessed for purity and concentration using NanoDrop spectrophotometer, ND-1000, Heathfield, UK. Gene expression analysis and calculation of factor scores were conducted as for PBMCs.

3.2.9. Missing data

For 13 At-Risk individuals, gene expression data were missing at random due to samples not being processed on the day. For comparisons with HC and SLE groups, only At-Risk individuals with complete data were presented. For prediction of progression, multiple imputation by chained equations was used to create 20 complete datasets, results of which were combined according to Rubin's rules (332).

3.2.10.   Statistical analyses

Associations between categorical variables were tested by Fisher's exact for independent samples and Stuart-Maxwell tests for paired samples. Continuous variables were compared either using Student's T-tests or analysis of variance (ANOVA) followed by pairwise Tukey tests. For other associations, Kendall's tau-b correlation was used if ties were present, otherwise using Pearson's correlation.

Receiver operator characteristic (ROC) curves were used to assess predictive strength and identify optimal thresholds for predicting progression to AI-CTD.

To assess baseline predictors of progression to AI-CTD at 12 months, all (imputed) putative variables were first evaluated using univariable analysis at the 10% level of significance. Only variables with p-value of <0.1 were

included in the multivariable analysis, using backward-elimination as well as penalised logistic regression by Lasso method (333). Leave-one-out cross validation (R package cv.glmnet) (334) identified the largest penalty coefficient lambda within 1 standard error of the value that minimised deviance in each imputed dataset; average coefficients from the best models were calculated. All analyses of IFN Scores were conducted using ΔCt scaling; results were then converted to relative expression ($2^{-\Delta Ct}$) or fold difference (FD) ($2^{-\Delta \Delta Ct}$).

Statistical analyses were performed using Stata v.13.1 (StataCorp College Station, Texas, USA), R version 3.3.3 (335) and GraphPad Prism v.7.03 (GraphPad, La Jolla, CA, USA) for Windows.

3.3. Results

3.3.1. Patient characteristics

A total of 135 At-Risk individuals were recruited in this study. Of these, 118 had at least 12 months follow-up and were analysed. Baseline characteristics are described in Table 3-2. The flowchart of participants is presented in Figure 3-1.

Table 3-2 Baseline characteristics of the 188 At-Risk of AI-CTD individuals

Characteristic	Values
Age, median (range) years	48 (20 - 84)
No. female patient (%)	104 (88)
Ethnicity, N (%)	
Caucasian	85 (72)
Indian/South Asian	20 (17)
African/Caribbean	12 (10)
Chinese	1 (1)
Positive ANA, N (%)	118 (100)
No. of positive ANA specificities, median (range)	1 (1 – 4)
Autoantibody positive specificities, N (%)	
anti-dsDNA	42 (36)
10 – 20 IU/mL	15 (13)
21 – 50 IU/mL	18 (15)
> 50 IU/mL	9 (8)

Characteristic	Values
anti-Ro	50 (42)
< 8 AI	24 (20)
≥ 8 AI	26 (22)
anti-La	9 (8)
anti-Smith	5 (4)
anti-Chromatin	17 (14)
anti-RNP	2 (2)
anti-Ribosomal P	0 (0)
anti-Sm/RNP	16 (14)
anti-Cardiolipin/anti-B2-Glycoprotein	5 (4)
Positive Lupus Anti-Coagulant, N (%)	4 (3)
Concurrent positive RF, N (%)	11 (9)
Low titre (< 50 iU/mL), N (%)	5 (4)
High titre (≥ 50 iU/mL), N (%)	6 (5)
*Concurrent positive anti-CCP antibody, N (%)	3 (3)
Low complement levels (C3 or C4), N (%)	8 (7)
No. of clinical criteria, N (%)	
0	20 (17)
1	98 (83)
Clinical criteria present, N (%)	
**Acute or Sub-acute cutaneous lupus erythematosus	27 (24)
Chronic cutaneous lupus erythematosus	1 (1)
Oral or nasal ulcers	4 (3)
Non-scarring alopecia	5 (4)
Arthritis	43 (36)
Serositis	1 (1)
Renal	0 (0)
Neurologic	0 (0)
Haemolytic anaemia	0 (0)
Leucopaenia or lymphopaenia	12 (10)
Thrombocytopenia	5 (4)
Glandular signs	0 (0)
***Family history of ARD, N (%)	43 (36)
Ever smoked, N (%)	45 (38)

* All patients had low anti-CCP antibody titre (<50 U/mL); ** Only 1 patient had sub-acute cutaneous lupus erythematosus lesion; *** First or second degree relative with autoimmune rheumatic disease. ANA: anti-nuclear antibody; ARD: autoimmune rheumatic disease; CCP: cyclic citrullinated peptide; RNP: ribonucleic peptide; Sm: smith
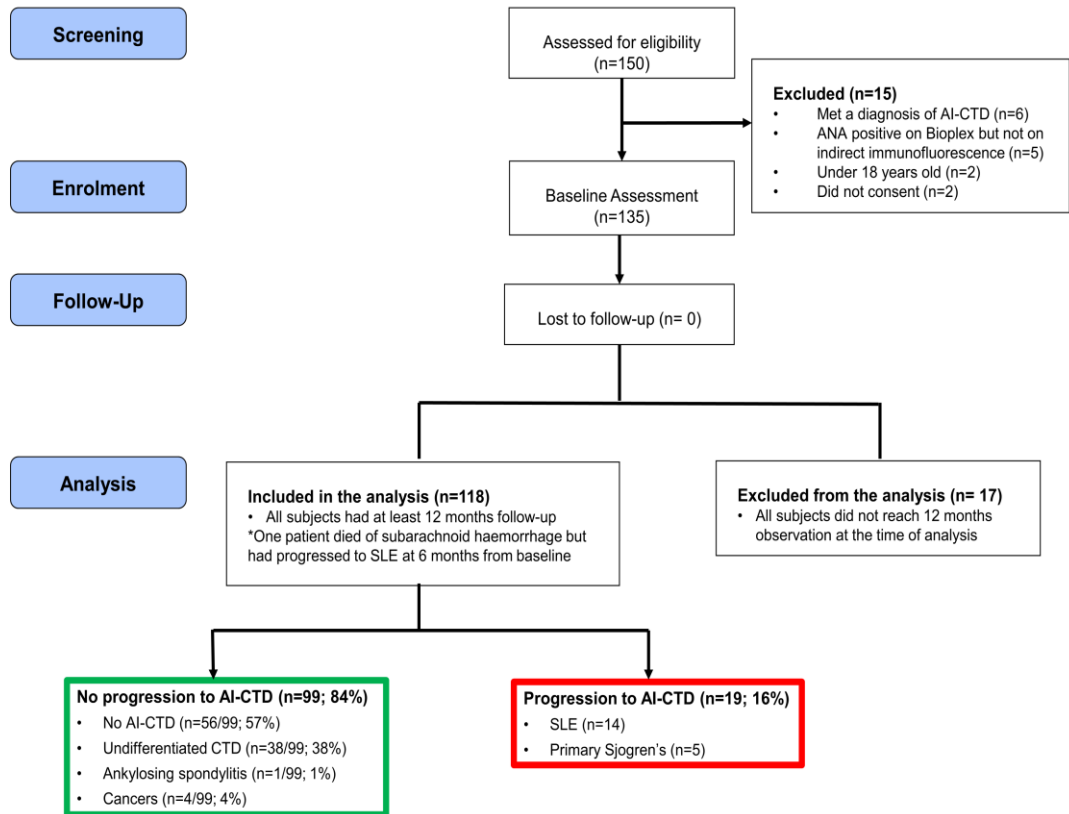
Figure 3-1 Flowchart of the participants in the PREVENT-CTD study in Leeds

Adapted with permission from the BMJ publishing group. Md Yusof MY et al. Prediction of autoimmune connective tissue disease in an at-risk cohort: prognostic value of a novel two-score system for interferon status. *Ann Rheum Dis.* 2018 Oct;77(10):1432-1439

3.3.2. Clinical outcomes at 12 months

At 12 months, 19/118 (16%) At-Risk individuals progressed to meeting criteria for an AI-CTD; namely SLE (n=14; 74%) and pSS (n=5; 26%) (336). For those who progressed, all had 1 clinical criterion at baseline. The number of clinical SLE criteria increased to 2 in 4/19 (21%), 3 in 9/19 (47%) and 4 in 6/19 (32%) (Stuart-Maxwell Chi-square=20.0, p<0.001) at 12 months. These details are presented in Table 3-3 and illustrated in Figure 3-2. Two patients developed internal organ involvement; pleural effusion and class III lupus nephritis.

On the other hand, 19/99 (19%) of the non-progressors had no clinical SLE criteria at both baseline and 12 months, 1/99 (1%) increased from 0 to 1, 41/99 (42%) decreased from 1 to 0 (indicating a remission of autoimmunity) and 38/99 (38%) had 1 criterion at both time-points (Stuart-Maxwell Chi-square=38.1, p<0.001).

Of note, 1/99 (1%) of non-progressors had ankylosing spondylitis (AS) while 4/99 (4%) of had cancers [lung=1, hepatocellular=1, prostate=1 and leiomyosarcoma=1].

Table 3-3 Clinical characteristics of At-Risk progressors at 12 months

Clinical criteria	Baseline (n=19)	12 months (n=19)
Mucocutaneous		
ACLE or SCLE	5/19 (26%)	13/19 (68%)
Mucosal ulcers	2/19 (11%)	8/19 (42%)
Alopecia	0	4/19 (21%)
Musculoskeletal		
Synovitis	9/19 (47%)	18/19 (95%)
Haematological		
Leucopaenia or lymphopenia	3/19 (16%)	7/19 (37%)
Thrombocytopaenia	0	1/19 (5%)
Glandular signs	0	6/19 (32%)
Serositis		
Pleural effusion	0	1/19 (5%)
Renal		
Class III nephritis	0	1/19 (5%)

ACLE: acute cutaneous lupus erythematosus, SCLE: subacute cutaneous lupus erythematosus
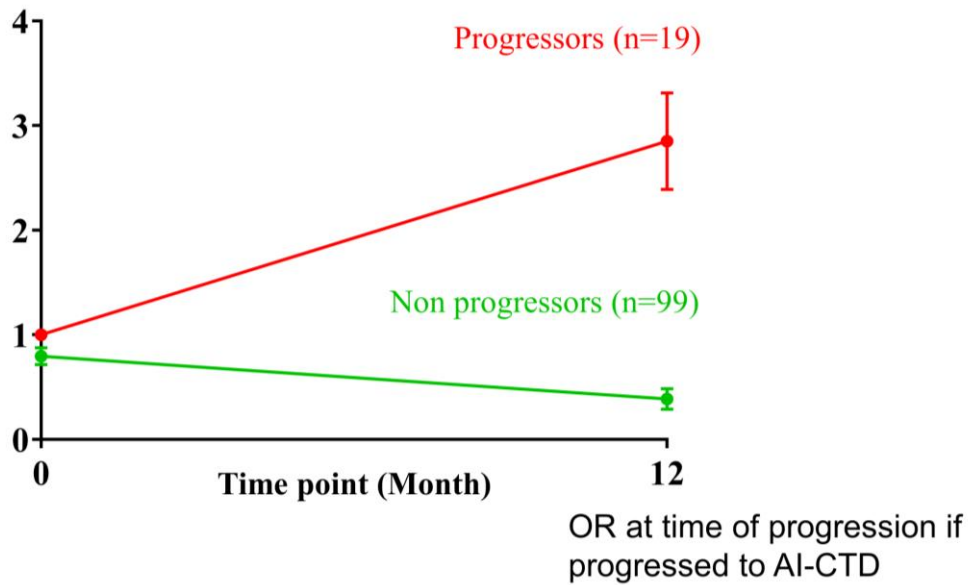
Figure 3-2 Mean number of clinical criteria at baseline and 12 months in the two At-Risk progressors groups

Mean number of clinical criteria increased at 12 months or at time of progression in At-Risk progressors as depicted in red-coloured graph while mean number of clinical criteria decreased at 12 months in At-Risk non-progressors (green). The error bars denote standard deviation. AI-CTD: autoimmune connective tissue disease

3.3.3. Blood IFN status in At-Risk differs from SLE

At baseline, IFN-Score-A differed among HC, At-Risk and SLE groups (ANOVA F=40.26; p<0.001). The score was increased relative to HC (n=49) in both At-Risk [n=105; FD (95% CI) 2.21 (1.22, 4.00), p=0.005] and SLE [n=114; 7.81 (4.33, 14.04), p<0.001], and was increased in SLE relative to At-Risk [3.54 (2.22, 5.63), p<0.001] (Figure 3-3A). On the other hand, although IFN-Score-B differed among these groups overall (F=63.35; p<0.001), the score did not differ between At-Risk and HC [0.98 (0.66, 1.46), p=0.993], but was increased in SLE to both HC [3.85 (2.60, 5.72), p<0.001] and At-Risk [3.93 (2.87, 5.37), p<0.001] (Figure 3-3B).

3.3.4. Relationships of IFN scores with autoantibodies, complement and lymphopaenia

Correlations between routine immunology markers (complement levels and lymphopenia) and IFN Scores were performed in observed data using reflected ΔCt so that higher IFN Scores represented greater expression. At baseline, there was no association between count of positive ANA specificities (i.e. anti-dsDNA, Ro, RNP etc.) and IFN-Score-A (n=105, Kendall's tau-b 0.13, p=0.084) or IFN-Score-B (tau-b 0.09, p= 0.234 (Figure 3-3C and 3-3D).

There was a weak negative correlation between C4 levels and IFN-Score-A; n=97, Pearson's r = -0.221, p=0.029 (Figure 3-3E) but not IFN-Score-B, r = -0.089; p=0.385. There was a weak negative correlation between lymphocyte count and IFN-Score-A; n=105, r = -0.230; p=0.018 (Figure 3-3F) but not IFN-Score-B; r = -0.127; p=0.195.

With respect to the relationships of IFN scores with autoantibodies titres, the titres of two antibodies that were mostly prevalent using Bioplex; anti-dsDNA and anti-Ro were divided into 3 and 2 groups respectively. The results showed that there were no differences in both IFN Scores among the 3 anti-dsDNA groups (Figure 3-4A and 3-4B). However, elevated levels of IFN-Score-A (FD 2.41 (95% CI 1.10–5.26) but not Score-B were found in the high titre ie: ≥8 AI anti-Ro antibody positive group (Figure 3-4C and 3-4D).
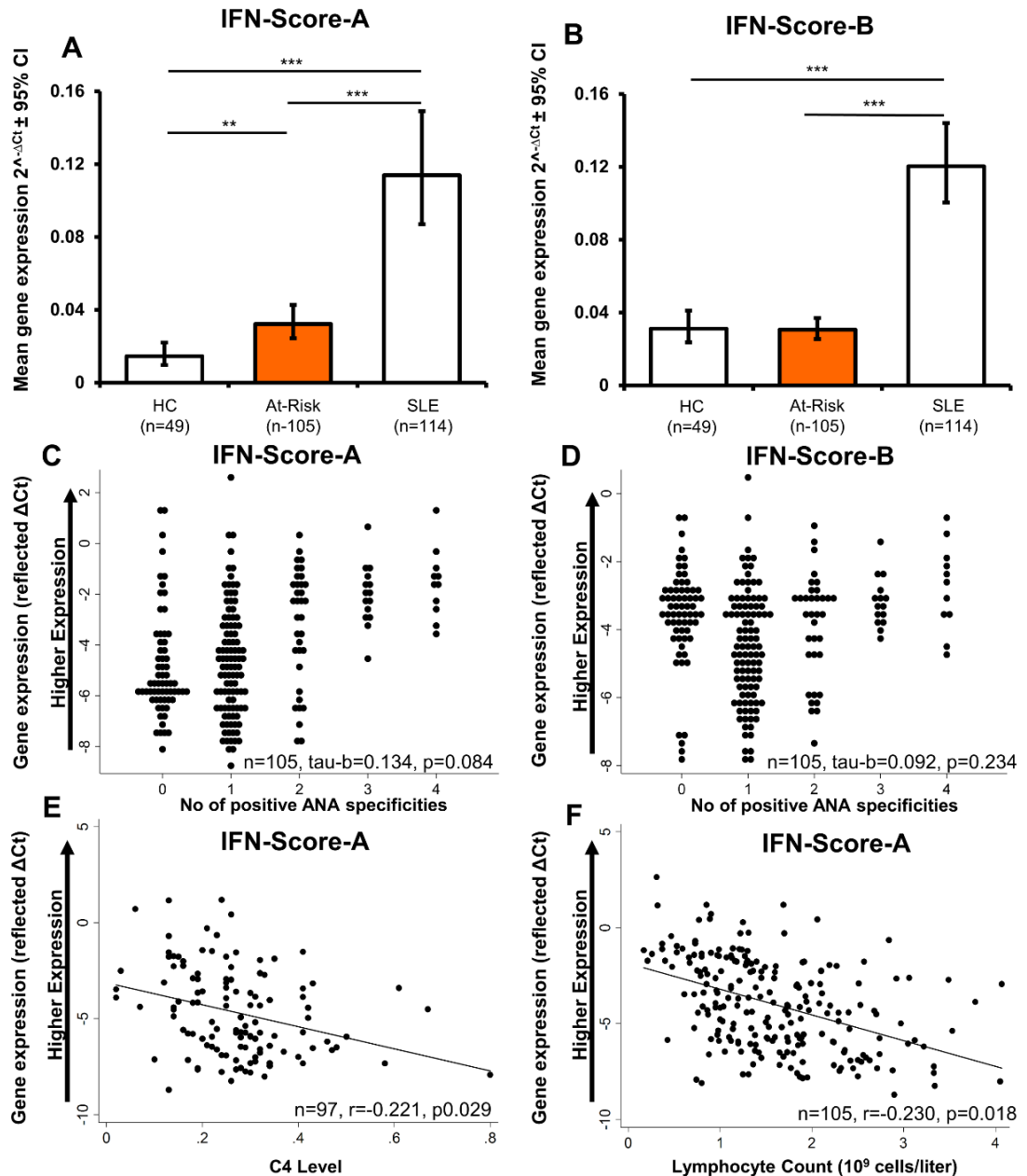
Figure 3-3 Pattern of baseline blood IFN scores and their relationships with clinical immunology markers

A) Baseline expression of IFN-Score-A was higher in At-Risk individuals compared to HC. B) However, there was no difference in IFN-Score-B between both groups. *** Highly significant (p<0.001), ** Moderate significant (0.001<p-value<0.01), *Significant (0.01<p-value<0.05). Error bars denote 95% confidence interval of the mean. C-D) Both IFN scores were not correlated with the count of positive ANA specificities and E-F) There were only weak correlations between IFN-Score-A and complement and lymphocyte count. Data for gene expression were expressed as reflected values for ΔCt so that higher IFN Scores represented greater expression. ANA: anti-nuclear antibody; C4: complement component 4; HC: healthy control; SLE: systemic lupus erythematosus.

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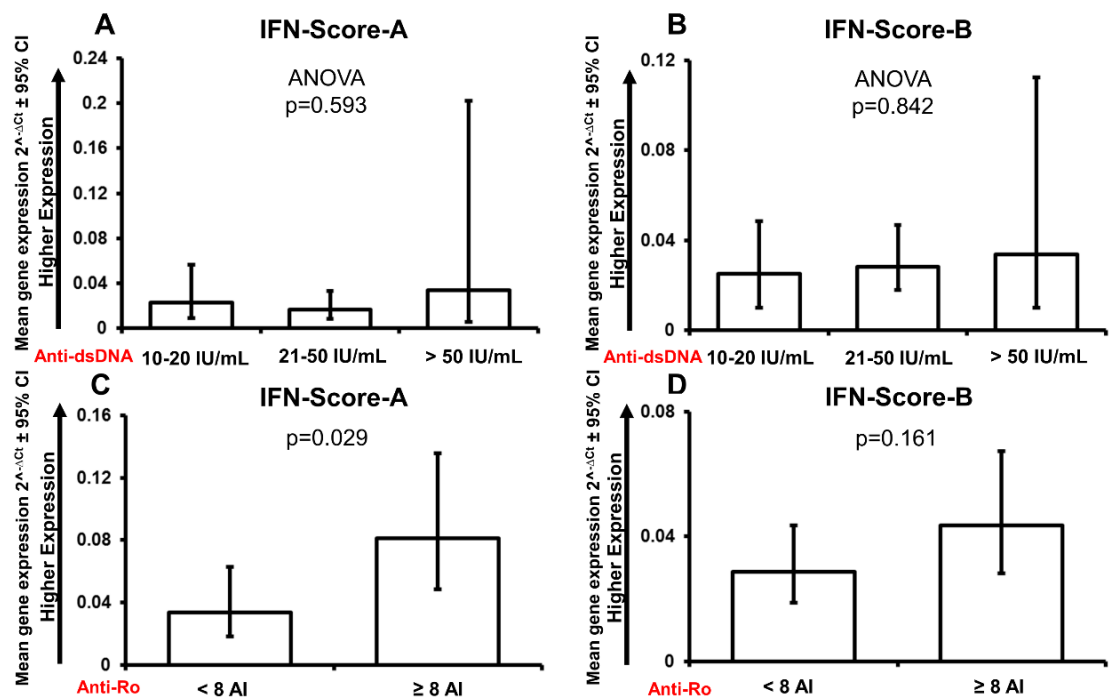
Figure 3-4 Comparison of baseline blood IFN scores based on autoantibodies titres

A-B) Anti-dsDNA titres were divided into 3 groups. IFN-Score-A and IFN-Score-B did not differ among the groups overall (p>0.1) at baseline C-D) Anti-Ro antibody titres were divided into groups. At baseline, IFN-Score-A was elevated in those with high titre i.e. ≥ 8 AI versus low titre; FD 2.41 (95% CI 1.10 – 5.26). There was a trend to increase in IFN-Score-B in those with high titre versus low titre; FD 1.52 (0.84 – 2.75). Error bars denote 95% confidence interval of the mean. ANOVA: analysis of variance; dsDNA: double stranded deoxyribonucleic acid; FD: Fold difference.

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3.3.5. Baseline IFN status in skin

Similar to the results obtained for PBMC, at baseline, only IFN-Score-A was increased in non-lesional skin biopsies in At-Risk individuals (n=10) versus HC (n=6); FD 28.74 (1.29, 639.48), p=0.036. There was no difference in IFN-Score-B; FD 1.82 (0.86, 3.86), p=0.100. As predicted, both IFN Scores were higher in SLE (active lesions) compared to either At-Risk or HC; all p<0.05.

3.3.6. Comparison of baseline IFN status between blood and skin

Expression of both IFN Scores were higher in At-Risk versus HC in both skin and PBMC, but FDs were greater in skin (Figure 3-5C). This could due to the small sample size for skin samples (paired skin-PBMC samples were not available).

3.3.7. Prediction of AI-CTD using baseline IFN scores in blood

When At-Risk were divided according to AI-CTD progression status at 12 months, both IFN scores differed among the groups overall (p<0.001). Both were elevated in At-Risk progressors (n=19) versus non-progressors (n=86), to a greater extent for IFN-Score-B [FD 3.22 (1.74, 5.95), p<0.001] than IFN-Score-A [2.94 (1.14, 7.54), p=0.018] (Figure 3-5A and B). Non-progressors did not differ from HC (n=49) for both scores; IFN-Score-B [0.79 (0.51, 1.23), p=0.520] and IFN-Score-A [1.82 (0.93, 3.53), p=0.096]. Neither IFN Score differed between At-Risk progressors and SLE (both p>0.1).

As the number of skin biopsies obtained in At-Risk individuals was small (n=10), no formal association between IFN Scores and progression could be determined in this study.

3.3.8. Baseline blood IFN-Score-B threshold of progression to AI-CTD

ROC curve analysis was used to assess the prognostic ability of baseline blood IFN Scores to predict progression to AI-CTD at 12 months. The results showed that the area under the ROC (AUROC) was greater for IFN-Score-B [0.82 (95% CI 0.73 to 0.92)] than IFN-Score-A [0.70 (0.57, 0.83); Chi-square=4.19, p=0.041. A cut-off of ≤5.01 ΔCt for IFN-Score-B maximised the Youden's index (sensitivity + specificity -1), which yielded 95% (95% CI 75% to 99%) sensitivity, 60% (50%, 70%) specificity, 35% (23%, 48%) positive

predictive value (PPV) and 98% (90%, >99%) negative predictive value (NPV). In order to design future prevention studies, for a rule-in biomarker, a high specificity indicator is warranted to exclude individuals with the lowest risk. Thus, we propose a cut-off of ≤3.90 ΔCt, which yielded 68% (46%, 85%) sensitivity, 80% (70%, 88%) specificity, 43% (27%, 61%) PPV and 92% (84%, 96%) NPV (Figure 3-5D).
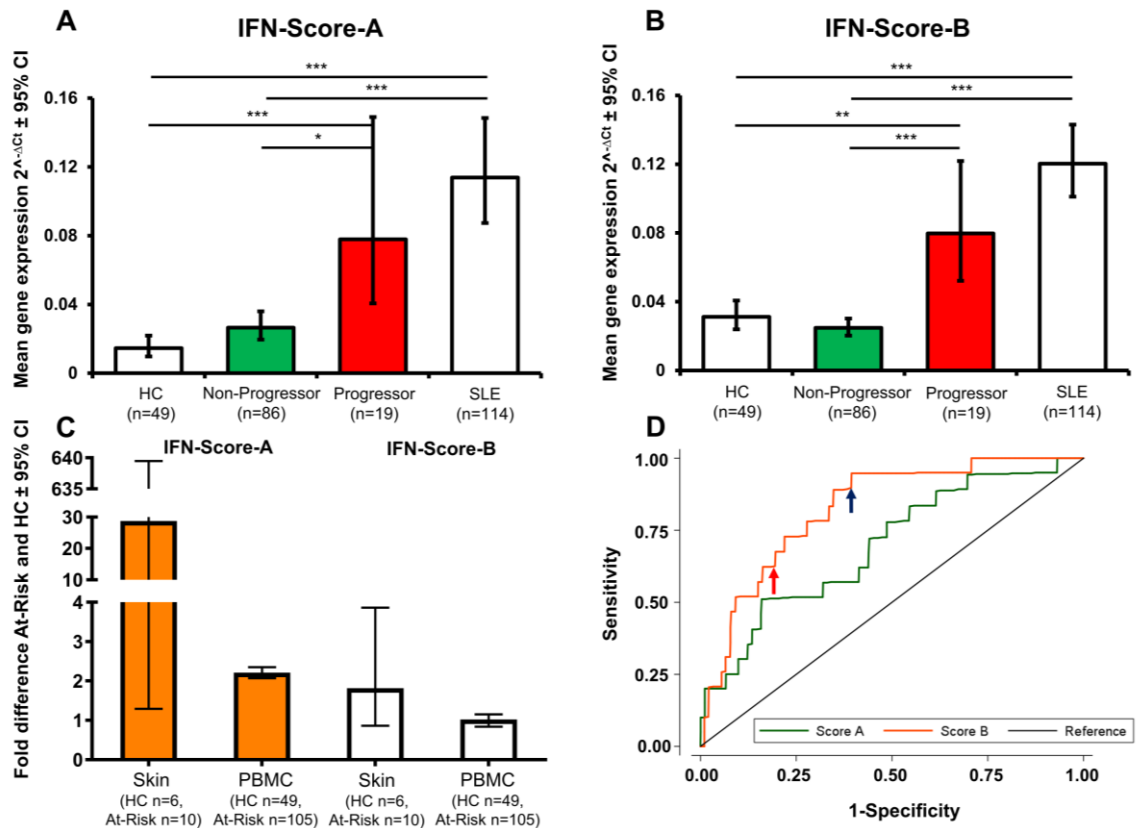
Figure 3-5 Baseline blood IFN scores as prognostic biomarkers

A-B) Baseline expression of both IFN-Score-A and IFN-Score-B were higher in At-Risk individuals who progressed to AI-CTD compared to the non-progressors, but to a greater FD in the latter. *** Highly significant (p<0.001), ** Moderate significant (0.001<p-value<0.01), *Significant (0.01<p-value<0.05). C) FDs for both IFN scores between At-Risk and HC were greater in skin than bloods. Error bars denote 95% confidence interval. D) The AUROC was significantly greater for IFN-Score-B than IFN-Score-A. The blue arrow denotes the optimal cut-off using Youden's index while the red arrow denotes the proposed cut-off for prevention study. FD: fold difference; HC: healthy control; PBMC: peripheral blood mononuclear cell; SLE: systemic lupus erythematosus.

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3.3.9. Baseline blood IFN Scores were lower in At-Risk without versus with one clinical criterion

Of 20/118 (17%) At-Risk individuals who had no SLE clinical criterion at baseline, none progressed to AI-CTD at 12 months. At baseline, FDs for both IFN scores differed among the groups overall (p<0.001). Both were lower in At-Risk with no criterion (n=17) versus with one criterion (n=88); all p<0.05 (Figure 3-6A and B).
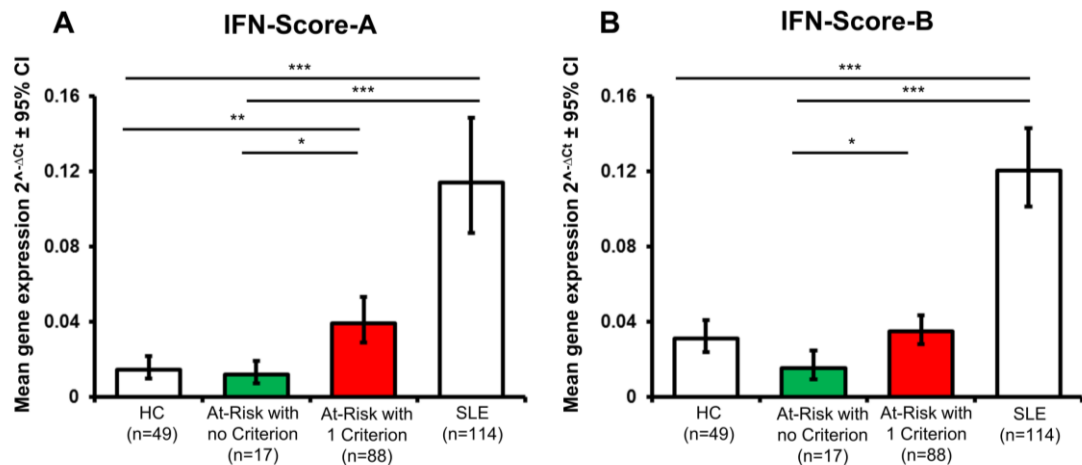
Figure 3-6 Comparison of baseline blood IFN scores based on SLE clinical criterion

A-B) At baseline, IFN-Score-A and IFN-Score-B differed among the groups overall (p<0.001) and both were elevated in At-Risk with one SLE clinical criterion versus without SLE criterion; FD 3.35 (95% CI 1.14 – 9.83); p=0.021 and 2.30 (1.11 – 4.78); p=0.018 *** Highly significant (p<0.001), ** Moderate significant (0.001<p-value<0.01), *Significant (0.01<p-value<0.05). Error bars denote 95% confidence interval of the mean. FD: fold difference; HC: healthy control; SLE: systemic lupus erythematosus.

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3.3.10. Musculoskeletal ultrasound

At baseline, one patient was not assessed using MSK-US. Of 117 At-Risk individuals with MSK-US available, 21(18%) had MSK-US-defined synovitis at baseline [GS≥2 only=13, PD≥1 with or without GS≥2=8]. Of 20 individuals who progressed, 7 (35%) had positive MSK-US at baseline versus 14% of non-progressors; p=0.050; PPV (95% CI) =33% (17%, 55%), NPV 86% (78%, 92%).

In this study, 43/118 of At-Risk individuals had clinical arthritis based on SLICC criteria (32) [8/43 (19%) had ≥2 joints with swelling or effusion whereas 34/43 (81%) had ≥2 joints with tenderness and early morning stiffness of ≥30 minutes] while 75/118 had no arthritis. In those without arthritis, MSK-US-defined synovitis was detected in 10/75 (13%) and 4/10 (40%) progressed to AI-CTD. On the contrary, in those with arthritis, only 11/42 (26%) had MSK-US-defined synovitis and 3/11 (27%) progressed to AI-CTD at 12 months. Sensitivity and specificity of physician-judged arthritis with MSK-US-defined synovitis were 52% and 68% respectively.

3.3.11. Analysis of baseline predictors of progression to AI-CTD

All putative predictors were associated with progression to AI-CTD at 12 months in imputed univariable analyses except for complement level and lymphocyte count (both p>0.1), which were excluded from multivariable analysis. In multivariable penalised logistic regression analysis, a family history of ARDs (OR 8.20, p=0.012) and IFN-Score-B (OR=3.79, p=0.005) were independently associated with progression to AI-CTD at 12 months. Penalised ORs remained substantive for these variables when all other variables were removed from model (Table 3-4). Results in complete data (n=100) were similar (data not shown).

Table 3-4 Penalised logistic regression for predictors of progression to AI-CTD at 12 months

Baseline predictors	No Progression n=99	Progression n=19	Imputed Univariable OR (95% CI), P-value	Imputed Multivariable OR (95% CI), P-value	Penalised coefficient, OR
Age, mean (SD)	49.0 (15.8)	39.6 (11.9)	0.96 (0.93, 0.99), p=0.016	0.97 (0.92, 1.02), p=0.232	0.000, 1.000
Ever Smoked, (%)	41.8 %	20.0 %	0.35 (0.11, 1.12), p=0.076	0.34 (0.06, 1.91), p=0.222	0.000, 1.000
Family history of ARDs, (%)	30.6 %	65.0 %	4.21 (1.53, 11.61), p=0.005	**8.20 (1.58, 42.53), p=0.012**	0.243, 1.275
No. of positive ANA specificities, median (IQR)	1 (1 - 1)	1 (1 – 2)	2.07 (0.97, 4.40), p=0.060	2.41 (0.71, 8.20), p=0.161	0.000, 1.000
Complement C4 level, mean (SD)	0.29 (0.12)	0.26 (0.08)	0.06 (0.00, 8.05), p=0.264	Excluded	Excluded
Lymphocyte count, mean (SD)	2.04 (0.77)	1.83 (0.67)	0.67 (0.34, 1.34), p=0.257	Excluded	Excluded
No. of Joints with MSK-US synovitis, median (IQR)	0 (0 – 0)	0 (0 – 2)	1.20 (0.97, 1.47), p=0.086	1.44 (0.98, 2.11), p=0.061	0.002, 1.002
Patient VAS, median (IQR)	36 (16 – 61)	47 (26 – 75)	1.02 (1.00, 1.04), p=0.079	1.01 (0.98, 1.04), p=0.484	0.000, 1.000
Physician VAS, median (IQR)	11 (3 – 31)	31 (15 – 47)	1.04 (1.01, 1.06), p=0.008	1.01 (0.97, 1.06), p=0.618	0.000, 1.000
IFN-Score-A (-ΔCt), mean (SD)*	-5.3 (1.9)	-3.8 (2.26)	1.43 (1.11, 1.84), p=0.005	0.87 (0.54, 1.39), p=0.560	0.000, 1.000
IFN-Score-B (-ΔCt), mean (SD)*	-5.3 (1.4)	-3.7 (1.0)	2.55 (1.60, 4.08), p<0.001	**3.79 (1.50, 9.58), p=0.005**	0.319, 1.376

* Analysis was made based on reflected ΔCt. Thus, the higher the number, the higher the gene expression to give positive values for odds ratio

ANA: anti-nuclear antibody; ARD: autoimmune rheumatic disease; IQR: interquartile range; MSK-US: musculoskeletal ultrasound; SD: standard deviation; VAS: visual analogue score;

3.4. Discussion

In this chapter, I have reported a unique cohort of At-Risk of AI-CTD individuals with longitudinal follow-up until progression to meeting classification criteria for clinical autoimmunity. My results show that IFN activity is strongly associated with progression independent of baseline clinical status, with measurement according to a two-score system we described being crucial. These results provide a rationale for diagnostic and preventative treatment pathways as well as assert the importance of IFNs in disease initiation.

The number of referrals of ANA-positive individuals to rheumatologists has increased over the last decade (337). Concerns are that these At-Risk individuals may be discharged prematurely from clinic, or be observed in an inefficient "watch and wait" fashion until the diagnosis is clear, by which time the potential to prevent disease and confer the most benefit may be lost. Therefore, by undertaking the largest prospective study of At-Risk individuals, which is the first to integrate clinical, imaging and immunological assessments (including skin), my findings offer a novel approach, biomarkers and have implications for future development of targeted therapies for this group of patients.

With regards to immunological assessment, within ANA-positive individuals, different immune phenotypes could be defined. At baseline, IFN-Score-A was elevated but not IFN-Score-B compared to HC. However, IFN-Score-B (and to a lesser degree, IFN-Score-A) were mostly elevated in individuals who progressed to AI-CTD. IFN-Score-A comprises many well-known ISGs that respond to various IFN-I subsets including IFN-α, -β –κ and -ω. In contrast, IFN-Score-B consists of ISGs that coincide with M3.4 and M5.12 modules of a previous microarray study (314). These ISGs were suggested to be responsive to IFN-II (IFN-gamma; IFN-γ), IFN-III (IFN-lambda; IFN-λ) as well as IFN-I. However, in our previous study, we could not demonstrate such a clear relationship between ISG subsets and subtypes of IFN, thus we could not exclude the influence of other inflammatory mediators on this pattern of gene expression (320). Some studies suggested that IFN-I contributes to priming cells to secrete IFN-II (338, 339). Conversely, a study that measured

IFN activity from serum postulated a sequential role of IFN-II augmentation that led to accumulation of autoantibody and subsequent elevations in IFN-α prior to SLE (340). Although this present study could not confirm which IFN pathways predominate, my findings suggested that progression to AI-CTD might not be exclusively driven by IFN-I but by a synergistic activation of ISGs induced by a range of IFNs and IFN-Score-B could act as a biomarker for more diverse immune activation.

At the tissue level, this is the first study that reports IFN activity in non-lesional skin of At-Risk individuals. Similar patterns of immune dysregulation were observed between skin and blood. Interestingly, markedly greater FDs in both IFN scores were found in the former compared to the latter, thus highlighting skin as a potential site of AI-CTD initiation.

Pertaining to imaging, only a third of the At-Risk individuals who had MSK-US-defined synovitis at baseline progressed to AI-CTD within 12 months. My findings also highlighted discrepancy in the assessment of arthritis between clinical examination and imaging. Notably, a small number of asymptomatic individuals with MSK-US-defined synovitis were identified and some of them progressed to AI-CTD. Although there was a trend in association between the presence or number of joints with MSK-US-defined synovitis and progression to AI-CTD, further work including longitudinal analysis is required to determine the role of MSK-US in assessing At-Risk individuals.

Together with a family history of ARD, IFN-Score-B from blood is independently predictive of progression as a prognostic biomarker. A cut-off level of IFN-Score-B with a moderate diagnostic accuracy in order to design future prevention study has been defined.

This study has some limitations. First, the cohort was recruited from secondary care as well as positive ANA detected by both Bioplex and indirect immunofluorescence, which might contribute to moderate-to-high pre-test probabilities for progression to AI-CTD. Thus, our results might not be generalised to all ANA-positive cases detected in a primary care setting. However, our cohort was quite heterogenous in terms of ethnicity and 17% of the patients had no SLE criterion at baseline. Next, individuals with scleroderma or myositis-specific only autoantibodies were excluded, which might lead to preponderance of progression to SLE or pSS. Remarkably, one

patient had a severe AS and required biological therapy. Importantly, 4% of non-progressors had cancers thus highlighting the need to be vigilant of paraneoplastic manifestation in ANA-positive individuals as well as diverse alternative diagnoses in general. Lastly, although large in the context of existing studies in this field, sample size was still relatively small for multivariable analysis. However, penalised logistic regression was used to minimise the risk of overfitting of data.

3.5. Conclusion

To conclude, a novel ISG score, IFN-Score-B and a family history of ARD predict progression from ANA-positivity to AI-CTD. Longitudinal analyses are in progress as well as a validation cohort. Once this biomarker is validated, the predictive value of IFN scores may allow us to identify patients with imminent AI-CTD for earlier intervention either using therapies that block IFNs or conventional immunosuppressants, in order to avoid irreversible organ damage and glucocorticoid exposure. Furthermore, patients with benign autoreactivity can be better identified.

3.6. Key messages

i. In At-Risk of CTD individuals (ANA-positive and limited inflammatory symptoms), 16% progressed to meet AI-CTD criteria at 12 months.

ii. Within these ANA-positive individuals, different immune phenotypes can be defined.

iii. Blood IFN-Score-B and a family history of ARDs are independent predictors of AI-CTD at 12 months.

iv. The predictive value of IFN scores may allow us to identify patients with imminent AI-CTD for earlier intervention to prevent from disease and avoid irreversible organ damage.

Chapter 4. Predicting and managing primary and secondary non-response to rituximab in systemic lupus erythematosus

4.1. Introduction

Rituximab, a chimeric anti-CD20 mAb remains an important treatment option for SLE patients with moderate to severe disease. A high degree of rituximab efficacy across a range of lupus manifestations has been reported in open label studies from single centre series (16, 341, 342), multicentre registries (14, 343, 344) and a systematic review of off-label use (13). Despite the success of these series, two phase III RCTs in non-renal lupus (204) and renal lupus (205) failed to meet their primary endpoints (defined in section 2.7.1) . The discrepancy in efficacy outcomes between RCTs and real world evidence has been attributed to aspects of trial design including the choice of appropriate and meaningful endpoints, the use of an active comparator, inclusion criteria and low statistical power, as detailed in section 2.6 previously (214).

In the EXPLORER trial of rituximab in SLE, *post-hoc* analysis showed that the primary endpoint was met in Hispanic and African-American subgroups (204). Despite efficacy reported from open label evidences, there are limited data on predictors or biomarkers of response to rituximab. Using HSFC, a protocol that was optimised for the detection of plasmablasts, our group previously discovered that the depth of B-cell depletion predicted response in 37 rituximab-treated SLE patients (16). Larger studies as well as identification of other clinical predictors of response to rituximab in SLE are needed to optimise its use and help design trials of alternative B-cell depleting strategies.

The effect of B-cell depletion with rituximab is not long-lasting and majority of the patients experience a relapse following B-cell repopulation (albeit with a variable interval). After an initial response to rituximab, repeat treatment has been reported to be effective to treat clinical relapse (341). However, there are limited data on long-term outcomes of these patients. Moreover, we have

observed cases of SLE patients who had previously depleted and responded to rituximab but subsequently developed (i) a severe infusion reaction >24 hours during the second infusion of a cycle; (ii) failure to deplete CD20+ (naïve and memory) B-cells and (iii) clinical non-response during repeat cycles. We called this phenomenon secondary non-depletion and non-response (2NDNR), which was suggestive of immunogenicity to rituximab. In this group of patients, further courses of rituximab would be detrimental in terms of safety and no longer be effective. Thus, alternative anti-CD20 mAbs, particularly humanised, could theoretically be used instead in order to overcome this anti-drug antibody phenomenon.

4.1.1. Hypotheses

i. B-cell depletion is an independent predictor of clinical response to rituximab in SLE.
ii. In patients who experience a clinical relapse after an initial response, repeat cycles of rituximab is effective and with no major safety signals.
iii. 2NDNR is associated with anti-rituximab antibodies.
iv. 2NDNR can be overcome using alternative humanised anti-CD20 mAbs.

4.1.2. Objectives

i. To assess factors predicting primary and secondary non-response to rituximab in SLE
ii. To validate B-cell depletion as a biomarker of response to rituximab in a second independent cohort
iii. To evaluate the efficacy and safety of repeat cycles of rituximab
iv. To evaluate management of 2NDNR using alternative humanised anti-CD20 agents

4.2. Methods

4.2.1. Candidate's roles in this project

In this work, the initial concept and design of the study were set by myself, Prof Emery and Dr Vital. I carried out the weekly Lupus and Vasculitis clinic in Leeds under the supervision of Dr Vital and Prof Emery and performed a

significant proportion of the clinical assessments. Peripheral blood B-cell subsets were measured using HSFC at the Haematological Malignancy Diagnostic Service, Leeds Teaching Hospitals NHS Trust. I performed the ELISA test on research samples for anti-rituximab antibodies under the supervision of my laboratory supervisor, Dr El-Sherbiny. I organised and entered half of the data onto the study database (the other half was done by Dr Shaw). I performed all statistical analyses and led the writing of the manuscript, while other co-authors revised the draft critically for important intellectual content and final approval of the manuscript prior to submission to journal for publication.

4.2.2. Design and Patients

A retrospective observational study was conducted of all patients with moderate to severe SLE who were treated with rituximab in Leeds between January 2004 and July 2016.

Inclusion criteria were (i) adults (>16 years old); (ii) fulfilling the revised 1997 ACR classification for SLE (31) and (iii) at least 6 months follow-up post-rituximab.

4.2.3. Ethical approval

The use of rituximab, ofatumumab and ocrelizumab were all approved by Leeds Teaching Hospitals NHS Trust Drug and Therapeutic Committee. Analysis of samples for anti-rituximab antibody was approved by the Leeds (East) Research Ethics Committee (REC), 10/H1306/88, and the committee confirmed that other aspects of the study did not require ethical approval in accordance with the UK National Health Service REC guidelines.

4.2.4. Treatment pathways

All patients received a first cycle of treatment consisted of 100 mg of methylprednisolone and 1000 mg of rituximab given intravenously on days 1 and 14. Further cycles of the same regimen were repeated on clinical relapse (as defined in section 4.2.4).

Continuation of a stable or a reduced dose of concomitant csDMARDs (including oral corticosteroid), was left to investigators' discretion with the aim

to stop glucocorticoid if MCR (as defined in section 4.2.4) was achieved at 6 months.

Of those who met the 2NDNR criteria (as defined in section 4.1), their treatment was switched from rituximab to humanised anti-CD20 mAbs either by using (i) 2x1000mg ocrelizumab (compassionate use from Roche UK) or (ii) 2x700mg ofatumumab (individual funding request to NHS England).

4.2.5. Clinical Data and outcomes

Disease activity was assessed using the BILAG-2004 index (172) at baseline and every 3 months thereafter. Clinical responses at 6 months were determined as follows: (i) MCR = improvement of all domains rated A/B to grade C/better and no A/B flare between baseline and 6 months; (ii) PCR = maximum of 1 domain with a persistent grade B with improvement in all other domains and no A/B flare and (iii) non-response (NR) = those not meeting the criteria for MCR or PCR. Relapse was defined as a new grade A or recurrence of ≥1 grade B following either MCR/PCR clinical response at 6 months. Global BILAG score was calculated as follows: grade A=12, grade B=8, grade C=1, and grades D and E=0 point (345).

4.2.6. Protocol for peripheral B-cell analysis using Highly Sensitive Flow Cytometry (HSFC)

As per standard of care for patients who received treatment with rituximab in Leeds, peripheral blood B-cell subsets (naïve, memory B-cells and plasmablasts) were measured using HSFC at the Haematological Malignancy Diagnostic Service, Leeds Teaching Hospitals NHS Trust at baseline, 6 months and every 6 months without knowledge of patients' clinical status other than time since rituximab. This accredited diagnostic clinical laboratory provides a regional and supra-regional diagnostic service and specialises in ARDs and haematological malignancies.

B-cell numbers and subsets were enumerated following standard cell surface staining techniques using a sequential gating strategy with 6-colour flow cytometry, counting for 500,000 events. B-cells were first identified using gating strategy with peridinin chlorophyll A protein–Cy5.5–conjugated CD19 and phycoerythrin–Cy7 (PECy7)–conjugated CD38 (BD Biosciences)

expression and light scatter characteristics. Fluorescein isothiocyanate–conjugated CD3 and PE-conjugated CD14 (BD Biosciences) were used to exclude contaminating events. Subsequently, B-cells were classified according to the expression of PE-Cy7–conjugated CD38 and allophycocyanin (APC)–conjugated CD27 (BD Biosciences) as either naive B-cells (CD19++CD27-), memory B-cells (CD19++CD27+) or plasmablasts (CD19+/-CD27++CD38++). APC-Cy7–conjugated CD45 (BD Biosciences) was used to identify total leukocytes for calculation of absolute B-cell subset numbers (234). The gating strategy for enumeration of B-cell subsets using HSFC is explained in Figure 4-1.

Complete B-cell depletion was defined as counts $<0.0001 \times 10^9$/L and repopulation as $\geq 0.0001 \times 10^9$/L.
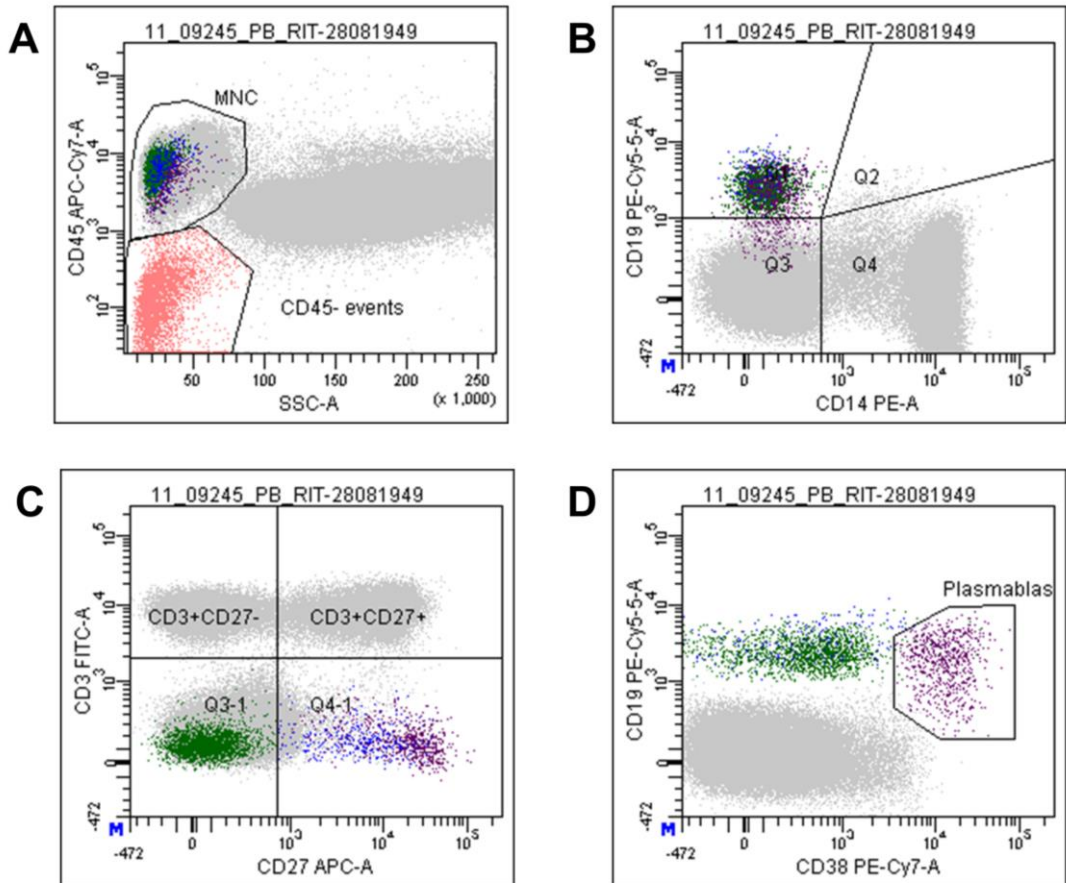
Figure 4-1 Gating strategy for B-cell subsets enumeration using HSFC

A) Plasmablasts have weaker CD19 expression. Therefore, the initial gate should be set around the MNCs rather than restrictive to lymphocyte. B-D) CD14 and CD3 markers were used to exclude contamination and T-cells respectively. Naïve B-cells were identified by MNC AND CD19+CD14- (Q1) AND CD3-CD27- (Q3-1). Memory B-cells were defined as MNC AND CD19+CD14- (Q1) AND CD3-CD27+ (Q4-1) AND CD38$^{weak/negative}$. Plasmablasts were identified as MNC AND CD19+CD14- (Q1) AND CD3-CD27+ (Q4-1) AND CD38++. HSFC: highly sensitive flow cytometry; MNC: mononuclear cells

4.2.7. Other laboratory assessments

Anti-dsDNA antibody titres were measured by ELISA until July 2012 and Bioplex 2200 Immunoassay (after July 2012). Complement levels (C3 and C4) and total serum immunoglobulin titres were measured by nephelometry.

Anti-rituximab antibodies were tested on a subset of patients with 2NDNR using the Promonitor®-Anti-Rituximab ELISA and these titres were compared to those with continued response to rituximab. A concentration >140 AU/mL was deemed a positive test (as determined by the manufacturer).

4.2.8. Safety

Safety assessments included severe adverse events (SAEs) and SIEs were recorded irrespective of possible association with SLE and/or therapy. SAEs were defined as those resulted in either hospitalisation that lasted more than 24 hours, flares requiring intravenous therapy, malignancies, life-threatening situations or death. Data for SIEs were gathered from hospital admission records using Patient Access Centre (PAS) system and was later confirmed with case notes.

4.2.9. Statistical Analyses

Descriptive statistics were summarised using mean with standard deviation or median with interquartile range for continuous variables where appropriate and proportion for categorical variables. The significance of the association between categorical variables was tested by Fisher's exact test while for continuous variables using Mann-Whitney U test. Multiple imputation was used to handle missing data. The imputed univariable and multivariable analyses of predictors of any response (MCR/PCR) and MCR at 6 months post-rituximab were analysed using binary logistic regression. All statistical analyses was performed using Stata 13.1 (StataCorp College Station, Texas, USA) GraphPad Prism v.7.03 (GraphPad, La Jolla, CA, USA) for Windows.

4.3. Results

4.3.1. Patient characteristics

Of 125 SLE patients who were treated with rituximab at our unit over the 12 years period, 117 patients with evaluable data at 6 months were studied. Eight patients were excluded (5 did not reach month 6 follow-up while 3 had left Leeds prior to the 6-month assessment).

Baseline characteristics are described in Table 4-1. One hundred and twelve (96%) had refractory and active disease as defined by BILAG ≥1A score and/or ≥2B scores. The remaining 5 had BILAG B in 1 domain only but was refractory to other conventional therapies as well as on maintenance with oral prednisolone ≥10mg daily. Total follow-up was 492 patient-years (246).

Table 4-1 Baseline characteristics of the 117 patients with SLE who were treated with rituximab

Characteristic	Values
Age at first rituximab infusion, median (IQR) years	39 (26-52)
No. female patient (%)	109 (93)
Ethnicity, N (%)	
Caucasian	80 (68)
Afro-Caribbean	11 (10)
South Asian	20 (17)
Others	6 (5)
SLE Disease duration at first rituximab, median (IQR) years	6 (2-11)
Positive ANA at diagnosis, N (%)	117 (100)
Antibody status at first rituximab infusion, N (%) positive	108 (92)
anti-dsDNA	56 (48)
anti-Ro	57 (49)
anti-La	18 (15)
anti-Smith	15 (13)
anti-Chromatin	19 (16)
anti-RNP	23 (20)
anti-Ribosomal P	6 (5)
anti-Cardiolipin/anti-B2-Glycoprotein	14 (12)
Prior CyC therapy, N (%)	63 (54)

Characteristic	Values	
Cumulative dose of CyC, mean ± SD gram	6.6 ± 4.2	
Number of prior immunosuppressant failure (including CyC but excluding glucocorticoid), median (range)	3 (0-9)	
Concomitant anti-malarials, N (%)	88 (75)	
Concomitant immunosuppressant, N (%)		
Azathioprine	19 (16)	
Methotrexate	16 (14)	
Mycophenolate Mofetil	39 (33)	
Prednisolone dose at first rituximab, median (IQR) mg	10 (3-20)	
ESR at first rituximab, median (IQR) mm/hour	29 (15-57)	
BILAG index score at baseline, N (%)		
≥1 A score	96 (82)	
No A score but ≥2 B scores	16 (14)	
BILAG domains at baseline, N (%)	Grade A	Grade B
General	9 (8)	12 (10)
Mucocutaneous	23 (20)	32 (27)
Neurological	17 (15)	17 (15)
Musculoskeletal	30 (26)	24 (20)
Cardiorespiratory	6 (5)	13 (11)
Gastrointestinal	6 (5)	0 (0)
Ophthalmic	0 (0)	0 (0)
Renal	34 (29)	0 (0)
Haematology	11 (9)	12 (10)
Global BILAG score, median (IQR)	21 (14-27)	
SLEDAI-2K score, median (IQR)	10 (6-14)	
SLICC Damage Index, median (IQR)	0 (0-1)	

ANA: antinuclear antibody; BILAG: British Isles Lupus Assessment Group; CYC: cyclophosphamide; dsDNA: double-stranded deoxyribonucleic acid; ESR: erythrocyte sedimentation rate; RNP: ribonucleic protein; RTX: rituximab; SLEDAI-2K: Systemic Lupus Erythematosus Disease Activity Index 2000; SLICC: Systemic Lupus International Collaborating Clinics

4.3.2. Treatment characteristics

Three hundred and eighteen cycles of rituximab were administered in total. Median (range) duration of response in rituximab responders for cycles 1–4 (C1–4) were 52 (26-423), 52 (26-299), 57 (27-184) and 50 (29-173) weeks respectively.

Concomitant cyclophosphamide was used in only 5 patients who presented with life-threatening disease.

4.3.3. Clinical and immunological response to first cycle rituximab

In cycle 1, there was a good overall clinical response to rituximab; 58 (50%) patients had MCR, 38 (32%) PCR and 21 (18%) were non-responders. The median global BILAG scores had reduced from 21 (IQR 14-27) pre-rituximab to 8 (IQR 1-10) at 6 months; $p < 0.001$.

Responses in individual BILAG domains are shown in Table 4-2. Although majority of domains improved, responses were more variable in the mucocutaneous and haematological domains. Mucocutaneous responses to rituximab have been described in detail previously (17). These long-term data showed a more consistence major response in lupus erythematosus non-specific lesions (LENS) and oral ulcers and non-response in chronic cutaneous lupus erythematosus (CCLE) [CCLE versus other lupus-specific lesions; $p = 0.022$]

The median serum anti-dsDNA titre had reduced from 109 (IQR 16-300) IU/ml pre-rituximab to 32 (IQR 7-116) IU/ml at 6 months; $p < 0.001$. Of 46 patients with low complement (C3 and/or C4) levels pre-rituximab, levels had normalised in 25/46 (54%) at 6 months.

Table 4-2 Responses in individuals BILAG domains at 6 months post-rituximab (cycle 1)

Domains	No cases at baseline (Grade A/B)	MCR	PCR	Severe Persistence (Grade A)	Moderate Persistence (Grade B)	Worsening	New Flare
General	21 (9/12)	19 (90)	0	2 (10)	0	0	0
Mucocutaneous	55 (23/32)	35 (64)	5 (9)	3 (5)	11 (20)	2 (4)*	3 (5)
ACLE/SCLE	34 (10/24)	24 (71)	3 (9)	2 (6)	4 (12)	2 (6)*	1 (3)
CCLE	12 (5/7)	3 (25)	2 (17)	1 (8)	6 (50)	0	2 (17)
LENS	7 (5/2)	7 (100)	0	0	0	0	0
Oral ulcers	12 (2/10)	11 (92)	0	0	1 (8)	0	0
Alopecia	13 (0/13)	9 (69)	0	0	4 (31)	0	0
Neurological	34 (17/17)	22 (65)	7 (21)	0	5 (14)	0	0
Musculoskeletal	54 (30/24)	41 (76)	8 (15)	4 (7)	1 (2)	0	0
Cardiorespiratory	19 (6/13)	17 (89)	0	0	2 (11)	0	0
Gastrointestinal	6 (6/0)	5 (83)	0	0	1 (17)	0	0
Ophthalmic	0	0	0	0	0	0	0
Renal	34 (34/0)	24 (71)	8 (24)	2 (5)	0	0	0
Haematology	23 (11/12)	17 (73)	2 (9)	2 (9)	2 (9)	2 (9)	0

* One patient with BILAG B for ACLE rash pre-rituximab had worsening of psoriasiform lesions (BILAG A) at 6 months post-rituximab. Hence, the total percentage for MCR, PCR, severe and moderate persistence in the mucocutaneous and ACLE/SCLE domains did not add up to 100%

ACLE: acute cutaneous lupus erythematosus; BILAG: British Isles Lupus Assessment Group; CCLE: chronic cutaneous lupus erythematosus; LENS: lupus erythematosus non-specific lesions; MCR: major clinical response; PCR: partial clinical response

4.3.4. Predictors of major clinical response to first cycle rituximab

Only B-cell depletion at 6 weeks post-rituximab increased the odds of any BILAG response (MCR/PCR) in multivariable analysis; adjusted imputed OR 13.93 95% CI 3.11-62.37; p=0.001 (Table 4-3).

As there was a high degree of response to rituximab in this cohort, we analysed predictors for MCR separately in order to identify patients who would respond best to therapy. In imputed univariable analysis, only younger age was associated with MCR to rituximab (OR 0.97 95% CI 0.95-0.99; p=0.031). While in imputed multivariable model, younger age (OR 0.97 95% CI 0.94-1.00; p=0.045) and B-cell depletion at 6 weeks post-rituximab (OR 3.22 95% CI 1.24-8.33; p=0.016) increased the odds of MCR to rituximab (Table 4-4).

Table 4-3 Multivariable analysis of predictors of any BILAG response (major/partial) to first cycle rituximab

Characteristics	No Response n=21	Major/Partial Clinical Response n=96	Univariable OR (95% CI), P-value (with multiple imputation)	Multivariable OR (95% CI), P-value (with multiple imputation)
Age, mean (SD) years	39 (14)	40 (16)	1.00 (0.97-1.03), p=0.833 per year	0.99 (0.95-1.04), p=0.785
White, N (%)	14 (67)	66 (69)	0.91 (0.33-2.48), p=0.852	0.56 (0.13-2.44), p=0.440
Anti-dsDNA titres, mean (SD) IU/ml	121 (217)	149 (233)	1.00 (0.99-1.00), p=0.464 per unit	1.00 (0.99-1.00), p=0.243
Anti-ENA positivity, N (%)	19 (90)	60 (62)	**0.18 (0.04-0.84), p=0.028**	0.21 (0.04-1.21), p=0.080
Low C3 and/or C4 titres, N (%)*	8 (38)	42 (44)	1.26 (0.48-3.33), p=0.640	-
ESR, mean (SD) mm/hour	45 (33)	39 (34)	1.00 (0.98-1.01), p=0.618 per unit	0.99 (0.97-1.01), p=0.525
Concomitant DMARDs, N (%)**	14 (67)	62 (65)	0.91 (0.34-2.47), p=0.856	-
Daily Prednisolone dose, mean (SD) mg	16 (13)	14 (13)	0.98 (0.95-1.02), p=0.390 per mg	0.98 (0.93-1.03), p=0.511
Total BILAG score, mean (IQR)	21 (11)	23 (11)	1.02 (0.97-1.07), p=0.486 per point	1.02 (0.96-1.09), p=0.487
Total B-cell counts, mean (IQR)***	132 (103)	116 (128)	1.00 (0.99-1.00), p=0.727 per unit	1.00 (0.99-1.01), p=0.986
B-cell depletion at 6 weeks post-rituximab, N (%)	3 (14)	65 (68)	**11.07 (2.97-41.20), p<0.001**	**13.93 (3.11-62.37), p=0.001**

* As there were high collinearity between concomitant DMARDs and B-cell depletion, low complement and Total B-cell counts and concomitant DMARDs and low complement, the last two variables were excluded in the multivariable analysis

** Concomitant DMARDs was defined as either using methotrexate, azathioprine, mycophenolate mofetil and/or other disease modifying anti-rheumatic drugs but excluded anti-malarials

***(count x 10^9 cells/L) for each subset multiply by 1000 prior to analysis

BILAG: British Isles Lupus Assessment Group; C3/C4: complement 3 or 4; DMARDs: disease modifying anti-rheumatic drugs; dsDNA: double-stranded deoxyribonucleic acid; ENA: extract nuclear antigen; ESR: erythrocyte sedimentation rate

Table 4-4 Multivariable analysis of predictors of major clinical response to first cycle rituximab

Characteristics	No Response/ Partial Response n=59	Major Clinical Response n=58	Univariable OR (95% CI), P-value (with multiple imputation)	Multivariable OR (95% CI), P-value (with multiple imputation)
Age, mean (SD) years	43 (17)	37 (14)	**0.97 (0.95-0.99), p=0.031 per year**	**0.97 (0.94-1.00), p=0.045**
White, N (%)	43 (73)	37 (64)	1.53 (0.70-3.34), p=0.292	0.92 (0.34-2.47), p=0.870
Anti-dsDNA titres, mean (SD) IU/ml	147 (230)	142 (230)	1.00 (0.99-1.00), p=0.879 per unit	1.00 (0.99-1.00), p=0.632
Anti-ENA positivity, N (%)	40 (68)	38 (66)	0.91 (0.42-1.99), p=0.812	0.90 (0.37-2.22), p=0.821
Low C3 and/or C4 titres, N (%)	25 (42)	24 (41)	0.97 (0.46-2.04), p=0.937	1.14 (0.41-3.13), p=0.801
ESR, mean (SD) mm/hour*	40 (32)	41 (36)	1.00 (0.99-1.01), p=0.827 per unit	-
Concomitant DMARDs, N (%)**	41 (69)	35 (60)	0.67 (0.31-1.43), p=0.301	0.43 (0.17-1.09), p=0.075
Daily Prednisolone dose, mean (SD) mg	13 (11)	16 (14)	1.02 (0.99-1.05), p=0.207 per mg	1.00 (0.97-1.04), p=0.713
Total BILAG score, mean (IQR)	21 (8)	24 (13)	1.03 (0.99-1.07), p=0.093 per point	1.02 (0.97-1.07), p=0.371
Total B-cell counts, mean (IQR)***	101 (95)	138 (150)	1.00 (1.00-1.01), p=0.161 per unit	1.00 (1.00-1.01), p=0.137
B-cell depletion at 6 weeks post-rituximab, N (%)	29 (49)	39 (68)	2.10 (0.95-4.62), p=0.065	**3.22 (1.24-8.33), p=0.016**

* As high collinearity was observed between ESR and Total B-cell counts, only the latter was included in the multivariable analysis
** Concomitant DMARDs was defined as either using methotrexate, azathioprine, mycophenolate mofetil and/or other disease modifying anti-rheumatic drugs but excluded anti-malarials
***(count x 10^9 cells/L) for each subset multiplied by 1000 prior to analysis
BILAG: British Isles Lupus Assessment Group; C3/C4: complement 3 or 4; DMARDs: disease modifying anti-rheumatic drugs; dsDNA: double-stranded deoxyribonucleic acid; ENA: extract nuclear antigen; ESR: erythrocyte sedimentation rate

4.3.5. Validation of association between complete B-cell depletion and clinical response

The published discovery cohort included 37 SLE patients (16). In this validation cohort, 67 subsequent and consecutive patients (with B-cell data available) were analysed. Similar to the discovery cohort, higher response rate was achieved in complete depletion compared to incomplete depletion groups; 93% versus 68%; p=0.011 in this validation cohort (Figure 4-2A).

4.3.6. Relationships between B-cell depletion and routine immunological markers

While there was no difference at baseline, patients with complete B-cell depletion had significantly lower anti-dsDNA antibody titres at 14 weeks (p=0.030) and 26 weeks (p=0.041) compared to those with incomplete depletion. Additionally, in the former, C3 and C4 levels were not different at 14 weeks (p=0.064 and p=0.148 respectively) but were higher at 26 weeks (p=0.020 and p=0.022 respectively) compared to the latter group. There was no difference in the count of anti-ENA specificities between the two groups at 14 and 26 weeks; all p>0.10.

4.3.7. Retreatment of first cycle non-responders

In RA, we showed that retreatment of incomplete depleters who were initial non-responders led to improved response rate in cycle 2 (240). In this present study, of 21 SLE patients who were cycle 1 non-responders, 9 were retreated with rituximab. The domains that persisted at grade A or B in cycle 1 were mucocutaneous (n=4), musculoskeletal (n=3), renal (n=2) and haematology (n=3). After retreatment, none of these patients responded. Moreover, 4 patients had clinical features that were suggestive of immunogenicity.

4.3.8. Retreatment of first cycle responders

Of the 96 SLE patients who were cycle 1 responders, 77 (with complete data on 72) were retreated on clinical relapse. Of these, 61/72 (85%) responded in cycle 2 (Figure 4-3). Numerically higher rate of B-cell depletion was achieved in cycle 2 compared to C1, 68% versus 58% respectively; p=0.206 and

depletion improved over subsequent cycle; cycle 3 versus cycle 1, 79% versus 58% respectively; p=0.022 (Figure 4-2B).

Twelve out of thirty-eight patients with PCR in cycle 1 were retreated at 6 months. Of these, MCR was achieved in 10/12 (83%) in cycle 2. One patient had worsening of arthritis while another had 2NDNR in cycle 2.

Of the 11 SLE patients who were cycle 2 non-responders, 9 met 2NDNR criteria. Therefore, the incidence of 2NDNR in this cohort was 9/77 (12%). In cycle 3, another 2 patients met 2NDNR criteria.
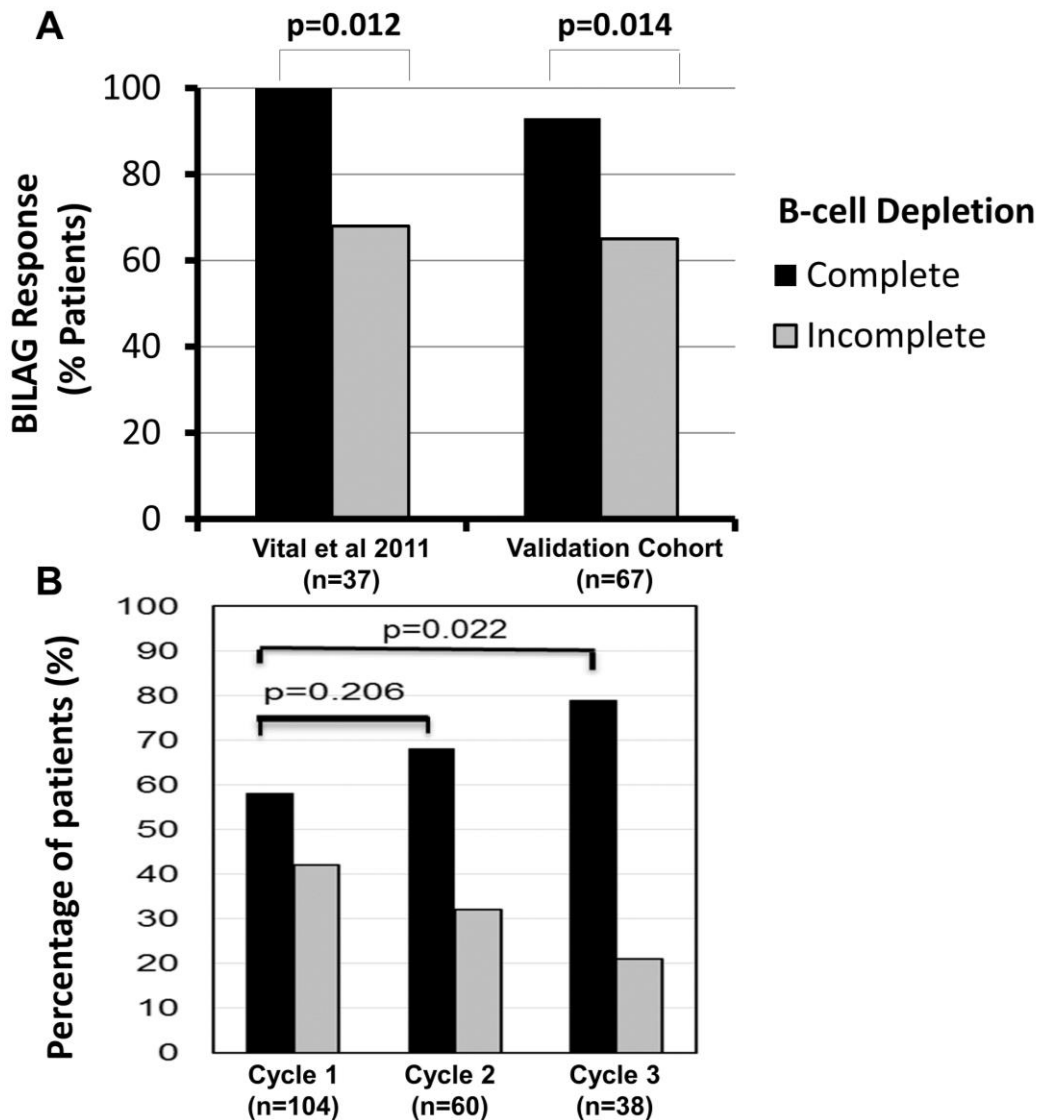
Figure 4-2 B-cell depletion as a biomarker of response and depletion over successive cycles

A) Similar to the discovery cohort, a higher response rate was achieved in complete depletion compared to incomplete depletion groups; 93% versus 68%; p=0.011 in the validation cohort B) There was an incremental increase in the rates of B-cell depletion over 3 cycles of rituximab. BILAG: British Isles Lupus Assessment Group.
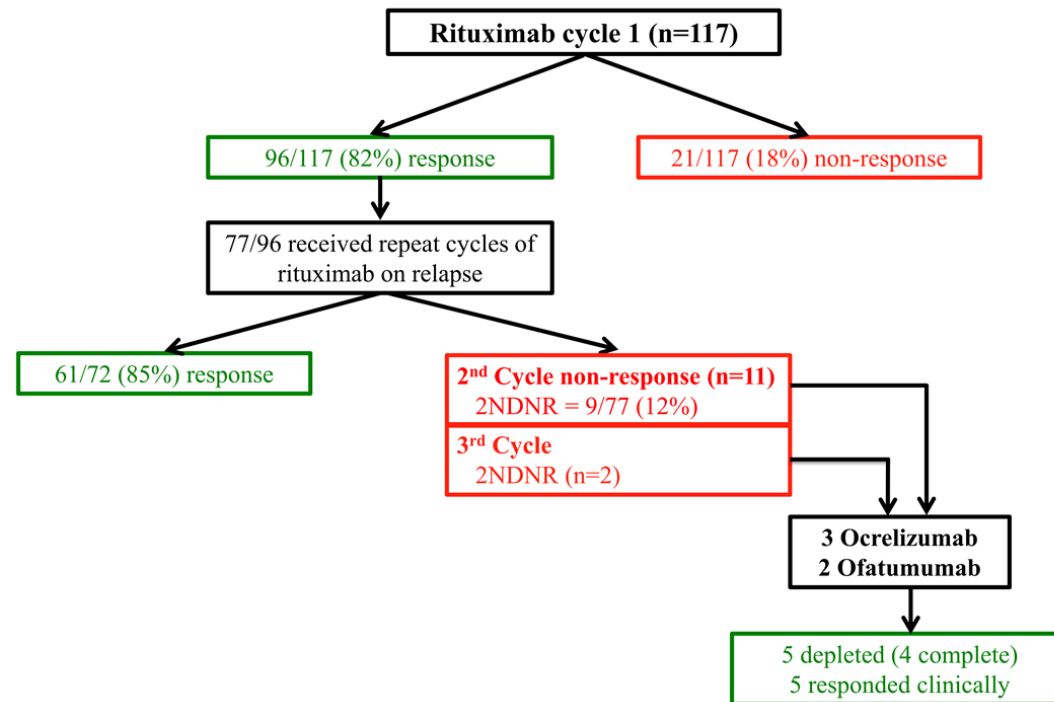
Figure 4-3 Efficacy of repeat cycles with rituximab in SLE

A high rate of initial clinical response to rituximab was observed in this cohort, 96/117 (82%). 77 responders who had clinical relapse were retreated in cycle 2. Of these, 61/72 (85%) continued to response in cycle 2. Of the cycle 2 non-responders, 9/11 met 2NDNR criteria. Five were switched to ocrelizumab or ofatumumab, resulted in depletion and response in all. 2NDNR: secondary non-depletion and non-response.

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4.3.9. Association of 2NDNR with anti-rituximab antibody

Anti-rituximab antibodies were tested in post-rituximab sera of 5/9 patients with 2NDNR. Of these, all 5/5 (100%) were tested positive. In contrast, of the 16 patients who were cycle 2 responders, 9/16 (56%) were also tested positive for anti-rituximab antibodies. The median anti-rituximab levels were higher in the former, 562 (IQR 394-9670) AU/ml compared to the latter group, 217 (IQR 0-409) AU/ml; p=0.024 (Figure 4-4).
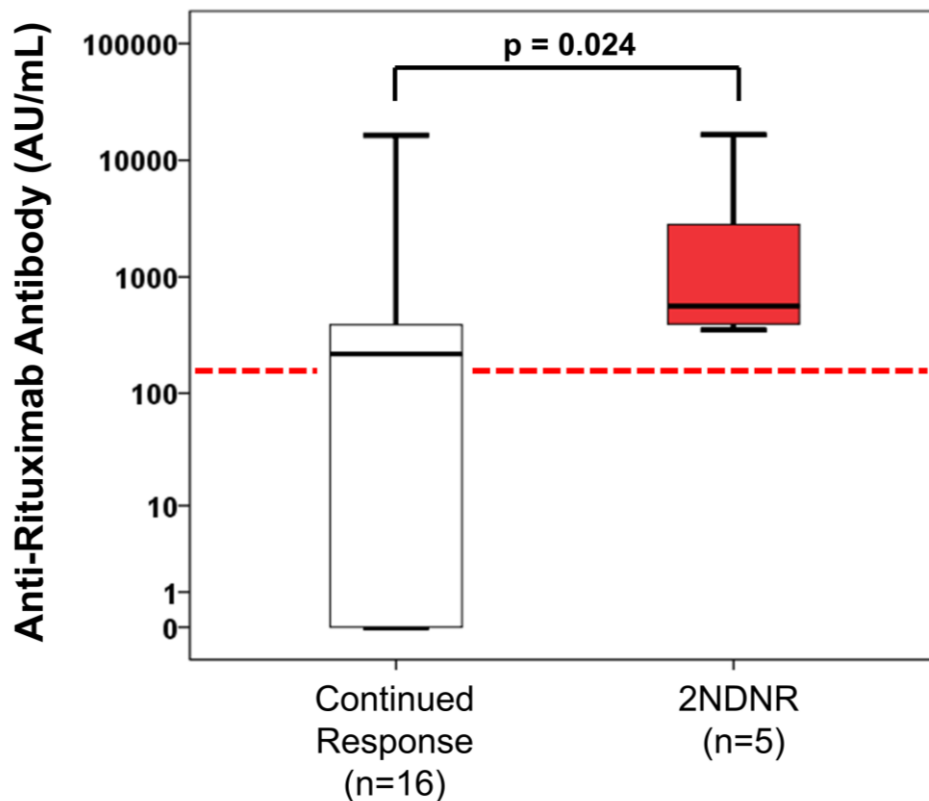
Figure 4-4 Patients with 2NDNR had higher anti-rituximab levels than those with continued response

> The phenomenon 2NDNR was associated with anti-rituximab antibody. Y-axis represents logarithmic scale with base 10 of the median anti-rituximab antibody level. Error bars denote interquartile range. The dotted red line represents normal cut-off of the anti-rituximab antibody ELISA test. 2NDNR: secondary non-depletion and non-response; ELISA: enzyme-linked immunosorbent assay

4.3.10. Factors associated with 2NDNR

Risk factors for 2NDNR were lack of concomitant DMARDs (p=0.023) and higher pre-rituximab plasmablasts (p<0.001) (Table 4-5). Concomitant corticosteroid dose, duration of response in cycle 1, clinical response category in cycle 1, pre-rituximab global BILAG score, pre-rituximab naïve- and memory B-cells were not associated with 2NDNR; all p>0.10.

Table 4-5 Factor associated with 2NDNR to rituximab

Characteristics prior to repeat rituximab	Continued response (n=61)	2NDNR (n=9)	P-value
Concomitant DMARDs, N (%)	41 (67)	2 (22)	**0.023**
Prednisolone, median (IQR) mg	5 (0-10)	5 (0-17.5)	0.729
Duration of response, median (IQR) weeks	50 (36-107)	62 (52-164)	0.239
Total BILAG score, median (IQR)	16 (12-21)	24 (12-27)	0.209
Partial clinical response in cycle 1, N (%)	24 (39)	3 (33)	0.731
Naïve B-cells, median (IQR) 10^9 cells/L	0.0349 (0.0071-0.0735)	0.0620 (0.0101-0.0950)	0.296
Memory B-cells, median (IQR) x 10^9/L	0.0019 (0.0010-0.0047)	0.0090 (0.0054-0.0394)	0.175
Plasmablasts, median (IQR) x 10^9/L	0.0011 (0.0004-0.0036)	0.0086 (0.0052-0.0227)	**<0.001**

2NDNR: secondary non-depletion and non-response; BILAG: British Isles Lupus Assessment Group; DMARDs: disease modifying anti-rheumatic drugs; IQR: inter-quartile range

4.3.11. Efficacy of switching to alternative humanised anti-CD20 antibodies

Following 2NDNR, treatment for 5 SLE patients were switched to humanised anti-CD20 mAbs (ocrelizumab=3 and ofatumumab=2). Post-treatment, complete depletion of total CD20+ cells were achieved in 4/5 patients while the remaining 1 had substantially low counts; 0.0016×10^9/L.

The median global BILAG scores had reduced from 24 (IQR 18-45) pre-treatment to 1 (IQR 0-8) post-treatment; p=0.008 (Figure 4-5A). The individual BILAG response is shown in Figure 4-5B and described in Table 4-6. One patient with class IV-G (active with moderate scarring) who had progressed into end-stage renal failure was treated with ofatumumab, mainly for severe thrombocytopenia with a view for renal transplantation preparation. Post-ofatumumab, her platelet had normalised from 45×10^9/L (pre-treatment), renal parameters were stable, and she successfully underwent live donor renal transplantation.
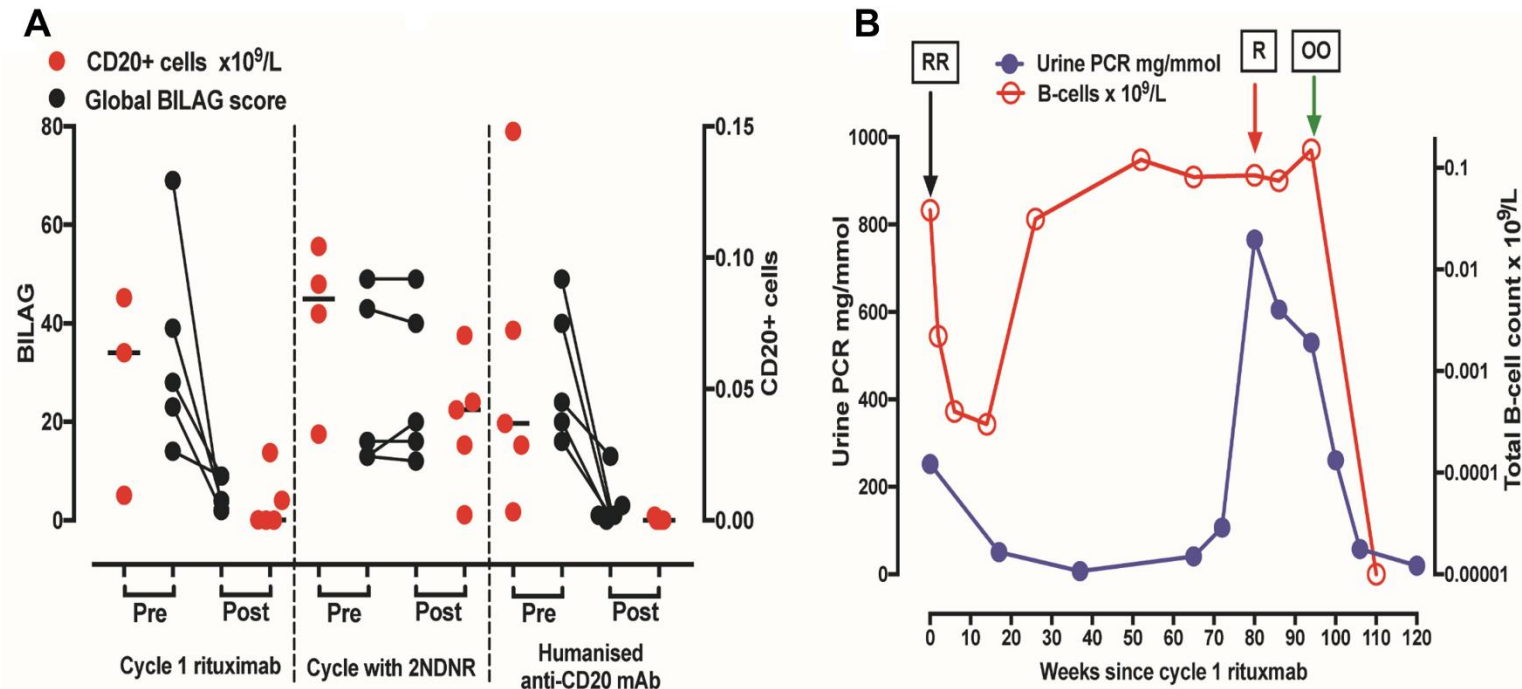
Figure 4-5 Efficacy of switching to humanised anti-CD20 antibodies

A) Global BILAG score and CD20+ B-cells are plotted for each patient. The black vertical line in the CD20+ B-cells figure represents the median. B) An example of a case where proteinuria was normalised following a switch to ocrelizumab. 'RR' represents 2 x infusions of rituximab, 'R' represents a single infusion as the patient cannot not complete the second due to severe infusion reaction and 'OO' represents 2 x infusions of ocrelizumab. Total B-cell counts were transformed to natural log. 2NDNR: secondary non-depletion and non-response; BILAG: British Isles Lupus Assessment Group; PCR: protein creatinine rato

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Table 4-6 Details of BILAG response following a switch to humanised anti-CD20 agents

Patient ID	Biologics	Pre-Treatment BILAG Activity	Pre-Treatment Global BILAG score	Post-Treatment BILAG Activity	Post-Treatment Global BILAG score	Pre-Treatment Total CD20+ B-cells x 10 ^{9}/L	Post-Treatment Total CD20+ B-cells x 10 ^{9}/L
01	OCR	B – General A – Mucocutaneous B – Musculoskeletal A – renal	40	D – General C – Mucocutaneous D – Musculoskeletal D – Renal	1	0.0032	0
02	OCR	B – General A – Renal	20	D – General D – Renal	0	0.1481	0
03	OCR	B – Neurological B – Musculoskeletal	16	D – Neurological C – Musculoskeletal	1	0.0724	0.0016
04	OFA	A – Renal A – Haematology	24	B – Renal C – Haematology	13	0.0369	0
05	OFA	B – General A – Mucocutaneous B – Neurological B – Musculoskeletal A – Renal C – Haematology	49	D – General C – Mucocutaneous D – Neurological D – Musculoskeletal C – Renal C – Haematology	3	0.0286	0

Note: 1 patient with severe SLE who had incomplete B-cell depletion and non-responder in cycle 1 was retreated with rituximab but subsequently developed immunogenicity. She was treated with ocrelizumab, resulted in enhanced depletion, biological response i.e. normalisation of anti-dsDNA and complement levels, as well as was able to be discharged home (after 3 months of prolonged hospitalisation). Unfortunately she died 5 months later due to multi-organ failure. This patient was not included in the above as she did not meet 2NDNR criteria.

2NDNR: secondary non-depletion and non-response; BILAG: British Isles Lupus Assessment Group, OCR: ocrelizumab; OFA: ofatumumab; SLE: systemic lupus erythematosus

4.3.12. Safety

138 SAEs were recorded in 54 patients who were treated with rituximab. Of these, 130 were hospitalisation episodes (median duration 5 (IQR 3-9) days), 3 malignancies and 5 deaths (Table 4-7). The causes of deaths were intracranial/subarachnoid haemorrhage=2, pneumonia=1, urinary sepsis=1 and multi-organ failure=1.

33 SIEs (6.7/100 patient-years) were recorded in 23 rituximab-treated patients, mostly due to chest infection (n=15). The 5 opportunistic infections recorded were *mycobacterium avium complex*=1, *pneumocystis jiroveci pneumonia*=1 in a patient who was simultaneously diagnosed as having human immunodeficiency virus (HIV) infection, cytomegalovirus=1, disseminated varicella zoster=1 and disseminated candidiasis. 36% (n=12) and 64% (n=21) of the SIEs occurred within 3 and 6 months respectively from the last rituximab infusion in any cycle. No cases of progressive multi-focal leukoencephalopathy (PML) were observed.

6 SAEs were recorded in 3 patients who were treated with ocrelizumab. Of these, 4 were SIEs (14/100 patient-years) which included one opportunistic infection with cytomegalovirus. One death occurred in a patient with severe SLE who died of multi-organ failure at 5 months post-ocrelizumab. No serious infection was recorded in the two patients who were treated with ofatumumab.

Table 4-7 Serious adverse events during treatment with anti-CD20 agents in SLE

	Rituximab (n=117)	Ocrelizumab (n=4)	Ofatumumab (n=2)
All severe adverse events			
No. of severe adverse events	138	5	5
No. patients with severe adverse events (%)	54 (46)	3 (75)	1 (50)
All serious infection, no. events	33	3	0
Pneumonia	15	1	0
Urinary tract infection	7	0	0
Opportunistic infections	4	1	0
Cellulitis/Skin abscess	3	0	0
Intra-abdominal abscess	1	1	0
Infectious diarrhoea	1	0	0
Necrotising fasciitis	1	0	0
Line infection	1	0	0
All other hospitalisation, no. events	97	1	5
SLE flare	31	0	0
Infusion reaction/serum sickness	12	0	0
Viral illness	8	0	0
Acute kidney injury	5	0	3
Bowel surgery/Hernia repair	5	0	0
Thromboembolism	4	0	0
Diverticulitis/Perforated colon	4	0	0
Orthopaedics surgery (Elective)	3	0	0
Kidney transplant	3	0	1
Acute coronary syndrome	2	0	0
Intracerebral haemorrhage	2	0	0
Palpitation/Atrial fibrillation	2	0	0
Avascular necrosis	1	0	0
Seizure	1	0	1
Kidney stones	0	1	0
Other medical	14	0	0
All malignancy, no. events	3	0	0
Renal transitional cell carcinoma	1		
Thymoma	1		
Uterine carcinoma	1		
Deaths	5	1	0

4.3.13. B-cell depletion and associated serious infection

As most of the SIEs occurred in cycles 1 and 2, (n=23 in 15 patients), we analysed the association between complete B-cell depletion and SIEs. After two cycles, there were no difference in the serious infection rates between complete and incomplete depletion groups; 8/98 (8.2%) and 7/73 (9.6%) respectively; p=0.789.

4.4. Discussion

The clinical challenges in the use of rituximab in SLE include defining subgroups of patients likely to respond to initial, subsequent cycles and establishing optimal repeat treatment strategy. By capturing data of all SLE patients who are treated with rituximab in this largest reported cohort as well as long-term follow-up to date, this study offers insights into pragmatic use of rituximab and has implications for the future development of targeted therapies.

In this study, the only consistent predictor of any and MCR to rituximab was B-cell depletion (as measured using HSFC) at 6 weeks post-rituximab. I have now validated this biomarker in an independent cohort. This underscores the immunomodulatory action of rituximab in terms of normalising autoantibody titres and complement levels without increasing the risk of severe infection. From treatment stratification perspective, my results provide the rationale for B-cell monitoring for as a biomarker for response prediction during therapy.

In this study, a high degree of clinical and immunological initial response to rituximab was confirmed in a wide range of SLE manifestations. Thus although not formally licensed, rituximab should remain an option in the treatment of patients with severe and refractory SLE. However, given the heterogeneity in clinical phenotype, it may be that there is no one-size-fits-all therapy. Clinical response was more varied for mucocutaneous manifestation and depended on the subtypes. In particular, CCLE was associated with poor clinical response. Although immune complex deposition is a common feature, non B-cell mechanisms through activation and apoptosis of keratinocytes and production of inflammatory cytokines and chemokines (274, 346, 347) are also

responsible in the pathogenesis of this lesion and consequently, should be targeted.

Regardless of initial response, about 12% subsequently developed 2NDNR in cycle 2. This phenomenon was associated with anti-rituximab antibodies. However, measuring anti-rituximab antibody alone was not enough to classify patients as 2NDNR as over half of the patients who were tested positive responded in that particular cycle. Instead, clinical features including severe infusion reaction, non-response and measuring B-cells were more meaningful. Lack of concomitant csDMARDs (excluding hydroxychloroquine only) and higher pre-rituximab plasmablasts predicted 2NDNR. Oral csDMARDs was decided at physicians' discretion but my data suggest they might have a role in preventing immunogenicity. The exact mechanism for the association of 2NDNR with plasmablast number is unknown but plasmablasts are markers for overall B-cell activation. Following initial depletion with rituximab, BAFF levels increase and promote the formation of plasmablasts (248). This early increase in plasmablasts enhances the formation of follicular T-helper cells, thus creating a positive feedback loop that perpetuates antibody-driven inflammation and may explain why some patients become refractory to rituximab in SLE (348).

Following 2NDNR to rituximab, switching to humanised alternative anti-CD20 mAbs restores depletion and response in SLE. Ocrelizumab and ofatumumab are both type 1 anti-CD20 mAbs. The primary endpoint was met in ocrelizumab-treated groups in RA trials (252) and was investigated in SLE (253). However, development in these indications was halted after an increase in opportunistic infections, some of which fatal were reported (254). All 3 patients in our study had MCRs and prolonged remission for over 5 years' period post-ocrelizumab. Ofatumumab is licensed for resistant CLL and has demonstrated efficacy in RA (255). Both patients in this present study responded well to ofatumumab included one who achieved complete depletion for the first time from B-cell depleting therapy. Moreover, a few case series have recently reported on its efficacy in non-renal and refractory lupus nephritis (349, 350). Alternatively, other anti-CD20 agents with enhanced ADCC may be more effective in SLE. *In vitro* obinutuzumab demonstrated that

enhanced depletion was achieved with this type 2 mAb, compared to rituximab (351).

This study has some limitations. First, an inter-observer variability could have occurred in BILAG assessments due to the lengthy follow-up duration as well as a cohort that was highly heterogeneous in lupus manifestations. However, the BILAG scores reflected the clinicians' intention-to-treat and the patients were managed in a dedicated single centre, thus allowing for consistency in assessment. Second, B-cells and laboratory data were missing in some cases. As these were deemed missing at random, multiple imputation was used to reduce potential bias in parameter estimation as well as enhancing generalisability of the results. Next, concomitant therapy with csDMARDs were used in more than 60% of the patients, thus efficacy could not be attributed to rituximab alone. Lastly, the lack of control group limits interpretation of efficacy and safety of rituximab.

4.5. Conclusion

In conclusion, treatment with anti-CD20 agents should be guided by B-cell monitoring with the aim of achieving complete depletion. 12% of the SLE patients lose depletion on repeat cycles of rituximab regardless of prior response and secondary non-depletion is associated with anti-rituximab antibodies. Concomitant csDMARDs may help to prevent this. If 2NDNR occurs, switching to humanised anti-CD20 mAbs restores depletion and response. Therefore, alternative humanised anti-CD20 antibodies may be more consistently effective in SLE treatment and several ongoing trials are addressing these issues.

4.6. Key messages

i.   Rituximab is effective for a wide range of SLE manifestations as well as no major safety signals were observed in long-term follow-up of this cohort study.

ii. B-cell depletion (as measured at 6 weeks post-rituximab) is an independent predictor of any or major clinical response to rituximab, which has been validated in a second cohort.

iii. 12% of patients with SLE lose depletion and response on repeat cycles regardless of prior response, and this 2NDNR phenomenon is associated with anti-rituximab antibodies.

iv. If 2NDNR occurs, switching to alternative humanised anti-CD20 antibodies restores depletion and clinical response.

Chapter 5. B-cell biomarkers in systemic lupus erythematosus and other B-cell mediated autoimmune rheumatic diseases

5.1. Introduction

Clinical efficacy of rituximab in RA has been associated with the level of both synovial membrane B-cell depletion (352, 353) and early peripheral blood plasmablast depletion as measured using sensitive assays (234); the latter possibly functioning as a surrogate marker. Following depletion with rituximab, repopulation of B-cells lead to a relapse in most patients (albeit after a variable interval after repopulation). However, the optimal retreatment strategy has not been clearly defined. The most common strategy is to give repeat cycles on clinical relapse. However, these relapses may give rise to further structural deterioration or, in severe CTD, may cause life- or organ-threatening disease for patients. An alternative approach is to use pre-emptive using fixed-intervals strategy (e.g. biannually), but this potentially exposes the patient to overtreatment and enhances the risk of adverse events as well as being expensive. Moreover, the time-to-relapse is specific to each patient and can be difficult to predict prior to treatment. To overcome this, one potential strategy may be to guide treatment decisions based on B-cell biomarkers; specifically monitoring for the depth of B-cell depletion and regeneration kinetics of B-cell subsets after rituximab administration.

In SLE, our group previously showed that initial complete B-cell depletion was associated with better initial clinical response (16), which was validated in my thesis (section 4.3.5) and subsequently published (246). However, factors leading to complete depletion is unclear. If this could be predicted, then modification of therapy to improve depletion would be made possible. Additionally, our group also reported that following B-cell depletion, there was a bimodal pattern of relapse i.e. earlier (within 18 months of first cycle rituximab) versus later relapse (>18 months). Earlier relapse requiring further rituximab therapy was predicted by a plasmablast count of $>0.0008 \times 10^9$/L at

6 months (the time of initial clinical response) whilst those with lower plasmablasts at 6 months had sustained response without retreatment. Validation of this as a biomarker is therefore needed to determine whether B-cell enumeration using HSFC can be used in clinical practice to guide retreatment decisions.

Simiarly, in RA, our group previously reported that the initial depth of B-cell depletion (measured after the first infusion of rituximab) was associated with clinical response; 96% complete versus 74% incomplete depletion at 6 months (p=0.020) (234). Furthermore, retreatment of initial non-responders with incomplete depletion led to 72% response rate in cycle 2 (240). These two aspects also need validation in a second cohort for this biomarker to be used in clinical practice.

Rituximab has been licensed by US FDA and EMA for remission induction in AAV since 2011 (258, 259). For this indication, retreatment strategy remains problematic. The fixed-intervals strategy either by administering 1g rituximab every 6 months or 2g rituximab every 12 months had led to secondary hypogammaglobulinaemia, increased rates of infection and cessation of therapy (261, 354). Despite evidence for the pathogenicity of ANCA (355), serum levels of these antibodies do not appear to be clinically useful as a biomarker of relapse. A meta-analysis of 18 studies demonstrated that ANCA titres were only modestly predictive of relapses (356). Thus, a reliable biomarker for relapse prediction is warranted and B-cells had not previously been analysed.

5.2. Hypothesis

Enumeration of B-cell subsets using HSFC will predict clinical response and relapse in SLE, RA and AAV patients treated with rituximab.

5.3. Objectives

i. To validate B-cell depletion after the first infusion as a predictor of response to rituximab (Project 1)

ii. To assess outcome of retreatment of first cycle non-responders in RA (Project 2)

iii. To assess factors contributing to complete B-cell depletion in first cycle rituximab for the treatment of SLE (Project 3)

iv. To validate the association of repopulation of plasmablasts at 6 months post-rituximab with clinical relapse in SLE (Project 4)

v. To assess the association of repopulation of B-cell subsets at 6 months post-rituximab with clinical relapse in AAV (Project 5)

vi. To compare utility of B-cell biomarkers to guide rituximab treatment decisions in RA, SLE and AAV

5.4. Methods

5.4.1. Candidate's roles in this project

In this work, the initial concept and design of the study were set by myself, Prof Emery and Dr Vital. I carried out the weekly Biologics, Lupus and Vasculitis clinic in Leeds under the supervision of Dr Vital and Prof Emery. I performed a significant proportion of the clinical assessments. Peripheral blood B-cell subsets were measured using HSFC at the Haematological Malignancy Diagnostic Service, Leeds Teaching Hospitals NHS Trust. I organised and entered half of the data (over 1000 patients) onto the study database (the other half was done by Dr Das, Dr Baptiste Candelier, Dr Eugenio and Dr Garcia-Montoya). I performed all statistical analyses and led the writing of the manuscript, while other co-authors revised the draft critically for important intellectual content and final approval of the manuscript prior to submission to journal for publication.

5.4.2. Design and patients

A retrospective observational study was conducted of all patients with ARDs, who were treated with at least a course of rituximab in Leeds between Jan 2002 and July 2016.

Inclusion criteria were (i) adults (>16 years old); (ii) fulfilling the classification criteria for RA (329), SLE (32) and AAV (357) and (iii) at least 3 months follow-

up post-rituximab. The published discovery cohorts included 60 RA patients in Project 1 (234) and 25 RA patients in Project 2 (240). For this validation study, we analysed the subsequent consecutive 180 patients with RA treated with rituximab in Leeds.

The published discovery cohort included 37 patients with SLE in Project 4 (16). For this validation study, the subsequent consecutive 78 patients with SLE treated with rituximab in Leeds were studied (see details and characteristics of patients in section 4.3.1). The total cohort was used to analysed predictors of complete B-cell depletion in SLE (Project 3).

For Project 5, the patients received rituximab if there was active severe disease despite either a course of cyclophosphamide lasting 3–6 months or maximum cumulative cyclophosphamide dose of 25g (358) or in circumstances where cyclophosphamide could not be given because of previous toxicities associated with therapy i.e. profound leukopenia and haemorrhagic cystitis and other relative contraindications such as a history of solid malignancy and issues with fertility (359). Blood from HCs were used as a negative control for comparison among diseases.

5.4.3. Ethical approval

This study did not require ethical approval because all treatment decisions were made prior to evaluation of data, in accordance with the UK National Health Service Research Ethics Committee guidelines. Peripheral B-cell analyses were performed as standard of care of patients receiving treatment with rituximab in Leeds and results were reported in the Trust server. The HCs' blood samples used for this study were collected under ethical approval, 04/Q1206/107, National Research Ethics Committee Yorkshire and Humber–Leeds East. The off-label use of rituximab prior to its approval in RA and AAV and commissioning by NHS England for SLE was all approved by the Leeds Teaching Hospitals NHS Trust Drug and Therapeutic Committee.

5.4.4. Treatment protocol

All patients received a first cycle of treatment consisted of 100 mg of methylprednisolone and 1000 mg of rituximab given intravenously on days 1 and 14. Further cycles of the same regimen were repeated on clinical relapse.

For Project 2, patients who had incomplete B-cell depletion and non-response in cycle 1 were given a further course of rituximab (using the same regimen as above) at 6 months.

Continuation of a stable or a reduced dose of concomitant DMARDs (including oral corticosteroid), was left to investigator's discretion with the aim to stop glucocorticoid if clinical response was achieved at 6 months.

5.4.5. Assessment schedule and follow-up arrangements

Comprehensive assessments including clinical, laboratory and B-cell biomarkers were performed at baseline, 3 months, 6 months and every 6-monthly then after. Participants were given a helpline number to contact for an additional flare visit to be organised if they had new or worsening inflammatory symptoms.

5.4.6. Clinical data and outcomes

For RA, disease activity was assessed using DAS-28 at baseline and every 3 months. Response at 6 months was defined according to the criteria of the European League Against Rheumatism (EULAR) (360).

For SLE, disease activity was assessed using the BILAG-2004 index at baseline and every 3 months thereafter. Criteria for clinical responses at 6 months were described in section 4.2.4.

For AAV, disease activity was assessed at baseline and every 3 months post-therapy using Birmingham Vasculitis Activity Score (BVAS 3.0) (361) without knowledge of B-cell results. Complete response (CR) was defined as BVAS = 0 while partial response (PR) was defined as 50% improvement in BVAS from baseline, both assessed at 6 months. Relapse was defined as an increase in the BVAS $\geq$ 1.

5.4.7. Peripheral B-cell analysis using HSFC

Peripheral blood B-cell subsets (naïve, memory B-cells and plasmablasts) were measured using HSFC at the Haematological Malignancy Diagnostic Service, Leeds Teaching Hospitals NHS Trust at baseline, 6 months and every 6 months without knowledge of patients' clinical status other than time since rituximab. The protocol for analysis is detailed in section 4.4.5.

Complete B-cell depletion was defined as counts $<0.0001 \times 10^9$/L and repopulation as $\geq 0.0001 \times 10^9$/L.

5.4.8. Other relevant laboratory assessments

Full blood count and C-reactive protein (CRP) were processed at a single accredited diagnostic laboratory.

The ANCA staining pattern; cytoplasmic (cANCA) or perinuclear (pANCA) was determined by indirect immunofluorescence. Measurement of antigen specificity for myeloperoxidase (MPO), proteinase-3 (PR3), anti-dsDNA and anti-ENAs were made using ELISA (until July 2012) and Bioplex 2200 Immunoassay (after July 2012). Complement levels (C3 and C4) and total serum immunoglobulin titres were measured by nephelometry.

5.4.9. Statistical analyses

Descriptive statistics were summarised using mean with standard deviation or median with interquartile range for continuous variables where appropriate and proportion for categorical variables. The significance of the association between categorical variables was tested by Fisher's exact test while for continuous variables using either Student's T-test or Mann-Whitney U test where appropriate. B-cell subsets over time among the three B-cell mediated diseases were compared ANOVA followed by pairwise Tukey tests. To account for missing data, multiple imputation by chained equations was used to create 20 complete datasets, results of which were combined according to Rubin's rules. The imputed multivariable analyses of predictors of complete B-cell depletion post-rituximab in SLE were analysed using binary logistic regression. Relapse-free survival time (measured in weeks) was calculated from the date of first rituximab infusion to either the date of rituximab re-treatment or the date of data last updated (July 2016). ROC curve analysis was used to measure sensitivity and specificity of optimal thresholds for investigations predicting time-to-clinical relapse in SLE. For AAV, relapse-free survival analysis for seven continuously distributed variables and six categorically distributed variables were analysed using univariate Cox-regression analysis. Analysis for other categorically distributed variables that were relevant for relapse prediction was calculated using Kaplan–Meier plot

and log-rank test. All statistical analyses was performed using Stata 13.1 (StataCorp College Station, Texas, USA) GraphPad Prism v.7.03 (GraphPad, La Jolla, CA, USA) for Windows.

5.5. Results

5.5.1. Project 1: Validation of depletion as a predictor of response in RA

Patient characteristics

Following the publication of the discovery cohort (234), data for the consecutive 180 patients with RA who were treated with rituximab were analysed (362). There was no difference in key clinical characteristics between the two cohorts. Baseline characteristics are summarised in Table 5-1.

Table 5-1 Comparison of clinical characteristics between the discovery and validation cohorts of RA patients treated with rituximab

Characteristics	Discovery Cohort (n=60)	Validation Cohort (n=180)
Mean age (SD), years	59.0 (15.2)	59.9 (13.7)
Female, n (%)	45 (75)	147 (81)
Median RA disease duration (IQR), year	12 (6 – 18)	10 (5 - 18)
RF and/or CCP antibodies positivity, n (%)	60 (100)	175 (97)
Previous TNF-inhibitors exposure, n (%)	40 (67)	108 (60)

CCP: cyclic citrullinated peptide; IQR: interquartile range; RA: rheumatoid arthritis; RF: rheumatoid factor; TNF: tumour necrosis factor

Validation of association between complete B-cell depletion and clinical response in RA

Similar to the discovery cohort, higher EULAR response (Good or Moderate) rate was achieved in complete B-cell depletion compared to incomplete depletion groups in RA; 77% versus 61%; p=0.036 in this validation cohort (Figure 5-1). Overall, combining results from both cohorts (n=240), we had confirmed in a large cohort of rituximab-treated RA patients that complete B-cell depletion after the first infusion of rituximab was associated with EULAR response in RA; 79% complete depletion versus 61% incomplete depletion groups, p=0.003.
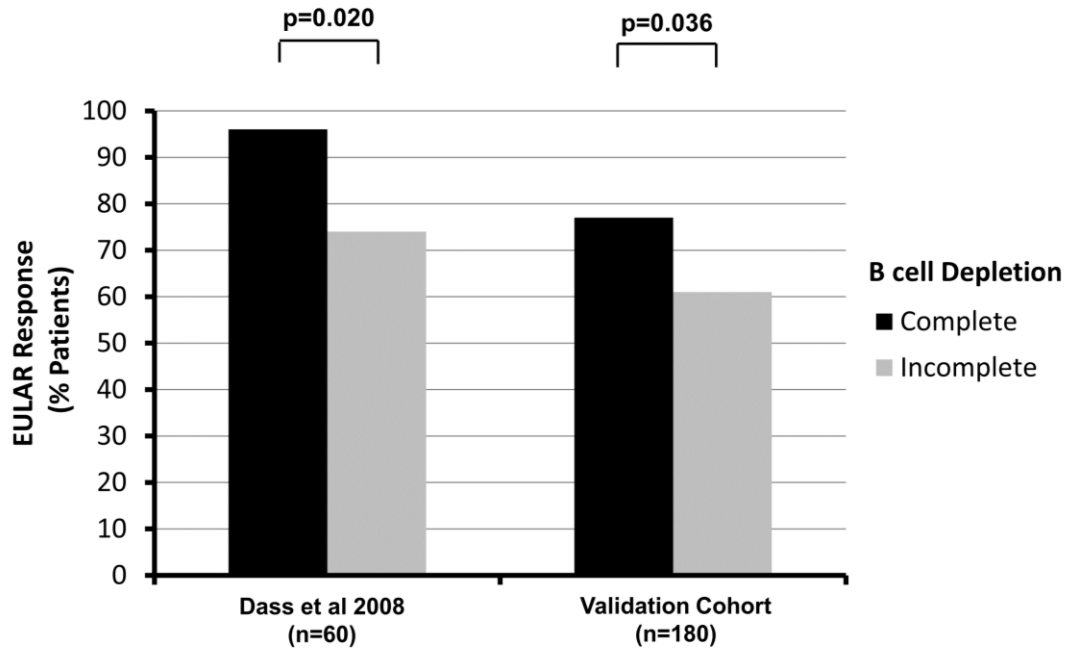
Figure 5-1 Association of complete B-cell depletion and EULAR response in RA

Similar to the discovery cohort, a higher EULAR response rate was achieved in complete depletion compared to incomplete depletion groups; 77% versus 61%; p=0.036 in the validation cohort. EULAR: European league against rheumatism; RA: rheumatoid arthritis

5.5.2. Project 2: retreatment of first-cycle non-responders in RA

Outcome of retreatment of first cycle non-responders in RA

Of 180 patients with RA who were treated with rituximab in this validation cohort, 30 patients who were cycle 1 non-responders and had incomplete depletion were retreated at 6 months. Of these, 20/30 (67%) had complete depletion and responded in cycle 2. This rate was about similar to the discovery cohort (240). In this validation cohort, non-responders in cycle 2 had a trend to higher plasmablasts at retreatment than responders; median count 0.0027 ×10^9/L and 0.0012 ×10^9/L respectively; p=0.145.

5.5.3. Project 3: Predictors of complete B-cell depletion to first cycle rituximab in SLE

Data for B-cell subsets were available for 104/117 (89%) of the patients. In imputed univariable analysis, higher anti-dsDNA titre (OR 1.00, 95% CI 0.99 to 1.00; p=0.038), normal complement levels (OR 0.41, 95% CI 0.18 to 0.91; p=0.028) and lower pre-rituximab plasmablasts (OR 0.88, 95% CI 0.80 to 0.98; p=0.015) were associated with complete B-cell depletion in cycle 1. While in the imputed multivariable model, only normal complement levels (OR 0.29, 95% CI 0.09 to 0.90; p=0.032) and lower pre-rituximab plasmablasts (OR 0.86, 95% CI 0.78 to 0.96; p=0.007) predicted complete B-cell depletion post-rituximab in cycle 1 (Table 5-2).

Table 5-2 Predictors of complete B-cell depletion post-first cycle rituximab in SLE

	Incomplete B-cell Depletion (n=44)	Complete B-cell Depletion (n=60)	Univariable OR (95% CI), P-value (with multiple imputation)	Multivariable OR (95% CI), P-value (with multiple imputation)
Anti-dsDNA titres, mean (SD) IU/ml	200 (282)	101 (166)	**1.00 (0.99-1.00), p=0.038**	1.00 (0.99-1.00), p=0.105
Anti-ENA positivity, N (%)	31 (70)	36 (60)	0.63 (0.27-1.44), p=0.273	0.75 (0.27-2.07), p=0.579
Low C3 and/or C4 titres, N (%)	25 (57)	21 (35)	**0.41 (0.18-0.91), p=0.028**	**0.29 (0.09-0.90), p=0.032**
Concomitant DMARDs, N (%)	25 (57)	43 (72)	1.92 (0.85-4.36), p=0.118	2.66 (0.98-7.27), p=0.055
Corticosteroid dose, mean (SD) mg	15 (11)	13 (14)	0.99 (0.96-1.02), p=0.339	1.02 (0.97-1.06), p=0.429
Total BILAG score, mean (IQR)	23 (11)	23 (11)	1.00 (0.97-1.04), p=0.938 per point	1.05 (1.00-1.11), p=0.064
Naïve B-cell counts, mean (SD)*	101 (105)	74 (79)	0.99 (0.99-1.00), p=0.202	0.99 (0.99-1.00), p=0.111
Memory B-cell counts, mean (SD)*	29 (31)	27 (70)	1.00 (0.99-1.01), p=0.889	1.00 (0.99-1.01), p=0.658
Plasmablast counts, mean (SD)*	8 (10)	3 (5)	**0.88 (0.80-0.98), p=0.015**	**0.86 (0.78-0.96), p=0.007**

*(count x 10^9 cells/L) for each subset multiply by 1000 prior to analysis. BILAG: British Isles Lupus Assessment Group; C3/C4: complement 3 or 4; DMARDs: disease modifying anti-rheumatic drugs; dsDNA: double-stranded deoxyribonucleic acid; ENA: extract nuclear antigen

5.5.4. Project 4: Validation of the association of repopulation of plasmablasts at 6 months post-rituximab with clinical relapse in SLE

At 6 months, B-cells were detectable in 81% of the C1 responders to rituximab. This time-point preceded all clinical relapses. As the median of duration of response to rituximab was 52 weeks, we divided the patients in this validation cohort (n=25 with B-cells data available) into two groups: (i) earlier relapse (≤12 months from first rituximab) and (ii) later relapse (>12 months). A 12-month relapse time was clinically significant as it indicated that a 6-monthly retreatment might not be necessarily needed in these patients. Similar to the discovery cohort (16), the ROC curve analysis indicated that a plasmablast count of >0.0008 x 10^9/L at 6 months yielded 73% (95% CI 45-92%) sensitivity and 90% (95% CI 56-99%) specificity in predicting earlier relapse; the AUROC of 0.86 (Figure 5-2A).

Of patients with plasmablasts >0.0008 x 10^9/L at 6 months, relapse rates within the next 6 and 12 months were 90% and 100% respectively. While of patients with plasmablasts ≤0.0008 x 10^9/L at 6 months, relapse rates within the next 6 and 12 months were 33% and 73% respectively (Figure 5-2B).

There were no differences in anti-dsDNA titres, the total BILAG score and memory B-cells at 6 months between the earlier versus later relapse groups, p=0.475, p=0.985 and p=0.414 respectively.
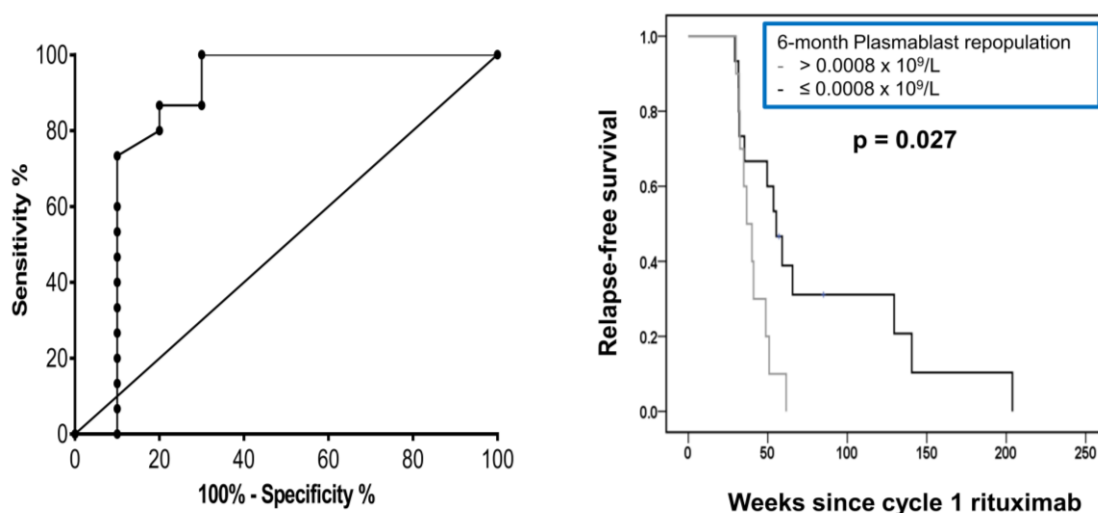
Figure 5-2 Predictive values of plasmablast repopulation at 6 months with clinical relapse to rituximab in SLE

A) ROC curve analysis indicated that a plasmablast count of >0.0008 x 10^9/L at 6 months demonstrated 73% sensitivity and 90% specificity in predicting earlier relapse. B) Similar to the discovery cohort, detection of plasmablasts >0.0008 x 10^9/L at 6 months predicted earlier relapse in this validation cohort. ROC: receiver operating characteristic

Adapted with permission from the BMJ publishing group. Md Yusof MY et al. Predicting and managing primary and secondary non-response to rituximab using B-cell biomarkers in systemic lupus erythematosus. *Ann Rheum Dis.* 2017 Nov;76(11):1829-1836

5.5.5. Project 5: association of B-cell subsets with relapse in AAV

Patient characteristics

A total of 37 consecutive patients with active severe AAV received treatment with rituximab. Only 35 patients were included in the analysis as two patients subsequently followed a pre-emptive re-treatment strategy guided by rising ANCA levels (162 patient-years follow-up). Baseline characteristics are described in Table 5-3.

Table 5-3 Baseline characteristics of the 35 patients with AAV treated with rituximab

Characteristic	Values
Median age at first rituximab infusion (IQR), years	56 (38-65)
Female patient, n (%)	18 (51)
Median AAV disease duration from diagnosis to first rituximab (IQR), months	27 (12-60)
Positive ANCA at diagnosis, No. (%)	
C-ANCA PR3	25 (71)
P-ANCA MPO	5 (14)
Immunofluoresence only	4 (11)
Negative but with a positive histology	1 (4)
Positive ANCA at first rituximab infusion, n (%)	23 (66)
Prior CyC therapy, n (%)	31 (89)
Cumulative dose of CyC, mean ± SD gram	12.9 ± 11.7
CyC contraindicated, n (%)	4 (11)
Previous therapies, n (%)	
Methotrexate	13 (37)
Azathioprine	13 (37)
Mycophenolate Mofetil	9 (35)
Plasma Exchange	2 (6)
Other	3 (9)
Median No. prior DMARDs including CyC but excluding steroid (IQR)	2 (1-2)
Organ system involvement at first rituximab infusion, n (%)	
Musculoskeletal and General (fever and myalgia)	17 (39)
Mucocutaneous	6 (17)
Eyes	4 (11)
Ear, nose and throat (ENT)	23 (66)
Chest	15 (43)
Abdominal	1 (3)
Renal	12 (34)
Nervous system	3 (9)
BVAS at first rituximab infusion, mean ± SD	10.5 ± 6.0

ANCA: Anti-neutrophil cytoplasmic vasculitis; BVAS: Birmingham Vasculitis Activity Score; C-ANCA: cytoplasmic ANCA; CYC: cyclophosphamide; DMARDs: disease modifying anti-rheumatic drugs; GPA: Granulomatosis with polyangiitis; IQR: inter-quartile range; P-ANCA: peri-nuclear ANCA; VDI: Vasculitis Damage Index

Clinical response

Response rates at 6 months (Complete/Partial) were high; above 83% in all first five cycles of rituximab. Response rates for cycles 1-5 were 33/35 (94%), 28/28 (100%), 17/20 (85%), 11/13 (85%) and 5/6 (83%) respectively. All BVAS non-responders responded to a subsequent cycle when retreated at minimum retreatment time. No patient had to permanently switch therapy due to non-response.

Association of complete B-cell depletion with BVAS response in AAV

There was a weak trend to association between complete peripheral B-cell depletion at 6 weeks post-rituximab and clinical response (major/partial) at 6 months, although this analysis was limited by the small number of non-responders; 97% complete versus 86% incomplete depletion; p=0.187 (Figure 5-3).
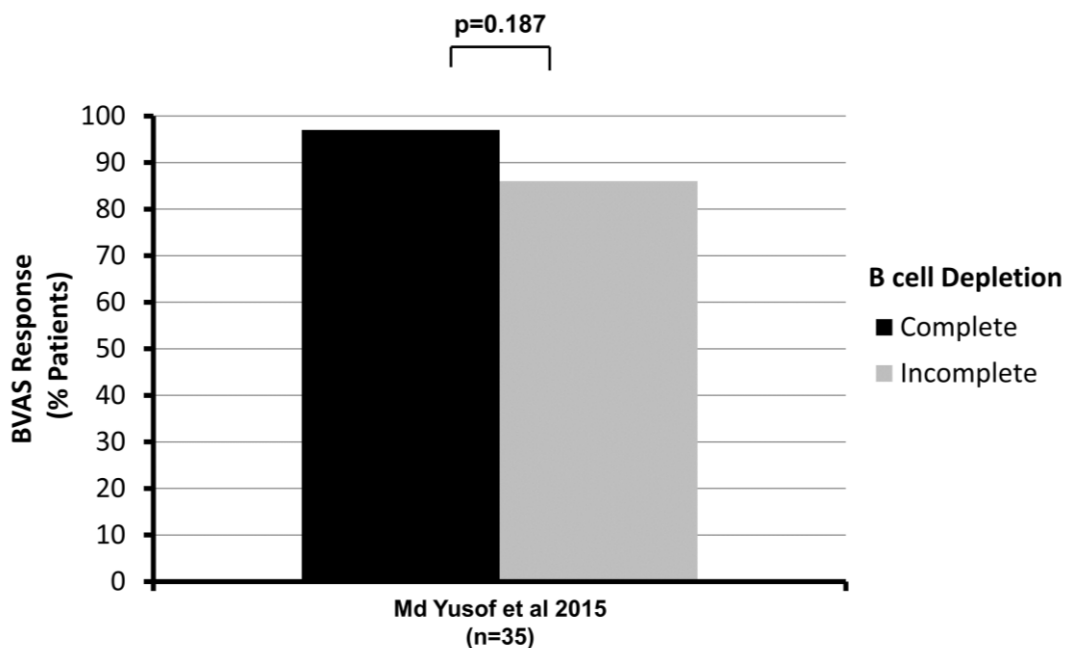
Figure 5-3 Association of complete B-cell depletion with clinical response in AAV

Despite a trend to association, there was no significant difference in the proportion of AAV patients who achieved a BVAS response according to their B-cell depletion status. AAV: anti-neutrophil cytoplasmic antibody-associated vasculitis; BVAS: Birmingham Vasculitis Activity Score

Comparison of B-cell kinetics in AAV with RA and SLE

Peripheral B-cells, which were measured before and after rituximab, were compared among age-matched RA (n=95), SLE (n=44) and AAV (n=35) patients (Figure 5-4).

At baseline, there was no difference in total B-cell counts among RA, SLE and AAV groups (ANOVA F=2.89; p=0.059) although there was a trend to higher total B-cell counts for RA relative to AAV [mean difference 0.0645×10^9/L (95% CI -0.0003 to 0.1292); p=0.051]. At 2 weeks post-rituximab, B-cell numbers were generally low and there was no difference in total B-cell counts among RA, SLE and AAV groups (ANOVA F=0.08; p=0.920).

At 6 weeks post-rituximab, although a large proportion of patients had complete B-cell depletion, there were differences among RA, SLE and AAV groups (ANOVA F=6.17; p=0.003). The total B-cell counts were higher for SLE relative to RA [mean difference 0.0060×10^9/L (95% CI 0.0018 to 0.0103); p=0.003] and for SLE relative to AAV [mean difference 0.0063×10^9/L (95% CI 0.0002 to 0.0124); p=0.040]. The rates for complete B-cell depletion for SLE, RA and AAV were 49%, 58% and 59% respectively.

At 26 weeks post-rituximab, there was no difference in total B-cell counts among RA, SLE and AAV groups (ANOVA F=2.89; p=0.059) although there was a trend to higher total B-cell counts relative to AAV in both SLE [mean difference 0.0182×10^9/L (95% CI -0.0017 to 0.0382); p=0.080] and RA [mean difference 0.0163×10^9/L (95% CI -0.0011 to 0.0337); p=0.070]. The rates for B-cell repopulation at 26 weeks for SLE, RA and AAV were 88%, 84% and 70% respectively.
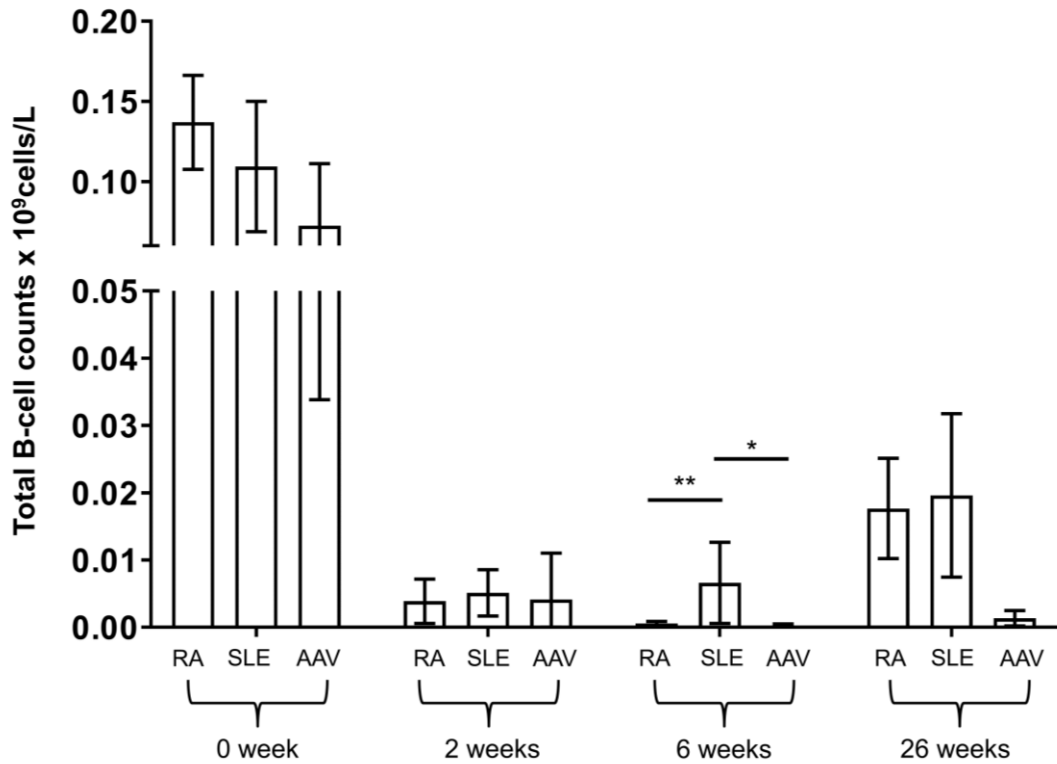
Figure 5-4 Comparison of B-cell kinetics before and after rituximab across the three B-cell mediated diseases

At baseline and 2 weeks post-rituximab, there were no differences in total B-cell counts among RA, SLE and AAV groups (ANOVA p=0.059 and p=0.920 respectively. However at 6 weeks post-rituximab, although a large proportion of patients had complete B-cell depletion, the total B-cell counts were higher for SLE relative to RA [mean difference 0.0060 x 10^9/L (95% CI 0.0018 to 0.0103); p=0.003] and for SLE relative to AAV [mean difference 0.0063 x 10^9/L (95% CI 0.0002 to 0.0124); p=0.040]. ** Moderate significant (0.001<p-value<0.01), *Significant (0.01<p-value<0.05). Bar charts represent mean total B-cell counts while error bars denote standard error of mean. AAV: anti-neutrophil cytoplasmic antibody-associated vasculitis; RA: rheumatoid arthritis; SLE: systemic lupus erythematosus

Predictors of relapse in AAV based on baseline characteristics

In AAV, using univariate cox-regression, only higher baseline memory B-cell numbers was a significant predictor of earlier relapse (HR: 1.01, 95% CI [1.00-1.03]) with p=0.040 (Table 5-4). However, this association was not reproduced in the subsequent cycles. Multivariable analysis was not performed since there was only 1 baseline significant predictor and a small sample size to prevent from overfitting of data.

Before the first rituximab infusion, AAV was characterised by naïve and memory B-lymphopenia compared to HCs, all p<0.05. These features which were suggestive of abnormal B-cell homeostasis or trafficking, were more marked in patients with raised CRP (Figure 5-5).

Table 5-4 Predictor of earlier relapse of AAV using univariate cox-regression analysis

Predictors	Hazard Ratio	p-value	95% Confidence Interval
Baseline Clinical Characteristics			
Age at baseline, years	0.995	0.635	0.903 – 1.017
Disease duration at baseline, months	1.005	0.170	0.998 – 1.012
BVAS, per point increase in score	1.001	0.964	0.939 – 1.038
CRP at baseline, mg/L	1.005	0.359	0.994 – 1.016
ANCA positivity at baseline (Y/N)**	1.038	0.918	0.508 – 2.121
Concomitant DMARDs (Y/N)	1.036	0.926	0.494 – 2.174
Clinical Characteristics at 26 weeks			
BVAS, per point increase in score	1.152	0.160	0.946 – 1.404
CRP, mg/L	0.976	0.152	0.944 – 1.009
ANCA positivity (Y/N)	1.258	0.550	0.555 – 3.021
PR3 titre, U/mL	1.007	0.695	0.973 – 1.042
MPO titre, U/mL	1.055	0.208	0.970 – 1.148
B-cell subsets, depletion and repopulation			
Naïve B-cells at baseline, cells/L*	1.004	0.329	0.996 – 1.012
Memory B-cells at baseline, cells/L*	**1.014**	**0.040**	**1.001 – 1.028**
Plasmablasts at baseline, cells/L*	0.884	0.187	0.736 – 1.062
Complete depletion at 6 weeks (Y/N)	1.088	0.813	0.540 – 2.192
Naïve B-cell repopulation at 26 weeks (Y/N)	**0.326**	**0.036**	**0.114 - 0.930**
Memory B-cell repopulation at 26 weeks (Y/N)	0.570	0.212	0.236 – 1.377
Plasmablasts repopulation at 26 weeks (Y/N)	1.413	0.439	0.588 – 3.391

*(count x 10^9 cells/L) for each subset multiply by 1000 prior to analysis

ANCA: anti-neutrophil cytoplasmic antibody-associated vasculitis; BVAS: Birmingham Vasculitis Activity Score; CRP: C-reactive protein; DMARDs: disease modifying anti-rheumatic drugs; MPO: myeloperoxidase; PR3: proteinase 3
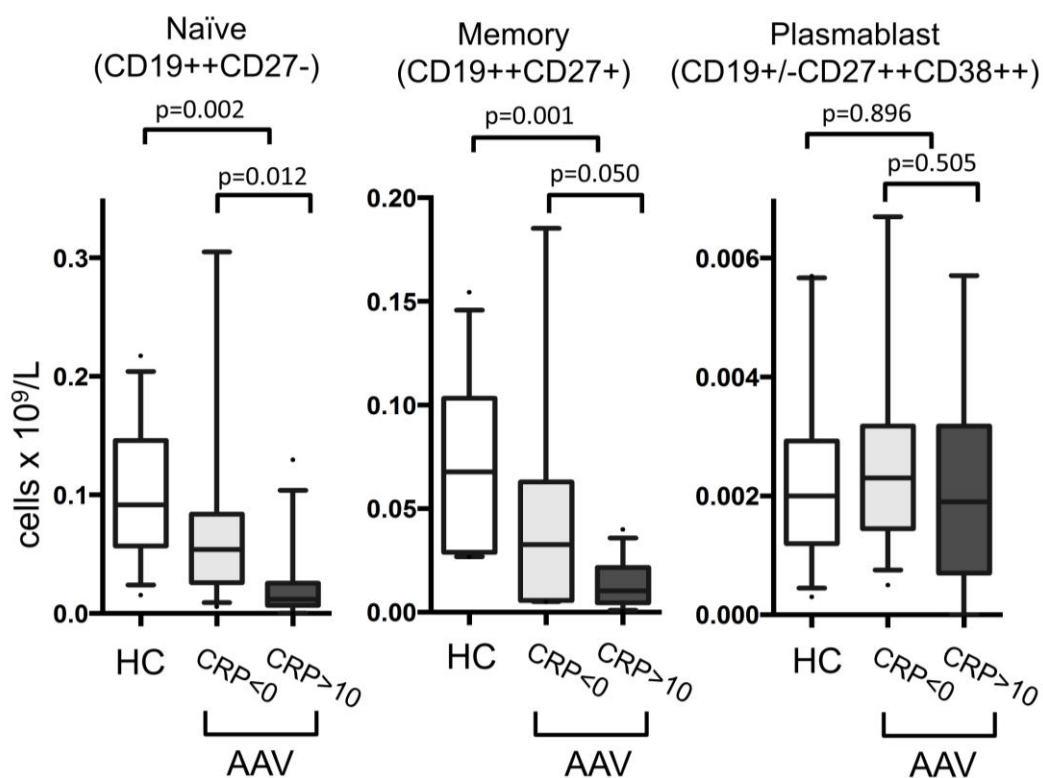
Figure 5-5 Comparison of B-cell subsets and their relationships with CRP levels

Prior to rituximab, active AAV was characterised by naïve and memory B-lymphopenia compared to healthy controls. This dysregulation was more marked in patients with severe systemic inflammation; raised CRP (CRP>10 mg/dL). Error bars denote interquartile range. AAV: anti-neutrophil cytoplasmic antibody-associated vasculitis; CRP: C-reactive protein; HC: healthy control

Reprinted with permission from the BMJ publishing group. Md Yusof MY et al. Repeat cycles of rituximab on clinical relapse in ANCA-associated vasculitis: identifying B cell biomarkers for relapse to guide retreatment decisions. *Ann Rheum Dis.* 2015 Sep;74(9):1734-8

Predictors of relapse post-rituximab

After the first cycle of rituximab, the median time to relapse (IQR) in patients with CR and PR were 94 (73-132) and 79 (62-91) weeks respectively. Patients with CR had a weak trend to time-to-relapse (albeit not clinically significant) compared to patients with PR (Log-rank (Mantel–Cox) test, $x2 = 1.675$, df = 1, $p=0.196$). The time-to-relapse for C1 and C2 of RTX showed moderately significant correlation ($r=0.490$, $p=0.020$). However, duration of C1 rituximab alone did not appear clinically useful in estimating duration of C2; the second cycle was >12 weeks longer than the first in 30% of cases, and >12 weeks shorter than the first in 45% of cases.

At 6 months, as reported above, B-cells were detectable in 70% of the patients. This time point preceded all clinical relapses. In order to analyse the effect of B-cell repopulation and relapse, we compared the time-to-relapse according to the presence or absence of each B-cell subset (naïve, memory, plasmablast).

There was a significant association between repopulation of naïve B-cells at 6 months and relapse; $p=0.010$ (Figure 5-6), but no association between memory B-cell ($p=0.399$) or plasmablast repopulation ($p=0.262$) and relapse. In order to better understand these results, we compared the baseline clinical characteristics of patients based on the presence or absence of naïve B-cells at 6 months. AAV patients without detectable naïve B-cells at 6 months had lower naïve B-cells and significantly higher CRP ($p=0.015$) at baseline, but no difference in age, disease duration, BVAS 3.0, PR3 or MPO titres. The relapse rates for this group at 12 and 18 months were 31% and 54% respectively.

On the other hand, of patients with detectable naïve B-cells at 6 months, the relapse rates at 12 and 18 months were 0% and 14% respectively. Similar trend was observed in subsequent cycles; C2 (n=12) and C3 (n=8): 0% relapse rates for both at 12 months.

The relationship between repopulation of naïve (CD19+CD27-) cells and later relapse, in a subset of patients (n=6) was further evaluated. We divided these cells into CD19+CD27-CD38- "mature naïve B-cells" and CD19+CD27-

CD38+ "transitional B-cells." Two distinct groups of transitional B-cell repopulation were observed; low frequency (1.5-5.0%) and high frequency (20.8-84.3%). Due to sample size, we were unable to confirm the association of these groups with duration of response.
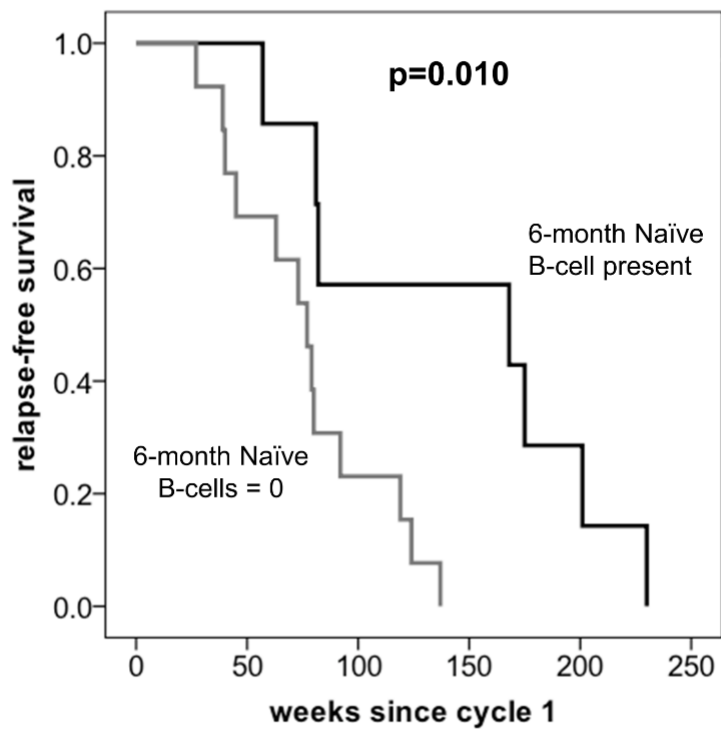
Figure 5-6 Association of naive B-cell repopulation at 6 month with later clinical relapse

Relapse-free survival according to repopulation of naïve B-cells at 6 months post-rituximab. Early repopulation of naïve B-cells at 6 months was associated with later clinical relapse.

5.5.6. Summary of evidence on the utility of B-cell biomarkers to guide treatment decisions with rituximab in SLE, RA and AAV.

For a biomarker to be used in clinical practice, it needs to be validated and reproduced (363). Based on the results presented in section 4.3.5 and in this chapter, the potential uses of B-cell biomarkers as measured using HSFC for response and relapse prediction in SLE, RA and AAV are summarised in Table 5-5.

Table 5-5 Current evidence and the proposed schedule for B-cell monitoring during rituximab treatment in SLE, RA and AAV

Disease	Discovery of Biomarkers	Validation of Biomarkers	Proposed monitoring in clinical practice
SLE	The depth of depletion (i.e.: depletion after the 2nd infusion) was associated with clinical response at 6 months; 100% complete vs 68% incomplete depletion; p=0.012. (n=38 patients) Ref: (16)	Validated in the subsequent n=46 patients. Confirmed association of complete depletion and response; 93% complete vs 65% incomplete depletion after 2nd infusion; p=0.014. Ref: (246)	Peripheral B-cell to be checked at 0 (first infusion), 2 weeks (2nd infusion), 6 weeks and 6 months post-rituximab.
	Earlier relapse post-rituximab was associated with plasmablast repopulation >0.0008 x 10^9/L at 6 months (p=0.024). (n=28 patients) Ref: (16)	Validated in the subsequent n=25 patients. Confirmed association of plasmablast repopulation (>0.0008 x 10^9/L) at 6 months with earlier relapse (p=0.027). Ref: (246)	
RA	Initial depth of B-cell depletion i.e. depletion after the first infusion was associated with clinical response at 6 months; 96% complete vs 74% incomplete depletion; p=0.020. (n=60 patients) Ref: (234)	Validated in the subsequent n=180 patients. Confirmed association of complete depletion and response: 77% complete vs 61% incomplete; p=0.036. Ref: (362)	Peripheral B-cell to be checked at 0 (first infusion) and 2 weeks (on the day of 2nd infusion) post-rituximab.
	Retreatment of initial non-responders with incomplete depletion in C1 led to 72% response rate in C2. (n=25 patients) Ref: (240)	Validated in the subsequent n=30 patients. Confirmed the strategy to re-treat patients who had incomplete depletion and non-response to C1. Response rate in C2 was 67%. Ref: (362)	
AAV	Naïve B-cell repopulation at 6 months was associated with a reduced risk of relapse (HR: 0.33, 95% 0.11-0.93, p=0.036). Relapse rates at 12 and 18 months were 0% and 14% with naïve repopulation and 31% and 54% without naïve repopulation. (n=35) Ref: (364)	Data collection for validation study is still ongoing.	Peripheral B-cell to be checked at 0 (first infusion) and 2 weeks (on the day of 2nd infusion) post-rituximab.

5.6. Discussion

In this chapter, I presented data on the use of B-cell biomarkers in terms of response and relapse prediction in RA, SLE and AAV, which had been validated in a second cohort for the first two indications. These data also offered insights about B-cell kinetics before and after treatment with rituximab and provided the rationale for B-cell monitoring to guide treatment decisions for efficient use of therapy.

Early studies indicated no correlation between the total B-cell numbers and clinical response to rituximab when they were measured using conventional cytometry (365). This could be attributed to the failure in enumerating persistence of plasmablasts in some patients, which required specialised flow cytometry protocols. This is important because their continuing presence in the blood indicates continuing B-cell activity at other sites (352). However, HSFC validated that patients who had complete B-cell depletion were associated with better outcome in RA. Moreover, the validation study, which demonstrated efficacy of retreatment of patients who had persistence B-cells and poor response to first cycle rituximab, provided an alternative strategy to manage this challenging group of RA patients who would otherwise be switched to a different agent, with uncertain efficacy and side effects. The assumption that patients who exhibited initial non-response to rituximab had a B-cell-independent disease was not supported by the results. Indeed, these findings highlighted that the full potential of B-cell depleting therapies was not maximised and that more RA patients than previously thought might benefit from rituximab if its use was refined.

In SLE, the degree of complete B-cell depletion (after the second infusion) as measured using HSFC was the least efficient in SLE compared to RA and AAV patients. This could be contributed to insufficient rituximab dose used (237) or intrinsic resistance to therapy (238). From treatment stratification perspective, by assessing patients (prior to rituximab) for higher plasmablasts and low complement levels, treatment modification could be employed to improve depletion either by increasing the dose or adding an extra infusion, as we previously showed in RA (236) for the former. While for those with low

complement levels pre-rituximab, treatment could be modified by combining rituximab with complement correction therapy such as fresh frozen plasma, as demonstrated in CLL (8, 9) or using other anti-CD20 mAb with enhanced ADCC such as obinutuzumab. At 6 weeks post-rituximab, complete depletion is a marker of good response to therapy. For those with incomplete depletion, close monitoring for response is required. At 6 months post-rituximab, repopulation of plasmablasts of $>0.0008 \times 10^9$/L increased the risk of clinical relapse within the following 6 months. Therefore, these patients could be considered for early retreatment in order to reduce the higher burden of B-cell numbers and enhance depletion in the subsequent cycle. Importantly, for those with plasmablasts of $\leq 0.0008 \times 10^9$/L at 6 months, monitoring for clinical relapse would appear to be an acceptable strategy.

In AAV, the results showed that active vasculitis was characterised by naïve lymphopenia compared to HCs, RA and SLE patients at baseline. There was no significant difference in lymphocyte count or relative B-cell numbers between patients who had received initial remission induction with cyclophosphamide versus rituximab, or comparing patients with or without concomitant csDMARDs at baseline (data not shown). Furthermore, the dysregulation of naïve B-cells was more marked in patients with more severe systemic inflammation (high CRP). These observations suggest that naïve lymphopenia is a biomarker of disease-associated B-cell activity rather than an effect of immunosuppressive therapy on B-cell homeostasis (366).

Patients with early relapse failed to repopulate with naïve B-cells in AAV. This differed to our results in SLE (16). In SLE, we observed sustained suppression of memory B-cells and plasmablasts despite repopulation of naïve B-cells that predicted longer responses to rituximab. Thus, undetectable naïve B-cells at 26 weeks in AAV might be an early indication of the recurrence of the naïve lymphopenia that characterised more severe disease before rituximab, and therefore served as an early sign of disease-associated B-cell activity. Alternatively, this subset might contain a population of regulatory B-cells (Bregs) that helped to maintain disease in remission. These have previously been described within transitional B cell subsets, $CD19^+CD24^{hi}CD38^{hi}27^-$ cells (367). Wilde et al. (368) demonstrated that interleukin (IL-10) competency was diminished during active and remission in AAV patients. Todd et al. (369)

concurred, but also reported an increase in frequency of the Bregs following rituximab despite 5 years of therapy. This hypothesis needs to be assessed in a prospective cohort study.

These studies have some limitations. First, the repopulation pattern of naïve B-cells at 6 months as a biomarker for relapse in AAV has not yet been validated in a second cohort for this to be applied in clinical setting. This is currently in progress as well as studies on the potential role of Bregs in AAV patients treated with rituximab. Second, although our cohort was one of the largest at a single centre reported in AAV, the sample size was still relatively small for a multivariable analysis to be conducted to identify the independent predictors of response and relapse to rituximab. Nevertheless, by linking analyses of B-cell subsets with clinical data in this longitudinal follow-up, this study was the first to report a B-cell biomarker for use in relapse prediction and that naïve lymphopenia might be a marker of B-cell-associated activity in AAV. Next, B-cell subsets were only measured in peripheral blood in the studies of RA, SLE and AAV. Therefore, this could explain the reason for a minority of patients with complete depletion in blood but exhibited poor response since persistence of B-cells could occur at other inflamed tissues (370, 371). Lastly, the lack of widespread availability of enumeration of B-cell numbers using HSFC may limit the generalisability of our findings. However, this flow protocol has been increasingly adopted in many institutions, which in turn strengthens its clinical applicability (372-374). Therefore, cost-effectiveness analysis of treatment decision based on these biomarkers is needed and may help its implementation in a wider population.

5.7. Conclusion

In conclusion, treatment of RA, SLE and AAV with B-cell depleting agents should be guided by B-cell monitoring using HSFC with the aim of achieving complete depletion. Close monitoring for clinical response is required for those with incomplete depletion post-rituximab. These patients may benefit from either an additional infusion or retreatment at 6 months in order to reduce the burden of high B-cell numbers. Following a clinical response to rituximab in SLE and AAV, relapse can be predicted by the repopulation of plasmablasts

of $>0.0008 \times 10^9$/L and the absent of naïve B-cell repopulation at 6 months respectively. Therefore, these patients with imminent relapse should be considered for early retreatment while monitoring may be all that required for those without these prognostic these biomarkers in order to promote judicious use of rituximab.

5.8. Key messages

i. Enumeration of B-cells (before and after therapy) using HSFC should be carried out in RA, SLE and AAV patients who are treated with rituximab.

ii. In RA, the association of complete depletion and clinical response and efficacy of retreatment of initial non-responders who had incomplete depletion in the previous cycle were validated in a second cohort.

iii. In SLE, repopulation of plasmablasts of $>0.0008 \times 10^9$/L at 6 months as biomarker of imminent relapse was validated in a second cohort.

iv. In AAV, relapse post-treatment may be predicted by absence of naïve B-cell repopulation at 6 months and that naïve B-lymphopenia may be a biomarker of disease activity.

Chapter 6. Candidate predictors for safety and rationale for immunoglobulin monitoring during rituximab treatment in systemic lupus erythematosus and other autoimmune rheumatic diseases

6.1. Introduction

Rituximab has been licensed for the treatment of moderate to severe RA for over a decade as well as approved for remission induction in AAV (223). Despite failure of RCTs in SLE (204, 205), pSS (375, 376) and autoimmune inflammatory myopathies (377) in meeting their respective primary endpoints, rituximab is commonly used in these indications and other various ARDs based on open label use, often in cases of severe, organ-threatening and refractory to other systemic therapies (16, 341, 378, 379). However, the immunomodulatory properties of biological DMARDs (bDMARDs) in general including rituximab have naturally raised safety concerns prompting careful evaluation in clinical trials and intensive post-marketing surveillance. Observations from these also provide insight into pathogenic basis of infectious disease (8). As described in section 2.7, long-term data from RCTs and LTE studies of rituximab in various ARDs had shown no major safety signals (204, 205, 256, 259, 380). However, these data need to be interpreted with caution since RCTs and their LTE studies selected patients who could tolerate the therapy while those with comorbidities were often excluded from trials. Therefore, real-world data from large cohort study is needed.

Infection events are frequent in the initial months after rituximab infusions, which may be secondary to the initial bolus of glucocorticoid given as pre-treatment (381). Subsequent cycles have either similar or lower rates of infections (382). However, data pertaining risk factors for infection identified through multivariable analysis are lacking and needed to discern patients at greatest risk.

B-cell depletion targeted at the CD20 molecule is temporary due to the sparing of stem cells. An initial expectation was that temporary clearance of B-cells

would be sufficient to eliminate pathogenic clones leading to sustained remission after repopulation with a new diverse population of B-cells. However for most ARDs, patients ultimately relapse albeit after a variable interval following B-cell repopulation, requiring repeat cycles to recapture response. Repeat cycles with rituximab is effective. However, attrition of plasma cells and low immunoglobulin levels may occur with repeat cycles. Data from the French registry reported that low IgG pre-rituximab increased the odds of SIE over the 12 months post-rituximab (262). Nevertheless, there is limited data on repeat treatment and outcome of patients with secondary hypogammaglobulinaemia. Additionally, evidence for immunoglobulin monitoring during rituximab treatment is also scarce.

Another potential concern is alteration of the B-cell repertoire during depletion with rituximab. The reconstitution of B-cell compartment following each cycle of depletion may not fully recapitulate the previous repertoire (although long-lived plasma cells are mostly spared from depletion). It is possible that long-term perturbation of the immune system in this fashion may lead to impaired host defence. Moreover, over the long run, the failure to mount adequate responses to variants of current pathogens or to new pathogens may put chronically B-cell depleted patients at risk for infection. Although the lack of association between depletion and SIE in the first 2 cycles of treatment for SLE was described in section 4.3.13, longer-term follow-up is needed.

6.1.1. Hypotheses

i. Low immunoglobulin subsets pre-rituximab are associated with increased odds of SIEs during the first 12 months of therapy and with repeat cycles.

ii. Low B-cell numbers pre-rituximab and B-cell depletion post-rituximab of any cycle are not associated with SIEs.

6.1.2. Objectives

i. To evaluate risk factors for SIEs within the first 12 months of therapy and with repeat cycles of rituximab for the treatment of SLE and other ARDs

ii. To assess the effect of attrition in immunoglobulin levels and B-cell depletion on the rates of SIEs

iii. To evaluate infection outcomes of patients with hypogammaglobulinaemia

iv. To assess efficacy of immunoglobulin replacement therapy in those with secondary hypogammaglobulinaemia

6.2. Methods

6.2.1. Candidate's roles in this project

In this work, the initial concept and design of the study were set by myself, Prof Emery, Dr Vital and Dr Savic. I carried out the weekly Biologics, Lupus and Vasculitis clinic in Leeds under the supervision of Dr Vital and Prof Emery. I performed a significant proportion of the clinical assessments. Peripheral blood B-cell subsets were measured using HSFC at the Haematological Malignancy Diagnostic Service, Leeds Teaching Hospitals NHS Trust. For safety data, I spent two months reviewing medical notes and results for all 700 patients single-handedly. I organised and entered all data onto the study database. In terms of statistical analyses, I carried out the descriptive statistics analyses, multiple imputation of missing data and multivariable analyses of prognostic predictors of serious infection in the first 12 months of therapy. Prof McElvenny checked my results and performed the complex mixed-effect logistic regression analyses. I am currently leading the writing of the manuscript, while other co-authors will revise the draft critically for important intellectual content and final approval of the manuscript prior to submission to journal for publication.

6.2.2. Design and patients

A retrospective observational study was conducted of the first 700 consecutive patients with ARDs, who were treated with at least a course of rituximab in Leeds between Jan 2002 and May 2015.

Inclusion criteria were (i) adults (>16 years old); (ii) fulfilling the classification criteria for any ARD including RA (329), SLE (32), AAV (383), pSS (326), autoimmune inflammatory myopathies (328), systemic sclerosis (327) and others (384, 385) and (iii) at least 3 months follow-up post-rituximab.

6.2.3. Ethical approval

This study did not require ethical approval because all treatment decisions were made prior to evaluation of data, in accordance with the UK National Health Service Research Ethics Committee guidelines. The off-label uses of rituximab in non-licensed indications were all approved by Leeds Teaching Hospitals NHS Trust Drug and Therapeutic Committee.

6.2.4. Treatment protocol

All patients received a first cycle of therapy consisted of 100 mg of methylprednisolone and 1000 mg of rituximab given intravenously on days 1 and 14. Further cycles consisted of the same regimen were repeated on clinical relapse. For RA, 19% of the patients received repeat treatment with half-dose regimen (500mg x 2) following a EULAR response in the previous cycle (360). This decision was left to investigator's clinical judgement.

Prior to approval of rituximab by NICE for remission induction in AAV in 2014 (386) and its commissioning by NHS England for SLE in 2013 (233), intravenous cyclophosphamide was commonly used as a remission induction agent for severe and refractory AAV and SLE cases. This was typically given for 3-6 months, adjusted for age, body weight and renal function. Following a subsequent relapse or non-response to cyclophosphamide, patients were treated with rituximab for remission induction in SLE and AAV.

Continuation of a stable or reduced dose of concomitant csDMARDs (including oral corticosteroid) was left to investigators' discretion with the aim

to stop glucocorticoid if clinical response for the respective disease was achieved at 6 months.

6.2.5. Clinical data and outcomes

Age, gender, disease duration, diagnosis, comorbidities including previous history of any type of cancer, chronic lung disease such as interstitial lung disease, bronchiectasis and asthma (with recurrent exacerbations), heart failure, diabetes, previous history of severe infection, number of previous csDMARDs, number of previous bDMARDs, previous treatment with cyclophosphamide, concomitant corticosteroid, corticosteroid dose, concomitant csDMARDs (excluding anti-malarials only), rituximab dose and time-to-rituximab retreatment were recorded.

SIEs were recorded irrespective of possible association with ARDs and/or therapy. Serious infections were defined as those resulted in hospitalisation for > 24 hours or required intravenous antibiotics. Data for SIEs were gathered extensively from hospital admission records using the PAS system, pathology results server and confirmed with case notes.

6.2.6. Laboratory assessments

Total serum immunoglobulin levels were measured by nephelometry before and after treatment with intravenous cyclophosphamide, at rituximab baseline and at 4-6 months after each cycle (normal range for IgM: 0.5-2.0 g/L; IgA: 0.8-4.0 g/L and IgG: 6.0-16.0 g/L). Secondary hypogammaglobulinaemia was defined as a level of IgM, IgA or IgG which was below its respective lower limit of normal (LLN) for at least 4 months following rituximab.

Peripheral blood B-cell subsets were measured using HSFC (as previously described in section 4.2.5) at baseline, week 2, week 6, months 6, and every 6 months without knowledge of patients' clinical status other than time since rituximab. Complete B-cell depletion was defined as counts ≤0.0001×10^9 cells/L and repopulation as counts >0.0001×10^9 cells/L.

6.2.7. Missing data

Some data for immunoglobulin levels and B-cells were missing at random due to samples not being processed on the day. Multiple imputation by chained

equations was used to create 10 complete datasets, results of which were combined according to Rubin's rules (332).

6.2.8. Statistical analyses

Descriptive statistics were summarised using mean with standard deviation or median with interquartile range for continuous variables where appropriate and proportion for categorical variables. Percentage change for each immunoglobulin classes between 4-6 months post-rituximab and pre-rituximab level of each cycle was calculated for cycles 1-5. The significance of the association between categorical variables was tested by Fisher's exact test while for continuous variables using either Student's T-test or Mann-Whitney U test.

To assess baseline predictors of SIEs in the following 12 months post-rituximab, all (imputed) putative variables were first evaluated using univariable analysis. Only variables with p-value of <0.25 were included in the multivariable analysis. The final model included variables that showed p-values <0.10 using a stepwise backward elimination method, and the respective ORs and 95% CIs were reported.

To analyse predictors of SIEs in repeat cycles of rituximab (cycles 1-5), mixed-effect logistic regression, a statistical model comprised both fixed and random effects were used to account for repeated measurements made on the same individual or statistical unit in this long-term follow-up (387). Only variables with p-value of <0.25 in univariable analysis were included in the multivariable analysis. The final model included variables that showed p-values <0.10 using a stepwise backward elimination method.

Statistical analyses were performed using Stata v.13.1 (StataCorp College Station, Texas, USA) and GraphPad Prism v.7.03 (GraphPad, La Jolla, CA, USA) for Windows.

6.3. Results

6.3.1. Patients and characteristics

Of 700 patients with ARDs who were treated with rituximab in Leeds, 550 patients were female, median age (IQR) at rituximab initiation was 58 (46–68)

years and median disease duration (IQR) 7.9 (3.4–15.0) years. Most patients had RA, n=506 (72%). Total follow-up: 2880 patient-years. Baseline characteristics are described in Table 6-1.

Table 6-1 Baseline characteristics of the 700 patients with ARDs treated with rituximab

Characteristic	Values
Median Age (IQR) in years	58 (46-68)
Female : Male	550 : 150
Median Disease duration (IQR) in years (IQR)	7.9 (3.4-15.0)
Diagnosis, n (%)	
Rheumatoid arthritis	506 (72.3)
Systemic lupus erythematosus	94 (13.3)
ANCA-associated vasculitis	49 (7.0)
Autoimmune myopathies	14 (2.0)
Primary Sjogren's syndrome	9 (1.3)
Systemic sclerosis	6 (0.9)
Anti-Phospholipid antibody syndrome (APS)	5 (0.7)
Mixed connective tissue disease	4 (0.6)
Cryoglobulinaemic vasculitis	2 (0.3)
IgG4-related sclerosing disease	2 (0.3)
Other CTDs	9 (1.3)
Biologic naïve, n (%)	364 (52)
Prior Cyclophosphamide, n (%)	142 (20.3)
Concomitant anti-malarials, n (%)	85 (12.1)
Concomitant csDMARDs, n (%)	515 (73.6)
Methotrexate	389 (55.6)
Mycophenolate mofetil	54 (7.7)
Azathioprine	35 (5.0)
Leflunomide	26 (3.7)
Cyclophosphamide	7 (1.0)
Sulfasalazine	4 (0.6)
Concomitant prednisolone, n (%)	303 (43.2)
Median daily prednisolone dose (SD), mg	5 (7)
Ever smoked , n (%)	300 (43)

Characteristic	Values
Median Immunoglobulin (IQR) g/L	
IgM (0.5-2.0)	1.22 (0.82-1.78)
IgA (0.8-4.0)	2.75 (2.05-3.72)
IgG (6.0-16.0)	11.7 (9.3-14.7)

ANCA: anti-neutrophil cytoplasmic antibody-associated vasculitis; csDMARDs: conventional synthetic disease modifying anti-rheumatic drugs; CTD: connective tissue disease; IQR: interquartile range; SD: standard deviation

6.3.2. Rate and sources of serious infection events

Overall, there were 284 SIEs recorded in 179 patients (9.9/100 PY) from this long-term follow-up study of over 13 years. The rates of SIEs were slightly lower in AI-CTD (9.4/100 PY) compared to RA (10.1/100 PY). The rates of SIEs generally remained stable over time and with multiple treatment courses (Figure 6-1).

Most SIEs were due to lower respiratory tract infection (LRTI); n=170 (61%). Sources of SIEs are illustrated in Figure 6-2.
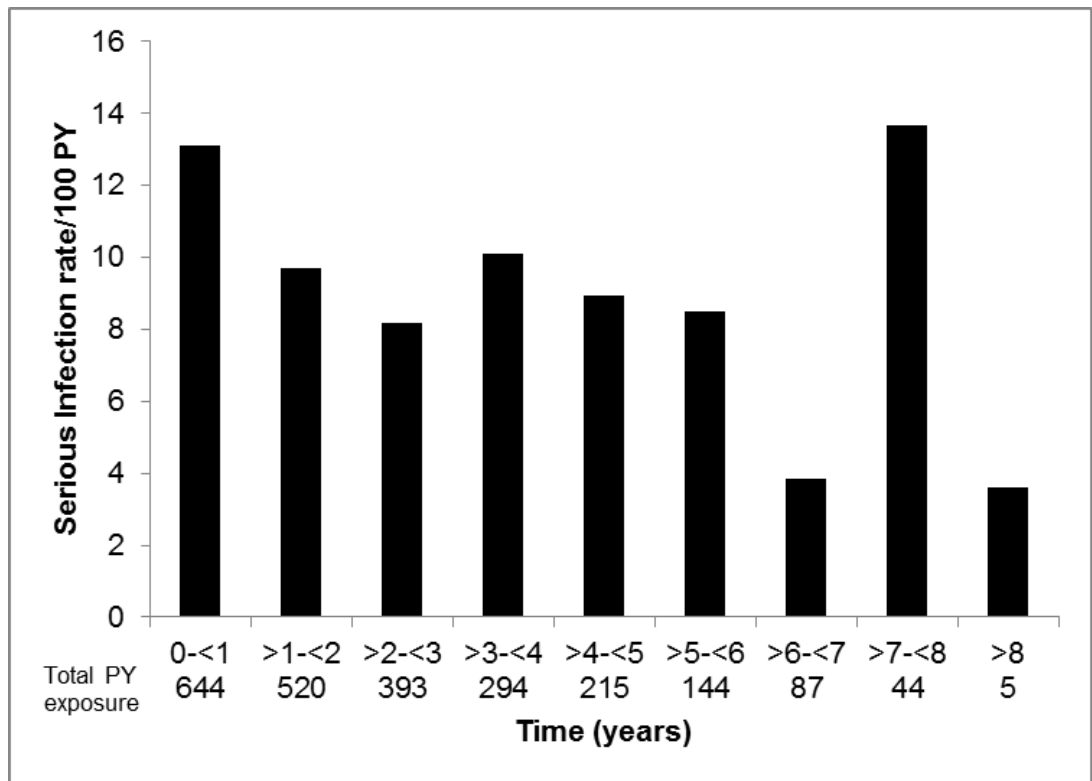
Figure 6-1 Serious infection events over time and with multiple cycles of rituximab

> The rates of serious infection events were comparable and stable over time and with multiple cycles of rituximab with the exception of at 7-8 years post-therapy. PY: patient-year
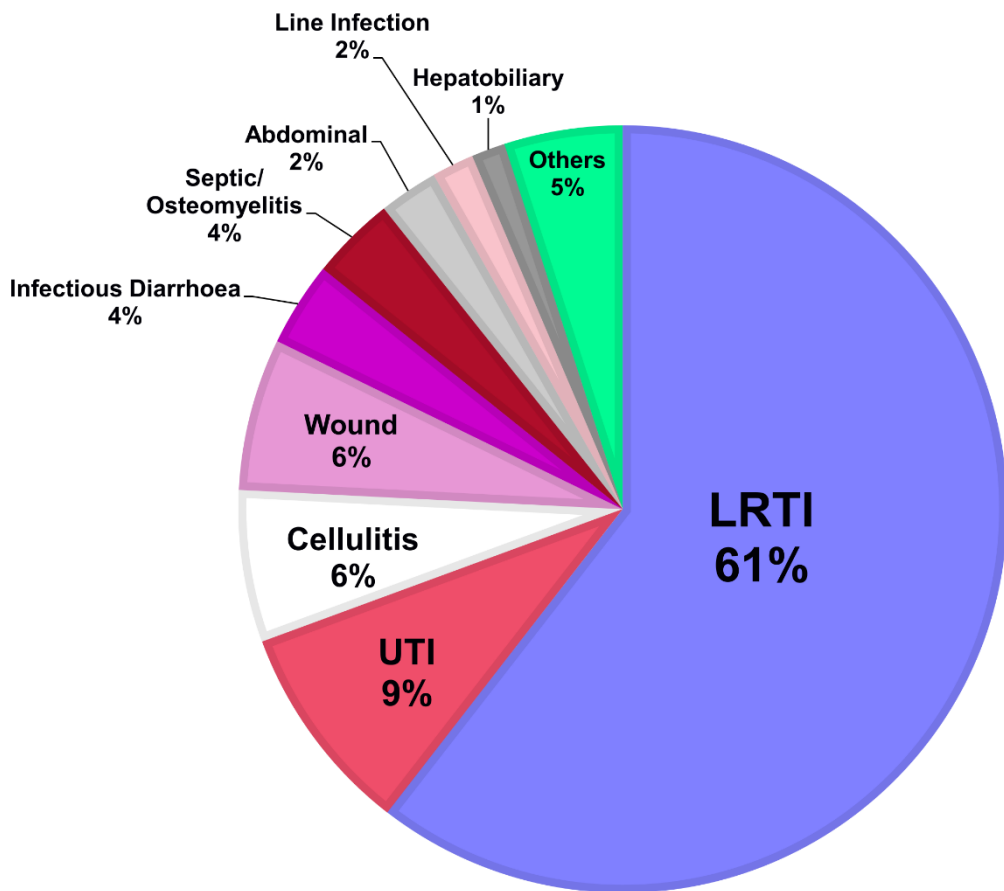
Figure 6-2 Sources of serious infection events

The pie chart above demonstrates the distribution of the sources of serious infection events, each is represented with different colours. The most common source for serious infections in this cohort was lower respiratory tract infection. LRTI: lower respiratory tract infection.

6.3.3. Opportunistic infections

There were 8 opportunistic infections recorded in this study. These were disseminated varicella zoster=2, mycobacterium=2, cytomegalovirus=2, disseminated candidiasis=1 and *pneumocystis jiroveci pneumonia* (PCP)=1. The last was observed in a patient who was subsequently diagnosed as having HIV infection. This was in the year 2005 when HIV test was not routinely tested in patients receiving rituximab at our unit.

6.3.4. Baseline predictors of serious infection within 12 months of rituximab initiation

Data for immunoglobulin levels were available for 667/700 (95%) while for B-cells were 597/700 (85%) of the patients. In the following 12 months from rituximab initiation, 89 SIEs were recorded in 75 patients. Univariable analysis with multiple imputation showed older age, ever smoked, previous history of cancer, chronic lung disease, diabetes, previous history of severe infection, concomitant corticosteroid, higher corticosteroid dose, low IgM, low IgA and low IgG increased odds of SIEs. While female gender reduced the risk of SIEs.

In multivariable analysis with multiple imputation, only previous cancer, chronic lung disease, previous history of severe infection and low IgG increased odds of SIEs in the following 12 months from first cycle rituximab. A diagnosis of RA was associated with lower risk of SIEs (Table 6-2).

Table 6-2 Baseline predictors of serious infection within 12 months of first cycle rituximab

	No severe infection N = 625	Severe infection N = 75	Univariable OR (95% CI), P-value (with multiple imputation)	Multivariable OR (95% CI), P-value (with multiple imputation)
Median age (IQR), years	57.1 (45.4-66.4)	64.5 (53.7-72.8)	1.27 (1.07-1.50) Per 10 years of age, P = 0.005	
Female, n (%)	497 (79.5)	53 (70.6)	0.62 (0.36-1.06), P = 0.080	
Median disease duration (IQR), years	7.9 (3.5-14.9)	8.4 (2.9-16.4)	1.0 (0.98-1.03) Per year, P = 0.517	
Ever smoked, n (%)	259 (41.4)	40 (53.4)	1.70 (1.00-2.61), P=0.051	
Previous cancer, n (%)	46 (7.4)	11 (14.7)	**2.16 (1.07-4.39), P = 0.032**	**2.85 (1.23-6.64) P = 0.015**
Chronic lung disease, n (%)	129 (20.6)	31 (41.3)	**2.71 (1.65-4.46) P < 0.001**	**1.68 (0.92-3.08) P = 0.092**
Heart failure, n (%)	11 (1.8)	2 (2.7)	1.53 (0.33-7.03) P = 0.585	
Diabetes, n (%)	33 (5.3)	9 (12.0)	2.45 (1.12-5.33) P = 0.025	
Diagnosis of RA vs CTDs, n (%)	456 (73.0)	50 (66.7) 25 (33.3)	**0.74 (0.44-1.24) P = 0.249**	**0.46 (0.24-0.86) P = 0.015**
Previous severe infection, n (%)	58 (9.3)	40 (53.3)	**11.17 (6.59-18.94), P < 0.001**	**10.65 (5.82-19.47), P < 0.001**
Previous cyclophosphamide, n (%)	123 (19.7)	19 (25.3)	1.38 (0.79-2.42) P = 0.252	
Previous biologics, n (%) 0 1 2 3 4	316 (50.5) 137 (21.9) 105 (16.8) 52 (8.3) 15 (2.4)	48 (64.0) 11 (14.7) 10 (13.3) 4 (5.3) 2 (2.7)	0.82 (0.64-1.04) Per number of previous biologics P = 0.102	
Concomitant corticosteroid, n (%)	256 (41.0)	47 (62.7)	2.41 (1.48-3.97) P < 0.001	
Median corticosteroid dose (IQR), mg	0 (0-7.5)	5 (0-10)	1.04 (1.01-1.07) P = 0.007	
Concomitant DMARDs, n (%)	470 (75.2)	44 (58.7)	0.47 (0.29-0.77) P = 0.003	

	No severe infection N = 625	Severe infection N = 75	Univariable OR (95% CI), P-value (with multiple imputation)	Multivariable OR (95% CI), P-value (with multiple imputation)
Low IgM (<0.5g/L), n (%)	42 (6.7)	15 (20.0)	3.47 (1.82-6.63) P < 0.001	
Low IgA (<0.8g/L), n (%)	11 (1.8)	4 (5.3)	3.14 (0.98-10.14) P = 0.055	
Low IgG (<6.0g/L), n (%)	20 (3.2)	14 (18.7)	**7.28 (3.48-15.25) P < 0.001**	**3.56 (1.47-8.63) P = 0.005**
Median naïve B-cell (IQR)*	72.2 (29.7-122.0)	43.3 (9.5-102.0)	1.000 (0.997-1.002), P = 0.728	
Median memory B-cell (IQR)*	18.5 (8.3-35.5)	10.7 (5.0-39.1)	0.999 (0.992-1.006), P = 0.743	
Median plasmablasts (IQR)*	1.9 (0.8-3.8)	1.9 (0.9-)	1.016 (0.991-1.043), P = 0.215	

*(count x 10^9 cells/L) for each subset multiply by 1000 prior to analysis

CTD: connective tissue disease; DMARDs: disease modifying anti-rheumatic drugs; IQR: interquartile range; RA: rheumatoid arthritis

6.3.5. Predictors of low IgG prior to first cycle rituximab

Since low IgG pre-rituximab was independently predictive of SIE, risk factors for development of low IgG were evaluated. In univariable analysis with multiple imputation, older age, previous history of cancer, previous history of severe infection, previous therapy with cyclophosphamide, concomitant corticosteroid and higher corticosteroid dose increased odds of low IgG at baseline. While a diagnosis of RA, biologic naïve or lower number of previous biologics, concomitant DMARDs, higher plasmablast counts reduced the risk of low IgG.

In multivariable analysis with multiple imputation, only older age, previous cancer, previous history of severe infection and previous therapy with cyclophosphamide increased the odds of low IgG at baseline. While a diagnosis of RA was associated with lower risk of low IgG (Table 6-3).

Table 6-3 Predictors of low IgG prior to first cycle rituximab

Variable	Low IgG (N=34)	Normal IgG (N=633)	Univariable OR (95% CI), P-value (with multiple imputation)	Multivariable OR (95% CI), P-value (with multiple imputation)
Median age (IQR), years	64.3 (56.0-75.5)	57.9 (45.6-67.5)	**1.37 (1.06-1.77) per 10 years of age, P = 0.018**	**1.48 (1.10-1.99) P = 0.010**
Female, n (%)	27 (29.4)	498 (78.7)	1.03 (0.44-2.41) P = 0.951	
Median Disease duration (IQR), years	7.0 (2.8-13.8)	8.1 (3.5-15.2)	0.97 (0.93-1.02) P = 0.224	
Ever smoked, n (%)	17 (50.0)	273 (43.1)	1.30 (0.64-2.61) P = 0.464	
Previous cancer, n (%)	6 (17.7)	49 (7.7)	**2.65 (1.05-6.72) P = 0.040**	**3.44 (1.19-9.94) P = 0.022**
Chronic lung disease, n (%)	10 (29.4)	143 (22.6)	1.48 (0.69-3.17) P = 0.317	
Heart Failure, n (%)	1 (2.9)	11 (1.7)	1.69 (0.21-13.38) P = 0.620	
Diabetes, n (%)	3 (8.8)	34 (5.4)	1.74 (0.51-5.97) P = 0.375	
Diagnosis of RA vs CTDs, n (%)	15 (44.1)	471 (74.4)	**0.27 (0.13-0.54) P < 0.001**	**0.31 (0.09-1.06) P = 0.062**
Previous Severe Infection, n (%)	13 (38.2)	85 (13.4)	**4.38 (2.10-9.14) P < 0.001**	**5.46 (2.25-13.22) P < 0.001**
Previous cyclophosphamide, n (%)	19 (55.9)	114 (18.0)	**5.94 (2.90-12.19) P < 0.001**	**5.22 (1.64-16.64) P = 0.005**
Previous biologics, n (%) 0 1 2 3 4	27 (79.4) 3 (8.8) 1 (2.9) 2 (5.9) 1 (2.9)	320 (50.6) 139 (22.0) 109 (17.2) 50 (7.9) 15 (2.4)	0.53 (0.33-0.86) Per number of previous biologics P = 0.011	
Concomitant corticosteroids, n (%)	23 (67.7)	269 (42.5)	2.78 (1.33-5.84) P = 0.007	
Median corticosteroid dose, (IQR) mg	5 (0-15)	0 (0-7.5)	1.04 (1.01-1.09) P = 0.024	
Concomitant DMARDs, n (%)	19 (55.9)	472 (74.6)	0.42 (0.21-0.85) P = 0.016	

Variable	Low IgG (N=34)	Normal IgG (N=633)	Univariable OR (95% CI), P-value (with multiple imputation)	Multivariable OR (95% CI), P-value (with multiple imputation)
Median naïve B-cell, (IQR)*	22.1 (3.4-67.1)	72.0 (28.8-124.8)	0.99 (0.99-1.00) P = 0.191	
Median memory B-cell, (IQR)*	9.2 (4.8-22.0)	18.4 (8.2-36.9)	0.99 (0.97-1.01) P = 0.415	
Median plasmablasts, (IQR)*	0.6 (0.0-1.4)	2.0 (0.8-4.0)	0.86 (0.74-1.01) P = 0.068	

*(count x 10^9 cells/L) for each subset multiply by 1000 prior to analysis

CTD: connective tissue disease; DMARDs: disease modifying anti-rheumatic drugs;

IQR: interquartile range; RA: rheumatoid arthritis

6.3.6. Effect of previous treatment with cyclophosphamide followed by rituximab on immunoglobulin levels

Immunoglobulin levels for patients with SLE and AAV who had received initial remission induction with intravenous cyclophosphamide followed by rituximab (after second relapse) were compared and illustrated in Figure 6-3. Remission induction with cyclophosphamide led to a profound decline in almost all immunoglobulin classes for both diseases (except IgM for SLE) at 6 months post-therapy. Mean IgA and IgG levels for SLE and AAV remained substantially lower than pre-cyclophosphamide levels at 18 months.

Following a second relapse, subsequent remission induction and maintenance were given using rituximab. As described in section 6.2.3, repeat treatment with rituximab was given using retreatment-on-relapse strategy in our cohort. Using this strategy, IgM, IgA and IgG levels remained stable up to 3 cycles in AAV. While in SLE, progressive reduction in all immunoglobulin classes over the 3 rituximab cycles were observed albeit their levels were still above the LLN.
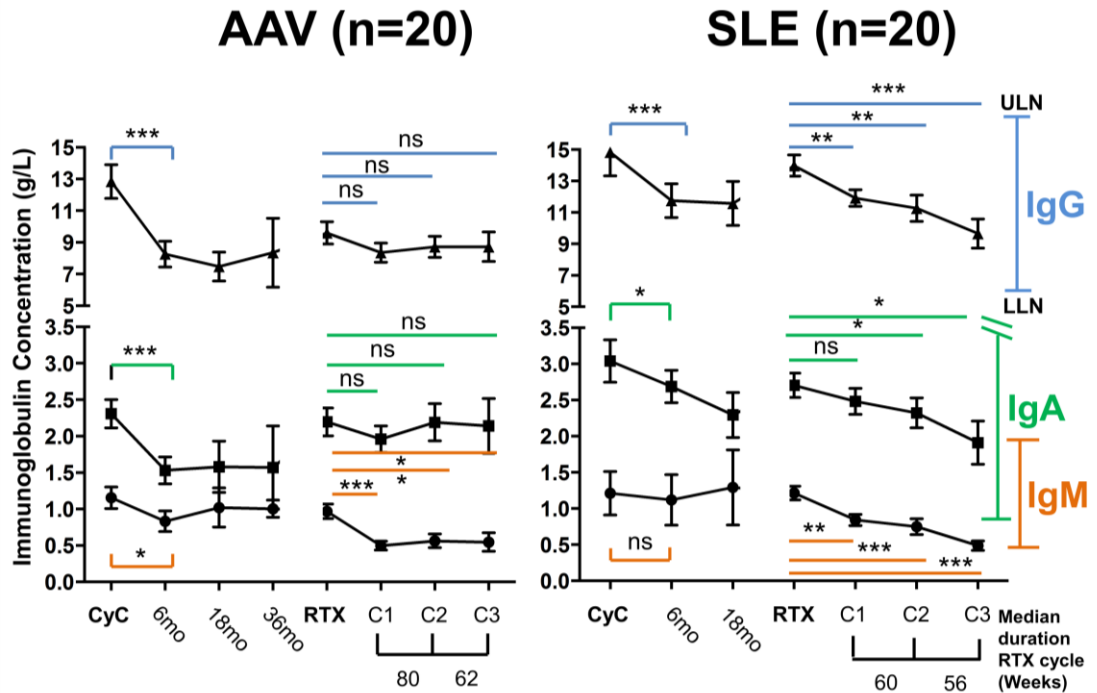
Figure 6-3 Trends of immunoglobulin levels following remission induction with cyclophosphamide and rituximab in SLE and AAV

Remission induction with cyclophosphamide led to a profound decline in almost all immunoglobulin classes for both diseases (except for IgM in SLE) at 6 months post-therapy. Mean IgA and IgG levels for SLE and AAV remained substantially lower than pre-cyclophosphamide levels at 18 months. Following a second relapse, subsequent remission induction and maintenance were given using rituximab, based on retreatment-on-relapse strategy at our unit. Using this strategy, IgM, IgA and IgG levels remained stable up to 3 cycles in AAV. While in SLE, progressive reduction in all immunoglobulin classes over the 3 rituximab cycles were observed albeit their levels were still above the LLN. *** Highly significant (p<0.001), ** Moderate significant (0.001<p-value<0.01), *Significant (0.01<p-value<0.05), ns: non-significant. The graphs for all immunoglobulin classes represent mean concentration and the error bars denote standard error of the mean. AAV: anti-neutrophil cytoplasmic antibody-associated vasculitis; C1-3: cycles 1-3; CyC: cyclophosphamide; LLN: Lower limit of normal, RTX: rituximab; SLE: systemic lupus erythematosus; ULN: Upper limit of normal

6.3.7. Infection outcomes in patients with low immunoglobulin levels

To assess infection risk in patients with ARDs who had hypogammaglobulinaemia, we divided them into 3 groups: i) low Ig levels prior to first cycle rituximab; ii) low Ig levels during rituximab therapy and iii) never developed low Ig. Only IgM and IgG were evaluated since frequency of IgA<LLN was the lowest; 32/670 (4.8%).

For IgM, rates of SIEs were similar in those with low IgM prior to first cycle rituximab, before development of low Ig (during therapy) and never developed low Ig. While the highest rate of SIEs was observed in those who developed IgM during rituximab therapy.

In contrast, the rates of SIEs were markedly higher in those with low IgG prior to first cycle rituximab and who developed low IgG during rituximab therapy compared to those before development of low Ig and never developed low IgG (Table 6-4).

Table 6-4 Summary of serious infection rates in patients with IgM and IgG levels <LLN for at least 4 months

	Group 1: Ig<LLN before 1st rituximab (n=57)	Group 2: Patients who developed Ig<LLN during rituximab (n=196)		Group 3: Those who never developed Ig<LLN during rituximab (n=482)
		Before Ig<LLN	During/After Ig<LLN	
IgM				
Total Exposure (PY)	235.5	538.1	528.2	1700.9
No of SIE	25	53	74	156
Rate of SIE/100 PY (95% CI)	10.6 (6.9 to 15.7)	9.8 (7.4 to 12.9)	14.0 (11.0 to 16.6)	9.2 (7.8 to 10.7)
IgG				
Total Exposure (PY)	122.1	123.5	136.0	2457.1
No of SIE	20	15	29	238
Rate of SIE/100 PY (95% CI)	16.4 (10.0 to 25.3)	12.1 (6.8 to 20.0)	21.3 (14.3 to 30.6)	9.7 (8.5 to 11.0)

LLN: lower limit of normal; PY: patient-year; SIE: serious infection event

6.3.8. Relationship between percentage of change in immunoglobulin level and serious infection

In addition to pre-rituximab Ig<LLN of any cycle as a predictor, for analysis of repeat cycles with rituximab, I also assessed the relationship between percentage change in immunoglobulin level and SIE. In cycles 1-5, higher percentage of change in all three immunoglobulin classes were associated with increased rates of SIEs (Figure 6-4).
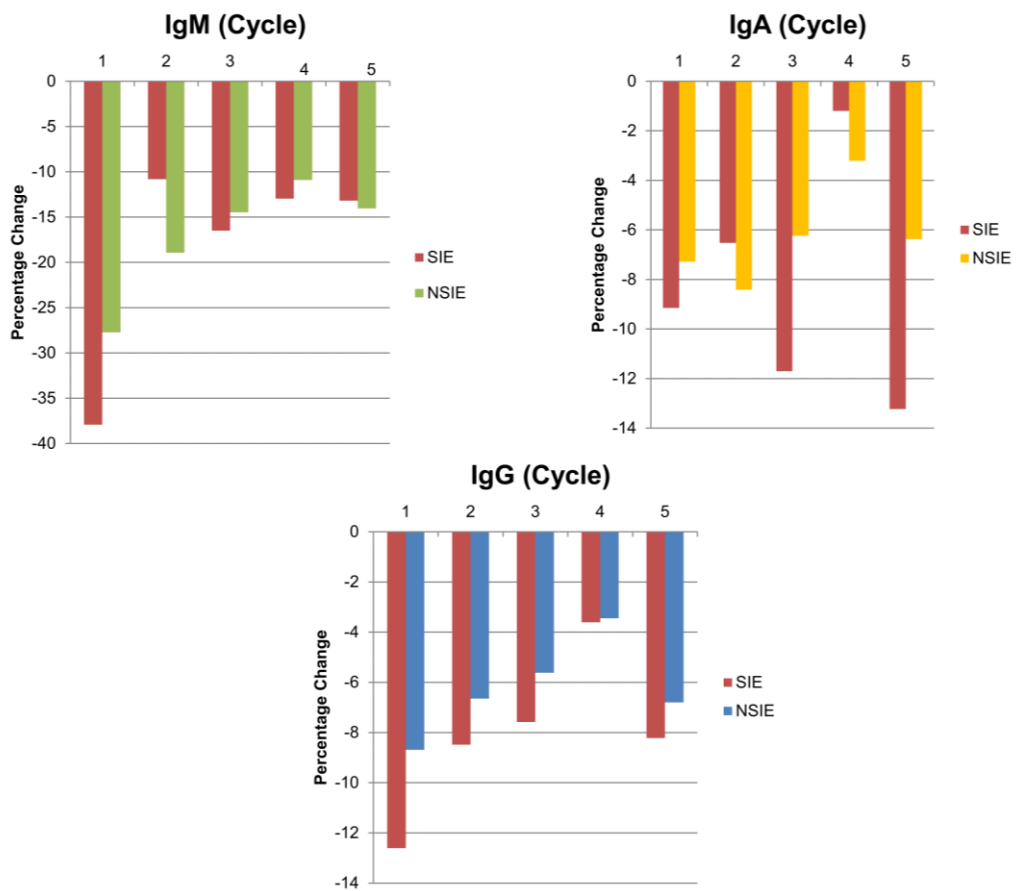
Figure 6-4 Percentage of change in IgM, IgA and IgG levels with serious infection

> Higher percentage of change in all three immunoglobulin classes could be associated with increased rates of SIEs over multiple cycles of rituximab. NSIE: no serious infection; SIE: serious infection event

6.3.9. Predictors of serious infection during repeat cycles of rituximab

For cycles 2-5, all plausible variables were analysed using mixed-effect logistic regression analysis. In multivariable analysis, baseline comorbidities including previous cancer, chronic lung disease and previous history of severe infection as well as pre-rituximab variables of any cycle such as higher corticosteroid dose, longer time-to-rituximab retreatment and larger percentage of change in IgM increased odds of with SIEs. Lower B-cell subsets numbers and depletion status were not associated with SIEs (Table 6-5).

Table 6-5 Predictors of serious infection within cycles 2-5 of rituximab

Variable	Serious Infection	No Serious Infection	Multivariable OR (95% CI), P-value
Mean age (SD), years	59.7 (15.3)	55.7 (14.5)	1.02 (1.00-1.04) P = 0.108
Female (%)	75.6	79.8	-
Previous cancer, n (%)	6 (17.7)	49 (7.7)	**2.35 (1.10-5.00) P = 0.026**
Chronic lung disease (%)	37.4	19.8	**2.04 (1.16-3.56) P = 0.013**
Heart Failure (%)	4.9	1.3	3.05 (0.79-11.72) P = 0.105
Diabetes (%)	12.2	6.5	1.92 (0.84-4.38) P = 0.119
Previous Severe Infection (%)	36.6	8.7	**5.71 (2.79-11.68) P < 0.001**
Concomitant DMARDs (%)	36.6	63.4	-
Mean corticosteroid dose, (SD) mg	4.22 (6.19)	2.05 (3.96)	**1.07 (1.02-1.12) P = 0.006**
Median time-to-rituximab retreatment (IQR), weeks	53.9 (39.0-86.4)	49.1 (36.1-64.0)	**1.01 (1.00-1.01) P = 0.013**
Median percentage of change in IgM (IQR), g/L	-18.5% (-36.3 to -2.6)	-15.4% (-25.8 to -5.9)	**1.01 (1.00-1.02) P = 0.027**
Median percentage of change in IgA (IQR), g/L	-10.0% (-20.3 to -1.9)	-7.1% (-14.6 to 0.7)	-
Median percentage of change in IgG (IQR), g/L	-8.9% (-20.8 to 2.63)	-6.3% (-14.1 to 1.1)	-
Median naïve B-cell (IQR)*	7.6 (0.2-45.5)	16.2 (1.5-51.1)	-

Median memory B-cell (IQR)*	1.3 (0.4-4.0)	1.4 (0.5-3.2)	-
Median plasmablasts (IQR)*	0.8 (0-2.4)	1.0 (0.3-2.1)	-
B-cell depletion at 6 weeks post-rituximab (%)	73.2	74.0	-

*(count x 10^9 cells/L) for each subset multiply by 1000 prior to analysis

DMARDs: disease modifying anti-rheumatic drugs; IQR: interquartile range

Median memory B-cell (IQR)*			
Median plasmablasts (IQR)*			
B-cell depletion at 6 weeks post-rituximab			

6.3.10.   Efficacy of immunoglobulin replacement therapy

Of 700 patients with ARDs who were treated with at least a cycle of rituximab, only 7 (1%) required immunoglobulin replacement therapy due to secondary hypogammaglobulinaemia and infection. These were RA=3, SLE=2, AAV=1 and APS=1. Median time (range) from first cycle rituximab to immunoglobulin replacement was 3.7 (0-7.2) years. Post-immunoglobulin replacement, 6/7 had IgG normalised, 3/7 had normal IgA and 2/7 had normal IgM. Number and severity of infection reduced in all patients. Median duration (range) of therapy was 3.7 (0.5-10) years. Cessation of immunoglobulin replacement was achieved in 1/7 of the patient.

6.4. Discussion

This report presents fundamental safety data on the use of rituximab for various ARDs from a real-world perspective. By carefully reviewing records of every patient to capture all infection episodes in the largest single centre cohort study to date, my findings provide insights on candidate predictors of SIEs as well as safety monitoring of rituximab.

In this study, a high rate of SIEs (9.9/100 PY) was observed. This is in comparison with data from RCTs and LTE studies of rituximab in RA (3.94/100 PY) (256), registries of rituximab in ARDs (ranging from 5.0 to 6.6 PY) (262, 388, 389) and other bDMARDs used in RA (ranging from 3.0 to 5.2/100 PY) (390-392). This high rate of SIE was contributed to a cohort which comprised patients with multiple comorbidities including chronic lung disease, previous history of cancer and previous history of serious infection. The presence of these could lead to channelling away of patients from using other bDMARDs to rituximab, thus led to an increase in reports of adverse events. Moreover, efficacy RCTs (and meta-analyses derived from them) are of limited value in identifying adverse events while LTE studies are not powered to and sub-select populations that tolerate therapy (393). For registry study, patients with recurrent infections were censored at the time of their first infection (262) while my study accounted for all recurrent episodes in the calculation of the rate of SIEs.

The finding from the French registry (262) that low IgG at baseline was associated with increased risk of SIE within 12 months of rituximab initiation was validated in this study. However, other important risk factors such as previous history of cancer and previous serious infection were not reproduced from their dataset. Importantly, my study offers advantages compared to this registry in terms of longer duration of follow-up, methods of dealing with missing data were better defined and the majority of data for immunoglobulin levels were available (95% versus 49% in registry). This study is also the first to identify predictors of SIEs with repeat cycles of rituximab including concomitant higher corticosteroid dose, longer time-to-rituximab retreatment, previous cancer and previous serious infection. Moreover, my findings provided a rationale for immunoglobulin monitoring, not only for below or above LLN as per consensus statement on the use of rituximab in RA (263), but also for percentage of change in immunoglobulin level with reduction in all Ig classes (in particular IgM) being associated with increased risk of post-treatment infection.

Other studies reported risk factors for secondary hypogammaglobulinaemia including lower pre-rituximab hypogammaglobulinaemia (394) and total dose of prior cyclophosphamide therapy (354). This study identified older age, previous cancer, previous history of severe infection and previous therapy with cyclophosphamide as predictors of low IgG at baseline. These factors as well as other predictors of SIEs as described in section 6.3.5 need to be taken into account when counselling patients before commencing therapy with rituximab from the perspective of safety. It is also important to note that development of secondary hypogammaglobulinaemia could not be solely attributed to rituximab. This study showed that remission induction for severe AAV and SLE with intravenous cyclophosphamide led to a marked decline in all immunoglobulin classes up to 36 months. Venhoff et al. demonstrated a single cycle of rituximab following cyclophosphamide further worsened the decline in IgM and IgG to below LLN levels (264). In contrast, using retreatment on clinical relapse strategy, IgM, IgA and IgG levels remained stable over time in AAV in this study. Meanwhile, reduction in all immunoglobulin classes from the use of rituximab in SLE (albeit their levels were still above the LLN) could be attributed to efficacy of rituximab. Overall then, retreatment on clinical

relapse strategy as implemented in this cohort led to a lower rate (1%) of patient who subsequently required immunoglobulin replacement therapy compared to fixed retreatment strategy i.e. every 6 to 12-monthly (ranging from 4-11%) (261, 354). Thus, this is an important consideration in guiding judicious use of retreatment with rituximab.

The continued efficacy of B-cell depletion in SLE and other ARDs depends on repeated cycles of rituximab to maintain depletion or low levels of B-cells. This is the first study to show that in a fully adjusted model, this significant manipulation of the humoral immune system appears safe pertaining to serious infection with repeated cycles of treatment.

This study has some limitations. First, this was a retrospective study, thus detailed information with regards to serious infections could be under-reported particularly those who lived outside the region. However, records for all patients were thoroughly reviewed in a systematic way and using various sources in order to enhance collection of data. Second, the lack of control group limits interpretation of safety of rituximab. Another potential limitation was heterogeneity of the patients, although this could also improve the generalisability of my findings. Next, multivariable analysis of predictors of SIEs based on repeated measurements, made on the same individual were complex to undertake. Nevertheless, this was overcome by using mixed effect logistic regression, which accounted for random effects on data. Lastly, concomitant therapy with csDMARDs and daily oral corticosteroid were used in 74% and 43% of the patients respectively, thus safety could not be attributed to rituximab alone.

6.5. Conclusion

In conclusion, risk factors that informed rituximab initiation were identified including previous cancer, chronic lung disease, previous history of severe infection and low IgG while a diagnosis of RA was associated with lower risk of SIEs. For repeat cycles of rituximab, risk factors that might inform cessation of therapy or modify using half-dose regimen from safety perspective were baseline comorbidities (similar as above) as well as pre-rituximab variables in any cycle such as higher corticosteroid dose, longer time-to-rituximab

retreatment and larger percentage of change in IgM. Finally, based on the data presented, immunoglobulin levels should be monitored at least 4-6 monthly and before any retreatment, particularly in those with comorbidities and low baseline immunoglobulin levels in order to identify those with the greatest risk for infection and who may require treatment with immunoglobulin replacement therapy.

6.6. Key messages

i. Patients with a previous history of cancer, chronic lung disease, previous severe infection and low IgG should be counselled regarding the risk of serious infection prior to commencing rituximab.

ii. Immunoglobulin levels should be monitored at least 4-6 monthly and before rituximab retreatment particularly in those with comorbidities, higher corticosteroid dose, longer time-to-rituximab retreatment and larger percentage of change in IgM in any cycle.

iii. Low B-cell subsets pre-rituximab and the degree of B-cell depletion post-rituximab were not associated with SIEs.

iv. More intensive B-cell depleting regimens have indicated the potential for more significant infection risk, thus an important consideration in guiding judicious use of retreatment with rituximab.

Chapter 7. TARGeted therapy using intradermal injection of Etanercept for remission induction in Discoid Lupus Erythematosus (TARGET-DLE)

7.1. Introduction

DLE is a chronic, autoimmune inflammatory skin condition and a form of CCLE. Chronic discoid lesions develop in up to 25% of patients with SLE but may also occur in the absence of any other clinical features of SLE (395, 396). Patients with DLE usually have only a negative or low-titre ANA (397). In these patients with positive autoantibodies, there is approximately 5 to 10% risk of eventually progressing to SLE, which usually tends to be mild (398). Hence the pathogenesis of DLE appears to be different from other systemic features of SLE, with a less clear role for circulating autoantibodies.

There is an unmet need for new therapies to control inflammation in DLE. A significant proportion of DLE patients (with or without SLE) are resistant to conventional therapies (272). There is no clinical guideline or algorithm on how to manage DLE patients who have refractory disease to the first line agents, i.e. anti-malarials. Combination therapy of anti-malarial agents and high dose oral steroid may be effective, but will lead to unacceptable complications from excess corticosteroid use including osteoporosis, metabolic consequences and increased risk of major cardiovascular events. Importantly, if left untreated, uncontrolled inflammation in DLE will lead to permanent disfiguring and irreversible scarring, thus posing a major cosmetic issue for the patient, which will significantly impair their quality of life (273, 399).

Targeted therapy based on immunopathogenesis of DLE is an attractive approach. As discussed in section 2.8, DLE may be exacerbated by B-cell depletion therapy (17). Moreover, the common occurrence of DLE in ANA-negative patients without lupus in other organs also suggests that B cell-targeted therapy may not be effective for this manifestation.

TNF is highly expressed in discoid lupus lesions and is implicated in the pathogenesis of DLE (400-402). A concern with systemic TNF-blocker administration is induction of pathogenic autoantibodies and flare of disease. Approximately 0.5-1.0% of patients treated with systemic TNF-blockers develop high affinity IgG autoantibodies to anti-dsDNA, that were associated with mild lupus-like syndromes (282). This could be overcome using a low-dose intra-lesional injection, which might be sufficient to neutralise the TNF in lesions. TNF-blockers have been administered using an intra-lesional injection in other TNF-mediated diseases such as Crohn's (403-405) and AS patients with refractory Achilles enthesitis (406), and appear safe and similarly effective to systemic administration.

Another important challenge is the problem with outcome measures as highlighted by the discussion around the negative clinical trials in SLE (214). The assessment of disease activity may be difficult owing to the concurrent infection and multiple comorbidities often present in these patients. Additionally, currently available instruments rely on subjective assessment (as described in section 2.3). Potential objective outcome measures to assess tissue response to therapy including an histology score of skin biopsy, optical coherent tomography (OCT), LDI (as described in section 2.3.8) and infrared thermography have not been utilised in a clinical trial.

Therefore, the TARGET-DLE trial addressed these problems by (i) administering an existing TNF-blocker, etanercept using a novel route of administration (intra-dermal), which would provide local concentration to neutralise TNF in tissue whilst minimised the effect to systemic immunity and (ii) measuring tissue response using the existing outcome measure; the modified limited SADDLE (ML-SADDLE) as well as objective measures such as skin biopsy, OCT, LDI and thermography. The concept and rationale of this study is illustrated in Figure 7-1.

The first proof-of-concept, open label trial to use existing drug, Etanercept but using a novel route of administration (Intra-dermal injection) for remission induction of discoid lupus (TARGET-DLE) [NCT02656082]

(n=25)
One index lesion was treated for 12 weeks

Local TNF-blockade 10mg

Discoid lupus erythematosus

- Safer
- More effective
- More cost effective

Skin   TNF   TNF   TNF

Systemic

B-cell targeted therapies ineffective

Systemic TNF-blockade 50mg

B cell

More antibodies
Worse SLE
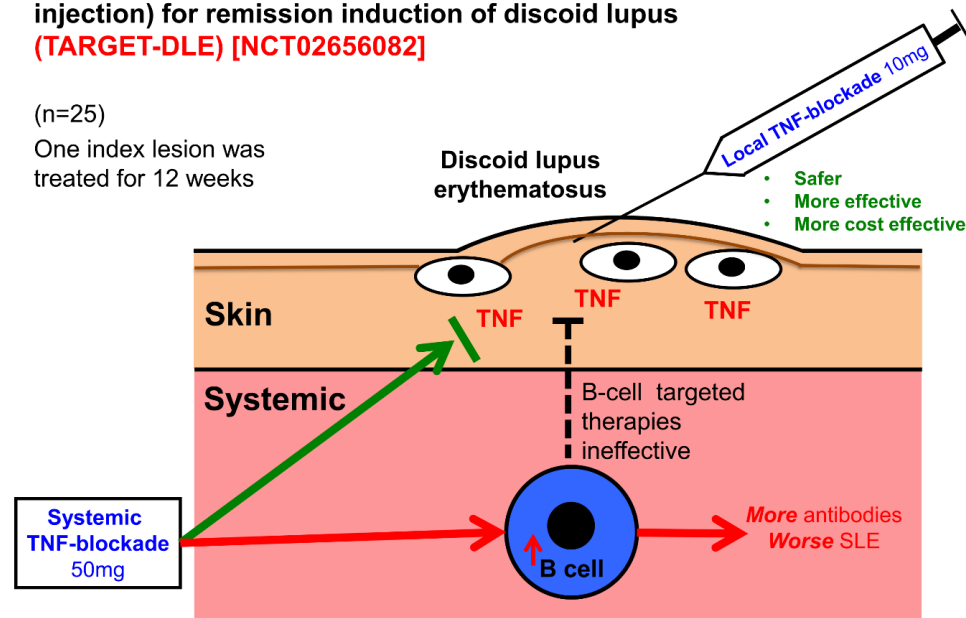
Figure 7-1 Concept and rationale of TARGET-DLE

TNF is implicated in the pathogenesis of DLE, thus should be targeted (green arrow). However, prolonged systemic administration of TNF-blockade therapy may activate B-cells (red arrow pointing upwards) by suppressing the production of Th1 cytokines, thereby driving the immune response towards Th2 cytokine production, IL-10, and IFN-α, a hypothesis called cytokine shift (407, 408). These cytokines then activate B-cells and lead to increase production of autoantibodies, which may render lupus worse or trigger a lupus-like syndrome. Therefore, I hypothesised that this induction of systemic autoimmunity could be minimised using intra-dermal injection of etanercept in DLE lesion. DLE: discoid lupus erythematosus; IFN-α: interferon-alpha; IL-10: interleukin-10; TNF: tumour necrosis factor; Th1: Helper T-cell type 1; Th2: Helper T-cell type 2

7.1.1. Hypothesis

Targeting TNF using an intra-dermal injection of etanercept is effective and safe for remission induction in DLE without inducing systemic autoantibody production.

7.1.2. Objectives

Primary

To assess the proportion of patients with active DLE that achieved the ML-SADDLE response (defined as reduction ≥20% in total activity from baseline) in the index lesion at Week 12 following treatment with weekly intra-dermal injection of etanercept.

Secondary

i. To assess other efficacy variables including higher hurdle endpoints such as ML-SADDLE 50 and 70 response rates, physician's VAS of global assessment of disease activity and daily oral corticosteroid requirement

ii. To evaluate patient-reported outcomes including Dermatology Life Quality Index (DLQI) and Patient's VAS

iii. To assess change in lesional OCT, LDI, thermography and histopathology score

iv. To report the safety of therapy in terms of AEs, adverse reactions (AR), serious adverse events (SAEs), serious adverse reactions (SARs), suspected unexpected serious adverse reaction (SUSAR)

v. To evaluate the effect of therapy to systemic immunity through development of SLE in patients with DLE only or worsening of SLE disease activities in patients with established SLE

vi. To assess whether intra-dermal delivery of administration is associated with accumulation of drug in systemic circulation

7.2. Methods

7.2.1. Candidate's roles in this project

In this work, the initial concept and design of the study were set by myself, Prof Goodfield, Dr Wittmann, Prof Emery and Dr Vital. As the principal investigator of this trial, I led the set-up of the study by writing the study protocol, preparing and submitting the appropriate documents for ethical, MHRA and research and innovation department approvals under the guidance of CTRU Leeds, trial coordinators (James and Huma) and chief investigator, Prof Emery. I recruited and consented all 25 participants to the study. I performed almost all clinical assessments including intra-dermal injection of etanercept, OCT, LDI, thermography and skin biopsy. The results for OCT were analysed by Dr Abignano while the skin biopsy was scored by Dr Edward. I designed the case report forms and entered a quarter of the data on the study database (the other three quarters were done by data entry personnel). In terms of statistical analysis, I performed all the statistical analyses, which were then checked by Dr Wilson. I am currently leading the writing of the manuscript, while other co-authors will revise the draft critically for important intellectual content and final approval of the manuscript prior to submission to journal for publication.

7.2.2. Study design

A prospective single arm, Simon's 2-stage minimax design with Hybrid adaptation, phase II open label trial was conducted in Leeds from 1 February 2016 to 31 December 2017. This study was registered with ClinicalTrials.gov, number NCT02656082.

Simon's 2-stage minimax design was chosen due to the advantage of allowing the minimum total number of patients needed to be treated with a new treatment that might be ineffective (409). While a hybrid adaptation of the 2-stage design was implemented to allow for recruitment to continue while the results of the first stage of recruitment were generated in the interim analysis (410).

7.2.3. Ethical approval

All patients provided informed written consent and this study was conducted in accordance with the principles of the Declaration of Helsinki. They also

consented for their images to be included in the study reports and publication.as long as their identities are protected (see Consent Form version 3.0 in Appendix). Ethical approval was gained from the National Research Ethics Committee Yorkshire and Humber, Sheffield [15/YH/0257] and the Medicines and Healthcare products Regulatory Agency [16767/0279/001-0001]. The University of Leeds was contracted with the administrative sponsorship.

7.2.4. Patients

Inclusion criteria

i.   Adults aged 18-80 years old.

ii. Had at least one active DLE lesion, either diagnosed by skin biopsy or confirmation by Dermatologist/ Rheumatologist.

iii. Patients with DLE only and SLE patients with DLE were included.

iv. Had refractory disease to an anti-malarial for at least 3 months as assessed by Dermatologist or Rheumatologist.

v.   Patients receiving anti-malarials must had been receiving them for at least 3 months prior to Screening, with a stable dose regimen for at least 28 days (±1 day) prior to Baseline (the first study drug administration)

vi. Ability to provide an informed consent.

vii. All male and female patients biologically capable of having children agreed to use a reliable method of contraception for the duration of the study and for a period of 3 weeks after their final dose of study drug. Acceptable methods of contraception were surgical sterilisation, oral, implantable or injectable hormonal methods, intrauterine devices or barrier contraceptives.

Exclusion criteria

i.   Any prior treatment with TNF-blockade therapies.

ii. Intramuscular or intra-dermal corticosteroid within 28 days of the Screening visit.

iii. Corticosteroid of greater than 10mg prednisolone daily equivalent, or change in oral steroid dose within 28 days prior to Baseline Visit.

iv.   A change in the dose of other immunosuppressant including methotrexate, azathioprine and mycophenolate mofetil within 28 days (±1 day) prior to Baseline Visit.

v.   Concomitant therapies with any alkylating agents (e.g. cyclophosphamide, chlorambucil), other immunosuppressant including sulfasalazine and leflunomide, other biological agent particularly anakinra and abatacept and other experimental drug. If patients were on any of these, they needed to be off therapies for at least 28 days prior to Baseline Visit to allow for washout.

vi.   Evidence of an immunosuppressive state, including an active HIV infection, agammaglobulinaemias, T-cell deficiencies or Human T cell Lymphotrophic Virus Type 1 (HTLV-1).

vii.   Chronic active infection such as hepatitis B or hepatitis C and tuberculosis. Patients with latent tuberculosis could be included if treated with chemoprophylaxis for at least 2 months before starting the study, and to continue chemoprophylaxis for a total of 6 months.

viii.   A history of cancer within the last 5 years except for squamous or basal cell skin carcinoma, which had been completely excised and treated cervical carcinoma *in situ.*

ix.   Demyelinating diseases.

x.   Moderate to severe heart failure based on New York Heart Association (NYHA) functional class III and IV.

xi.   Pregnancy.

xii.   Breastfeeding.

xiii.   Planned surgery within the study period which was expected to require omission of study medication of 28 days or more.

xiv.   Receipt of live attenuated vaccine within 28 days prior to Baseline Visit.

7.2.5. Treatment

The investigational medicine product (IMP) used in this study was etanercept (Enbrel). Etanercept is a recombinant human TNF-receptor fusion protein. It interferes with the inflammatory cascade by binding to TNF, thereby blocking its interaction with cell-surface receptors. The usual route of administration in its licensed indications i.e. RA, juvenile idiopathic arthritis, psoriatic arthritis,

psoriasis and axial spondyloarthropathies is via subcutaneous injection, given every week (280). However in this study, a novel route of administration using an intra-dermal delivery of the drug to DLE lesion for remission induction was investigated.

The non-IMP used was any anti-malarial agent including hydroxycholoroquine 200mg daily, chloroquine 150mg daily or combination therapy with hydroxychloroquine 200mg and mepacrine 100mg (alternate days). Patients would have had to receive any of the therapy above for at least 3 months. This non-IMP was continued during the trial as well as after the study had been completed at 12 weeks, for maintenance of disease control.

One index lesion was identified (i.e. the lesion with the highest ML-SADDLE score at baseline) and treated with weekly intra-dermal injection of etanercept for up to 12 weeks. The same lesion was injected at each time point.

If remission (as defined by modified limited SADDLE activity score = 0) was achieved earlier than expected, the study treatment would be ceased. The injection was administered by the investigators or qualified research nurses at the Day Case Unit (Ward 5), Chapel Allerton Hospital, Leeds.

Selection of dose and dose modification

The usual dose of an intradermal administration of a therapy such as corticosteroid is 0.2ml per injection, with repeated injections used to cover a larger lesion. Etanercept was available in a 10mg vial, which was made up to 1ml so that each 0.2ml dose would contain 2mg.

We estimated that this dose would adequately neutralise typical concentrations of TNF in DLE lesion. The estimated TNF concentration in an inflamed tissue would be up to 500-5000 ng/mL (411). The dose of TNF-blocker required to neutralise this would be 100 times the concentration of TNF. We estimated that a dose of 500µg of etanercept would be adequate to treat 1ml of inflamed tissue. When adjusting for residual volume retained in the syringe and backflow of volume out of the skin, the following dosing guide (Table 7-1) was used, with multiple injections spread across a larger lesion.

Table 7-1 Intra-dermal injection of etanercept dosing guide

Lesion radius (cm)	Volume of inflamed skin for 0.5cm thickness (cm^3)	Estimated Etanercept concentration required (mg)	Volume of Etanercept to be injected (ml) [10mg/ml]	Number of 0.2ml doses required
1	1.57	0.80	0.1	0.5
1.5	3.53	1.80	0.2	1
2	6.28	3.14	0.3	1.5
2.5	9.81	4.90	0.5	2.5
3	14.14	7.10	0.7	3.5
3.5	19.24	9.62	1.0	5.0

For safety and tolerability purposes, the first dose acted as a test dose using etanercept 1mg dose irrespective of the size of the lesion. As etanercept was used for an unlicensed condition in this study, we had capped a ceiling therapy of 10mg per injection at one treatment visit for a discoid lesion ≥3.5 cm radius. This is in line with clinical practice where up to 10mg of triamcinolone (corticosteroid) is injected intra-lesionally to discoid lupus at one session (412).

Prior and concomitant medications

Concomitant medications were kept to a minimum during the study. However, if these were considered necessary for the patients' welfare and were unlikely to interfere with the investigational products, they could be given at the discretion of the investigator and recorded.

Prohibited prior medications

- Any prior treatment with TNF blockade therapies.
- Intramuscular or intra-dermal corticosteroid within 28 days of the Screening visit.

Permitted concomitant medications

If the patients were prescribed oral prednisolone for maintenance, the dose must had been ≤10mg (or equivalent) and were stable for at least 28 days prior to Baseline visit.

Those who were prescribed anti-malarials must had been receiving them for at least 3 months prior to Screening, with a stable dose regimen for at least 28 days (±1 day) prior to Baseline visit.

Permitted other concomitant csDMARDs include methotrexate, azathioprine and mycophenolate mofetil. The patients must had been on a stable dose of this DMARDs for at least 28 days (±1 day) prior to Baseline visit.

Prohibited concomitant medications

- Any topical corticosteroid preparation
- Any alkylating agents (e.g. cyclophosphamide, chlorambucil).
- Certain csDMARDs including sulfasalazine and leflunomide.
- Any other bDMARDs particularly anakinra and abatacept.
- Any experimental drug.

7.2.6. Vaccination with live attenuated vaccines.Assessment

Study schematic

The study schematic of TARGET-DLE trial is summarised in Figure 7-2.

Summary schedule of study assessments

The schedule of study assessment of the 15-week study is summarised in Table 7-2.
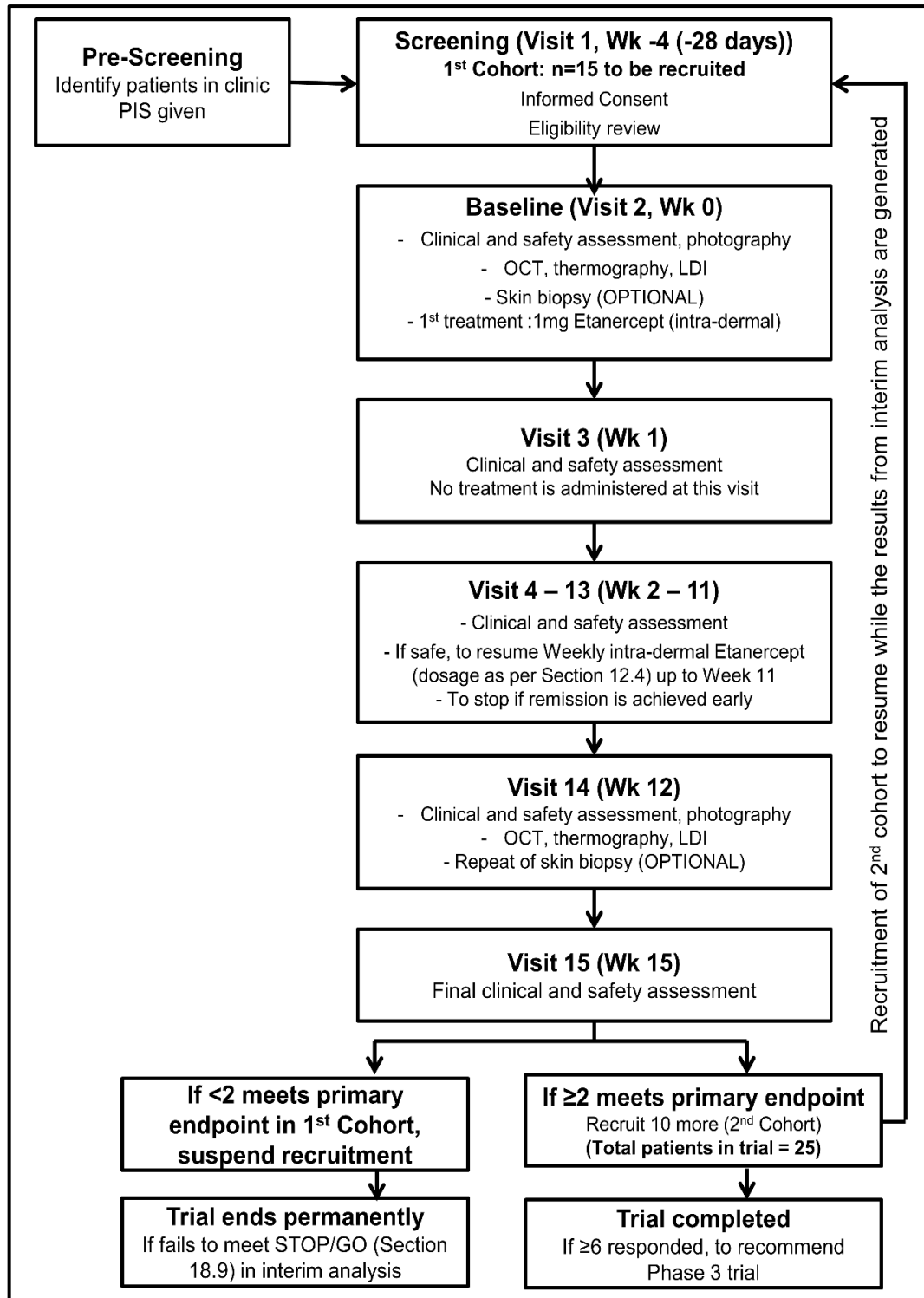
Figure 7-2 Study schematic of TARGET-DLE trial

A flow chart of the trial from pre-screening to screening, baseline, treatment and end of study visits.

Table 7-2 Summary schedule of study assessments for TARGET-DLE trial

Assessment (Procedure/Activity)	Screening Visit 1	Baseline Visit 2	Clin. Visit 3	Clin. Visit 4	Clin. Visit 5	Clin. Visit 6	Clin. Visit 7	Clin. Visit 8	Clin. Visit 9	Clin. Visit 10	Clin. Visit 11	Clin. Visit 12	Clin. Visit 13	Clin. Visit 14	Clin. Visit 15
Week (Wk)	Wk -4 (-28 days)	Wk 0	Wk 1	Wk 2	Wk 3	Wk 4	Wk 5	Wk 6	Wk 7	Wk 8	Wk 9	Wk 10	Wk 11	Wk 12	Wk 15
Treatment - Etanercept intra-dermal injection[a]		x		x	x	x	x	x	x	x	x	x	x		
Informed Consent	x														
Inclusion / Exclusion Criteria	x	x													
Demographic Data	x														
Medical/Surgical History	x	x												x	x
Concomitant Medication	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
Adverse Event check		x	x	x	x	x	x	x	x	x	x	x	x	x	x
Physical examination & Vital signs	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
Urinalysis	x	x							x						x
Pregnancy test (urine)	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
Chest X-ray (if not already done within 6 months)	x														
HIV screen	x														
Hepatitis screen	x														
Quantiferon test	x														
Immunology (ANA,anti-dsDNA, ENAs and ACA)	x								x						x
Complement (C3 and C4)	x								x						x
Immunoglobulins	x								x						x
Haematology	x								x						x
Biochemistry	x								x						x
Etanercept level[b]		x				x									
Blood for exploratory biomarkers		x													
Medical photography of the skin lesions[c]		x												x	x
Skin Biopsy[d]		x												x	
Optical Coherence Tomography (OCT)		x												x	
Infrared thermography		x												x	
Laser Doppler Imaging (LDI)		x												x	
Modified Limited SADDLE		x	x	x	x	x	x	x	x	x	x	x	x	x	x
BILAG 2004 (for SLE patients with DLE)		x							x						x
SLEDAI (for SLE patients with DLE)		x							x						x
Physician's Global Assessment (VAS)		x												x	x
Patient's Global Assessment (VAS)		x												x	x
Dermatology Life Quality Index (DLQI)		x												x	

7.2.7. Primary efficacy variable

SADDLE score (189) was chosen as the primary efficacy variable instead of other instruments such CLASI (183) because it accounted for the three important morphologies of DLE; erythema, scaling and induration (413). This instrument has been described in details in section 2.3.6.

In this study, a ML-SADDLE score was used; limited to only one index lesion and the efficacy was judged based on total score in activity only.

7.2.8. Secondary efficacy variables

Physician's VAS for global assessment of disease activity

The investigator rated the overall disease activity status of the patient on the day of the visit, with respect to the DLE signs and symptoms and the functional capacity of the patient, using a 100mm VAS where 0 was "very good, asymptomatic, and no limitation of normal activities" and 100 was "very poor, very severe symptoms which were intolerable, and inability to carry out all normal activities."

Requirement for daily oral corticosteroid

The patients reported daily oral prednisolone intake at each visit. Tapering of oral corticosteroids after Week 3 (Visit 5) to a target dose of ≤5 mg/day prednisolone equivalent was encouraged during the study. Steroid dose adjustments should be avoided during Weeks 9 to 12 (Visit 11 to 14).

A temporary increase in oral corticosteroids up to a maximum of 25% above Baseline levels was allowed, if needed, at the discretion of the investigator should the patients develop skin flare with therapy. Flare was defined as an increase in disease activity in skin compared to previous assessment in a patient previously improving or stable, requiring a change in treatment. Those who had increment in oral corticosteroids >25% of Baseline levels were considered non-responders.

BILAG-2004 (For SLE patients with DLE)

This index had been described in details in section 2.3.1. This assessment was only undertaken in SLE patients with DLE at Baseline, Week 7 and Week 15.

SLEDAI-2K (For SLE patients with DLE)

This index had been described in details in section 2.3.2. Similar to BILAG-2004 index, this assessment was only undertaken in SLE patients with DLE at Baseline, Week 7 and Week 15.

Dermatology Life Quality Index (DLQI)

The DLQI was the first dermatology-specific health-related quality of life (HRQoL) questionnaire developed in 1994 (414). This instrument consisted of 10 questions concerning patients' perception regarding the impact of skin diseases on different aspects of their health related quality of life over the last one week. The items of the DLQI encompassed aspects such as symptoms and feelings, daily activities, leisure, work or school, personal relationships and the side effects of treatment.

Each question was scored on a 4-point Likert scale: Not at all/Not relevant=0, A little=1, A lot=2 and Very much=3. Scores of individual items (0-3) were added to yield a total score (0-30).

Patient's global health assessment (VAS)

The patients rated the global assessment of their DLE disease activity on the day of the visit in response to the question "Considering all the ways your DLE affects you, please mark a vertical line on the scale below for how are you feeling today?" using a 100mm VAS where 0 was "very good, no symptoms" and 100 was "very poor, very severe symptoms."

Immunological assessments

Blood samples for measurement of immunological parameters were collected at the time points specified in the schedule of study assessments (Table 7-2).

- ANA
- anti-dsDNA
- anti-ENAs (anti-SM, anti-RNP, anti-Ro, anti-La and anti-chromatin antibodies)
- anti-cardiolipin antibody (ACA)
- Complement levels (C3 and C4)

Optical Coherence Tomography

"Virtual skin biopsy" using OCT, is a quantitative imaging biomarker that is useful for monitoring disease activity in inflammatory skin diseases (415-417). This test produces two-dimensional (2D) images of optical scattering from internal tissues, that enable visualisation of micromorphological structures at the epidermis and the upper dermis, thus provides information on the severity of the disease and on treatment effects (417, 418). The reasons for choosing OCT as a secondary efficacy variable in this trial were because it provided an instant, direct imaging of tissue morphology at much higher resolution (~ 10 μm) than other imaging modalities such as magnetic resonance imaging (MRI) or ultrasound, a non-invasive and non-contact test, as well as no ionising radiation was involved (419, 420).

In this study, the OCT scans were performed by a rheumatologist using the VivoSight machine (Michelson Diagnostics) which comprised four parallel Swept-Source OCT systems, using a laser with central wavelength of 1310 and 150 nm laser sweep. For each index lesion that was scanned, the handheld OCT probe was used to capture 100 OCT 6 mm B-scans with an inter-frame spacing of 4 μm. The resulting image (4×0.4×2 mm) was reviewed in real-time before being stored for later analysis.

Previous studies have shown that the OCT parameters correlated with the histopathology of cutaneous lupus in skin biopsy: (i) thickening and disruption of the entrance signal correlated with hyperkeratosis (ii) thinning of layer below the entrance signal correlated with atrophy of epidermis (iii) patchy hyporeflective zones in the epidermis correlated with lymphocytic infiltrates in the upper dermis and (iv) wide signal free cavities in the upper dermis correlated with dilated vessels in the upper dermis (421). At the end of this study, the OCT images were scored by an independent rheumatologist, who

was blinded to the patient's clinical status. These four OCT parameters were graded using a scale of 0-3; 0=none, 1=slight, 2=moderate and 3=strong.

Laser Doppler Imaging

This objective outcome measure had been described in section 2.3.8. An area with the highest ML-SADDLE score and non-lesional area were evaluated using a high resolution LDI system (moorLDI2-IR, Moor Instruments UK) by a rheumatologist; who was trained in the operation of the LDI and was blinded to the patient's clinical information.

All scans were performed in a designated assessment room after the patients were acclimatised to room temperature (22⁰ Celcius) for 15 minutes. Images were acquired at a distance between 40-70 cm from the selected areas using a bandwidth between 250Hz-15KHz and the scan speed of less than 5ms/pixel. The region of interests were selected and analysed using Moor LDI2-IR version 5.0 software. The relative difference in the mean perfusion between active and non-active CLE lesions was calculated and expressed in perfusion unit (PU).

In a previous study for which I am the first author, we reported that LDI correlated better with histology score compared to ML-RCLASI and physician's VAS of photograph. LDI was also shown to be responsive to clinical change with a cut-off of 20% reduction in the relative difference in the mean perfusion PU to be the most predictive (198).

Infrared thermography

Thermography is a non-invasive technique that detects infrared radiation to provide an image of the temperature distribution across skin surface. This skin temperature image is influenced by the state of the skin vasculature or heat generated in deeper tissues (inflamed). This tool has been used to assess disease activity in cutaneous manifestation of connective tissue disease (422).

The protocol for thermography and LDI was nearly identical, so these two tests were done at the same time. The only difference was that the former detected the temperature of the skin whereas the latter studied blood flow (perfusion) to the skin. Two areas were evaluated using the FLIR C2 compact thermal

imaging system; active DLE lesion and non-lesion areas by a rheumatologist, who was blinded to the patient's clinical information. The temperature difference between these lesions was calculated in real-time and expressed in Celcius.

Histology score from skin biopsy

The patients were invited to undergo skin punch biopsies at Baseline and post-treatment (Visit 14, Week 12). A separate section in the Consent Form was provided for the consenting patients.

Two x 4mm biopsies were obtained from the DLE lesion of the consenting patients, of which ½ x 4mm was fixed in 10% formalin before staining with haematoxylin and eosin whilst another ½ x 4mm was kept in Michel's transport medium for immunofluorescence staining. The samples were rated in real-time by a histopathologist, with over 10 years' experience in reporting DLE cases and who was blinded to the patient's clinic status. Since there was no standardised histological scoring system for DLE, the histopathologist scored the biopsy based on their classic histological features including (i) interface dermatitis; (ii) inflammatory cell infiltrate in a perivascular, periappendageal or subepidermal location; (iii) vacuolar alteration of the basal layer; (iv) thickening of the basement membrane; (v) follicular plugging; (vi) the presence of immunofluorescence and (vii) dermal mucin deposition (423). The first two parameters were rated using a graded scale of 0-2; 0=absent, 1=mild and 2=strong while the remaining five parameters were rated using a binary scale; 0=absent, 1=present, with a maximum total score possible of 9. Finally, since these parameters were not weighted for clinical significance, an overall grade of activity score was then assigned for each biopsy sample using a graded scale of 0-2; 0=non active, 1=mild and 2=active. This histology grade was used as a gold standard for measuring DLE activity and for comparison with other instruments.

The remaining 4.0 mm biopsy sample was cryopreserved using the optimum cutting temperature compound and stored at the University laboratory, Chapel Allerton Hospital for later analysis and future research.

Photograph of DLE

The photograph of the index lesion was taken at baseline and post-therapy using a macro digital camera, Canon EOS 600D.

Pharmacokinetics

The pharmacokinetic (PK) profile of etanercept was assessed to determine whether intra-dermal route drug delivery led to accumulation of etanercept in systemic circulation. Blood samples for determination of serum etanercept were collected at two time points: (i) before the first dose at Baseline and (ii) trough levels (prior to treatment at Week 4) as specified in the schedule of study procedures (section 7.2.5.2). These bloods were stored as serum at the University laboratory, Chapel Allerton Hospital. At the end of the study, these serum were tested for etanercept concentration using the Promonitor® Etanecept ELISA according to the manufacturer's instructions.

Safety

Safety variables including AEs, ARs, SAEs, SARs and SUSARs were recorded at each visit throughout the study period. All AEs were graded according to the National Cancer Institute's Common Terminology Criteria (version 4.0) (424).

Laboratory measurements

In addition to the blood obtained for immunological tests, other laboratory measurements collected in this study were haematology, biochemistry, urine pregnancy testing (for women of child bearing potential) and urinalysis.

7.2.9. Withdrawal criteria

The patients were permitted to withdraw from the study at any time, without prejudice to their continued care.

They could be withdrawn from the study for the following reasons:

- At their own request – they might (i) withdrew from having treatment only but were happy to be followed up; or (ii) withdrew consent for further trial treatment and follow-up, but were willing to have any available follow-up information collected from healthcare records; or (iii) withdrew from further

trial treatment, and follow-up information to be collected.

- At the request of their legally authorised representative.
- If, in the opinion of the investigator or the Data Monitoring and Ethics Committee (DMEC), continuation in the study was detrimental to the patient's well-being.

They must be discontinued from study medication based on the following circumstances:

- Pregnancy or constant failure to use a medically acceptable form of birth control in the 4 months of the study period (every attempt must be made to follow up patients who became pregnant to determine the outcome of the pregnancy).
- Grade 3 or 4 systemic toxicity (424) or SAEs thought to be related to study treatment and not alleviated by symptomatic treatment after cessation the patient's medication of up to 4 weeks.
- Serious infection requiring parenteral (intravenous, intramuscular) antimicrobial agent or hypotension suggestive of impending sepsis syndrome.
- Acute or re-activation of tuberculosis or hepatitis infection.
- Confirmed blood dyscrasia or a demyelinating disorder (such as multiple sclerosis or optic neuritis).
- Progression to SLE in patients with DLE only.
- Worsening in BILAG-2004 in organ systems other than mucocutaneous compared to baseline in SLE patients with DLE.
- The patients' compliance. If they were to miss the treatment by 4 or more consecutive injections, then they would be withdrawn from further therapy.

7.2.10.   Primary endpoint

Since the patients had DLE lesions that were refractory to the standard therapy with anti-malarial agents, there was no other proven effective second line agent for this condition. Therefore, a relatively low hurdle was set. Hence, treatment with intra-dermal injection of etanercept was deemed successful if there was a decrease of 20% from baseline at Week 12 in the ML-SADDLE score. This was also in line with response criteria for other ARDs such as RA

and psoriatic arthritis where a reduction of 20% from baseline in the ACR disease activity index was used as primary endpoints in clinical trials (425, 426).

Therefore, the primary endpoint for this study was at least 6 patients achieving the ML-SADDLE 20 response (defined as reduction ≥20% in total activity from baseline) at Week 12 for a phase 3 trial to be recommended.

7.2.11. Secondary endpoints

i. Proportion of patients with ML-SADDLE-50 and 70 response.

ii. Change in physician's VAS and daily oral prednisolone requirement at Week 12.

iii. Change in patient-reported outcomes; DLQI and patient's VAS at Week 12.

iv. Change in the total score of OCT parameters at Week 12.

v. Change in the difference in temperature between active DLE and non-active areas using thermography at Week 12.

vi. Change in the difference in perfusion between active DLE and non-active areas using LDI at Week 12.

vii. Change in the overall grade of histology score of skin biopsy at Week 12.

viii. Incidence of AEs, ARs, SAEs, SARs and SUSARs.

ix. New development or worsening of positive auto-antibodies titres: ANA, anti-dsDNA, anti-ENAs and ACA at Week 7 and 15.

x. Change in complement (C3 and C4) levels to below the normal limit (if normal at baseline) at Week 7 and 15.

xi. For SLE patients with DLE, change in disease activity as assessed using the BILAG-2004 score and SLEDAI-2K indices at Week 7 and 15.

xii. Proportion of patients with detectable trough etanercept level in serum post-therapy.

7.2.12.   Statistical analyses

Sample size calculation and STOP/GO criteria

Based on the current evidence (272) and from clinical experience, after treatment failure to an anti-malarial agent, there is no second line agent which is effective. Most patients would have exhausted various csDMARDs such as acitretin, thalidomide, retinoids, dapsone, methotrexate and mycophenolate mofetil; all with limited benefit.

Therefore, we considered that if intra-dermal injection of etanercept could reduce the ML-SADDLE score by 20% or more from baseline score in 30% or more patients who were refractory to other treatments, then this would be worthwhile to assess further in a phase III trial. However, if the response rate was lower than 10%, then intra-dermal injection of etanercept in DLE should be rejected from further consideration.

Using a maximum significance level of 5% and power of 80% in a minimax design required 15 patients to be recruited in the first cohort. If 2 or more patients were considered responders from the interim analysis, then a second cohort of 10 patients would be recruited. While the outcomes data for each of the first 15 patients were collected, recruitment of second cohort of patients would continue. This accrual would stop if a total of 25 patients had been recruited prior to a complete evaluation of the results from the first cohort. Should this occur, then study would be treated as per a single-stage design with no interim analyses required. Otherwise, a formal interim analysis would take place.

During the interim analysis, if there were less than two responders, then the accrual of second cohort would be suspended. The interim analysis would take into account data from the second cohort of patients that had been collected. In this circumstance, the stopping rule for permanently terminating accrual could be calculated using the formula:

$$c^* \approx r_1 \left(1 - (n^*/n_2)\right) + r_2 \left(n^*/n_2\right);$$

where c^* was the maximum number of responders required for trial termination, r_1 was the maximum number of responders for terminating the trial in the first stage based on the original minimax design, n^* was the number

of patients (with complete data) that had been accrued in the second cohort, r2 was the total maximum number of responders in the first and second stages that would result in a phase III trial not going ahead based on the original minimax design, and n2 was the number of patients in the second cohort.

For example, if there were less than 2 responders when the first 15 patients were analysed while 5 further patients had completed follow-up in the second cohort, then the criteria for permanently stopping recruitment in the interim analysis, C^* would be = $1(1-(5/10))+5(5/10) = 3$. Hence, if 3 or fewer of the 20 patients responded to treatment in the first cohort, then further recruitment would be permanently terminated.

Once the second cohort had been recruited and the study was completed, if 6 or more of the combined 25 patients were considered responders, a phase III RCT could be recommended.

Missing data

For the primary and secondary efficacy analyses, complete data might be missing if the patients met the early discontinuation of study criteria as per section 7.2.8. The effect that any missing data might have on results would be assessed using sensitivity analysis of augmented data sets. Dropouts (essentially, those who withdrew consent for continued follow-up) or missing data for laboratory components in BILAG-2004, SLEDAI and safety analysis were included in the analysis by multiple imputation method for missing data if appropriate.

Analyses

All data including patients who withdrew from the therapy were included in the final analysis (full analysis set). Descriptive summary statistics including number of patients, mean, standard deviation, median, 25% and 75% quartiles were reported for all continuous variables. Frequency distributions were provided for categorical data. The number of cases that met the primary and secondary endpoints were summarised using proportion and 95% CIs. Continuous variables were compared either using Student's T-tests or Mann-Whitney U tests whilst Fisher's exact test was used for categorical variables. Correlation between two continuous variables was assessed using Pearson's

correlation. To evaluate responsiveness to therapy, predictive values of each of the three ML-SADDLE response criteria were evaluated against LDI and were reported in terms of sensitivity, specificity, PPV, NPV and their respective 95% CIs.

Line listings of all AEs, SARs, SAEs and SUSARs were provided. Statistical analyses were performed using IBM SPSS Statistics v21.0 (IBM Corp, Armonk, New York, USA) and Stata v.13.1 (StataCorp College Station, Texas, USA) for Windows.

7.3. Results

7.3.1. Study summary

All 25 patients were recruited within the 18-month period set for this study. Of this, 17 patients completed the primary efficacy assessment [Did not attend Week 12 visit=1, early withdrawals=7 (personal choice=2, AE=2, worsening of DLE=1, non-compliance to protocol=1 and pregnant=1)]. The flowchart of participants is illustrated in Figure 7-3.
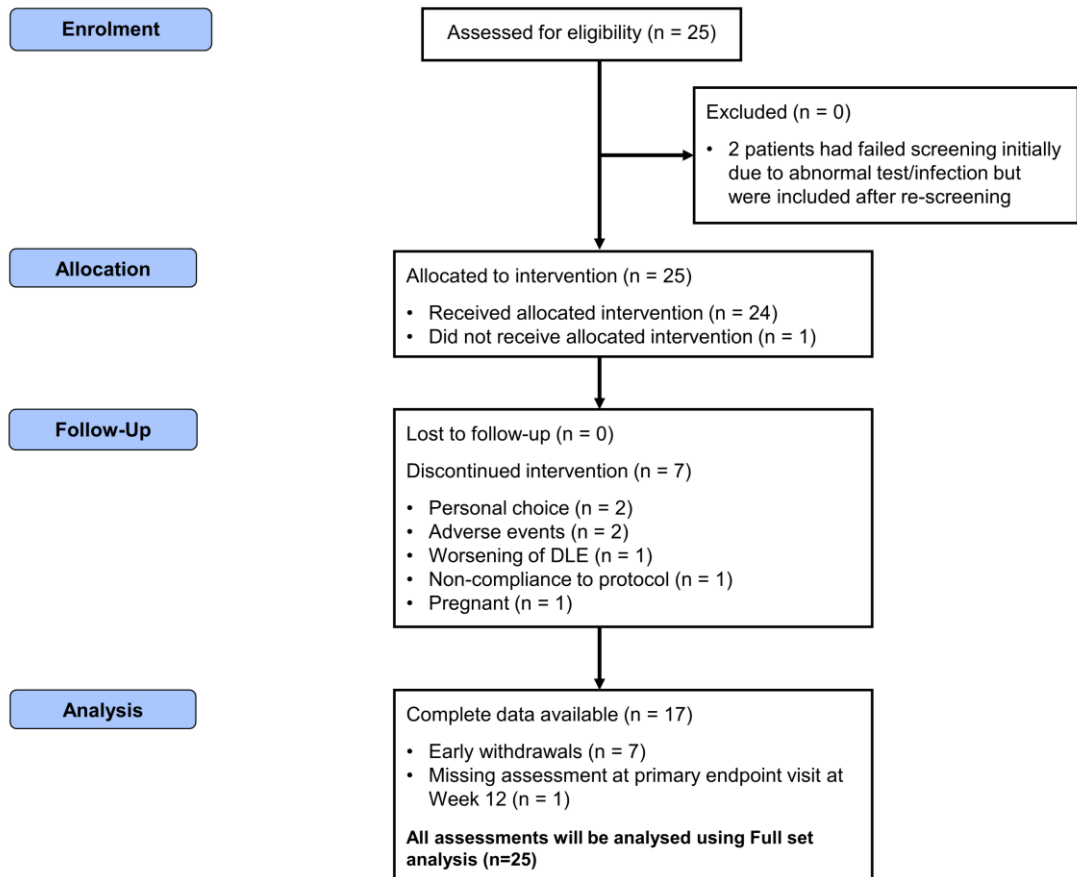
Figure 7-3 CONSORT flow diagram of TARGET-DLE trial

All 25 patients were recruited into the study over a period of 18 months. Of this, 24/25 received the intervention. 7 patients discontinued treatment early. At the end of the study, complete data were available for 17 patients for per protocol set analysis. However, for primary endpoint assessment, the results will be reported based on full analysis set.

7.3.2. Patient characteristics

Baseline characteristics are summarised in Table 7-3. Notably, this cohort comprised resistant DLE patients with median (range) number of previous systemic therapies (csDMARDs and bDMARDs) of 5 (1-16).

Table 7-3 Baseline characteristics of the 25 patients recruited in TARGET-DLE trial

Characteristic	Values
Mean Age (SD), Years	47 (12)
Female : Male	18 : 7
Race, n (%)	
Caucasian	18 (72)
South Asian	6 (24)
Afro-Caribbean	1 (4)
Median DLE duration (Range), Years	10 (0.5 – 26.5)
Previous positive skin biopsy for DLE, n (%)	19 (76)
Concurrent SLE, n (%)	6 (24)
ANA positive, n (%)	9 (36)
anti-dsDNA	2 (8)
anti-Ro	3 (12)
anti-La	2 (8)
anti-Sm	1 (4)
anti-Chromatin	1 (4)
anti-RNP	0
anti-Ribosomal P	0
anti-Sm/RNP	1 (4)
anti-Cardiolipin/anti-B2-Glycoprotein	0
Low C3 or C4 complement levels, n (%)	1 (4)
Concomitant csDMARDs excluding anti-malarials, n (%)	6 (24)
Methotrexate	2 (8)
Thalidomide	2 (8)
Azathioprine	1 (4)
Mycophenolate mofetil	1 (4)
Concomitant anti-malarial agents, n (%)	14 (56)
Concomitant prednisolone, n (%)	7 (28)

Characteristic	Values
Median no. previous cs and bDMARDs (Range)	5 (1 – 16)
Family history of ARDs, n (%)	6 (24)
Ever smoked, n (%)	20 (80)
Current	15 (60)
Previous	5 (20)

ARD: autoimmune rheumatic disease; bDMARDs: biological disease modifying anti-rheumatic drugs; cs: conventional synthetic; DLE: discoid lupus erythematosus; dsDNA: double-stranded deoxyribonucleic acid; RNP: ribonucleic peptide

7.3.3. Treatment characteristics

In terms of feasibility of therapy administration, 10/25 (40%) adhered to all 11 injections planned. The median percentage treatment compliance was 80%. Details of treatment characteristics are described in Table 7-4.

Table 7-4 Treatment characteristics and compliance to therapy

Treatment characteristic	Values
Anatomical site for injection i.e. lesion with the highest pre-treatment SADDLE score; n	24*
Scalp	13
Cheek	4
Upper back	2
Forehead	2
Nose	1
Ear	1
Arm	1
Patients who completed all 11 intra-dermal injections included the test dose, n (%)	10 (40)
Median percentage treatment compliance i.e. number of injections received/expected number of injections received, (range)	80 (18 – 100)
Reason for treatment interruption, n	
Concurrent infection	12
Early withdrawals	7
Logistics issues	4
Other Personal reasons	4
Dose modification that violated the trial protocol, n	0

* One patient withdrew prior to receiving first treatment, hence total number was 24

SADDLE: Score of Activity and Damage in Discoid Lupus Erythematosus

7.3.4. Primary endpoint

Since all 25 patients were recruited prior to complete evaluation of the results from the first cohort i.e. the first 15 patients, no formal interim analysis was undertaken. Therefore, this study was treated as per a single-stage design.

In the full analysis set, the primary endpoint was met with 13/25 (52%, 95% CI 31-73) meeting the ML-SADDLE 20 response rate at week 12. The mean ML-SADDLE had reduced from 5.2 (SD 1.6) pre-treatment to 2.5 (1.8) post-treatment; mean difference 2.7 (95% CI 1.7 to 3.7); $p<0.001$. At week 15, the ML-SADDLE 20 response rate was sustained at 52% (95% CI 31-73).

7.3.5. Secondary endpoint: Efficacy

For higher hurdle endpoints, the ML-SADDLE 50 response rates at week 12 and week 15 week were both 12/25 (48%, 95% CI 27-69) whilst the ML-SADDLE 70 response rates for week 12 and week 15 were 5/25 (20%, 3-37) and 6/25 (24%, 6-42) respectively.

Of 7 patients who were on daily oral prednisolone at baseline, none of them had their dose reduced or increased at week 12. At week 15, 2/7 had their dose doubled by the medical team since they were hospitalised due to infections. Of 18 patients who were not on daily oral prednisolone at baseline, none of them required treatment with steroid throughout the trial.

There were significant improvements at week 12 from baseline for most of the key secondary efficacy endpoints including physician's VAS, patient-reported outcomes (DLQI and patient's VAS) and objective outcome measures (LDI and thermography).

Although there was a trend to improvement in the total OCT score, mean difference between pre-treatment and post-treatment was not significant. These results are shown in Table 7-5 whilst photographs and LDI images of those who had improved with therapy are depicted in Figures 7-4 to 7-6.

Thirteen patients underwent skin biopsy procedures at baseline. Of these, 6/13 had paired pre- and post-biopsy samples. Of those with paired biopsy samples, 2/6 had histology score improved, 2/6 remained the same and 2/6 had worsening score at week 12.

There was a trend to correlation between total histology score and ML-SADDLE score at baseline, r=0.50; p=0.085.

Table 7-5 Results of secondary efficacy endpoints

Endpoint	Baseline	Week 12	Mean difference (95% CI), p-value
Physician VAS, mean (SD) mm	53.1 (16)	23.2 (20)	29.9 (19.4 to 40.4), p<0.001
Patient VAS, mean (SD) mm	56.9 (28)	29.7 (28)	27.2 (12.2 to 40.1), p<0.001
DLQI, mean (SD)	11.4 (7)	6.5 (6)	4.9 (2.6 to 7.1), p<0.001
LDI, mean (SD) perfusion unit (PU)	495.1 (224)	376.2 (223)	118.9 (23.7 to 214.0), p=0.018
Thermography, mean (SD), ºC	1.92 (1.17)	1.08 (1.05)	0.84 (0.30 to 1.39), p=0.005
OCT score, mean (SD)	4.4	3.7	0.7 (-0.3 to 1.7), p=0.144

DLQI: Dermatology Life Quality Index; LDI: laser doppler imaging; OCT: optical coherence tomography; SD: standard deviation; VAS: visual analogue score

Figure 7-4 Photographs of Patent 05 who responded to intra-dermal injection of etanercept

Photos of a patient who responded to the therapy. Red arrow denotes the index lesion, the site where the injection was given

Figure 7-5 Photographs of Patient 07 who responded to intra-dermal injection of etanercept

This patient met the ML-SADDLE 70 response at Week 12. This was an exceptional case where her scarring alopecia did improve with therapy. The red arrow denotes the index lesion where the injection was given. ML-SADDLE: Modified Limited Score of Activity and Damage in Discoid Lupus Erythematosus
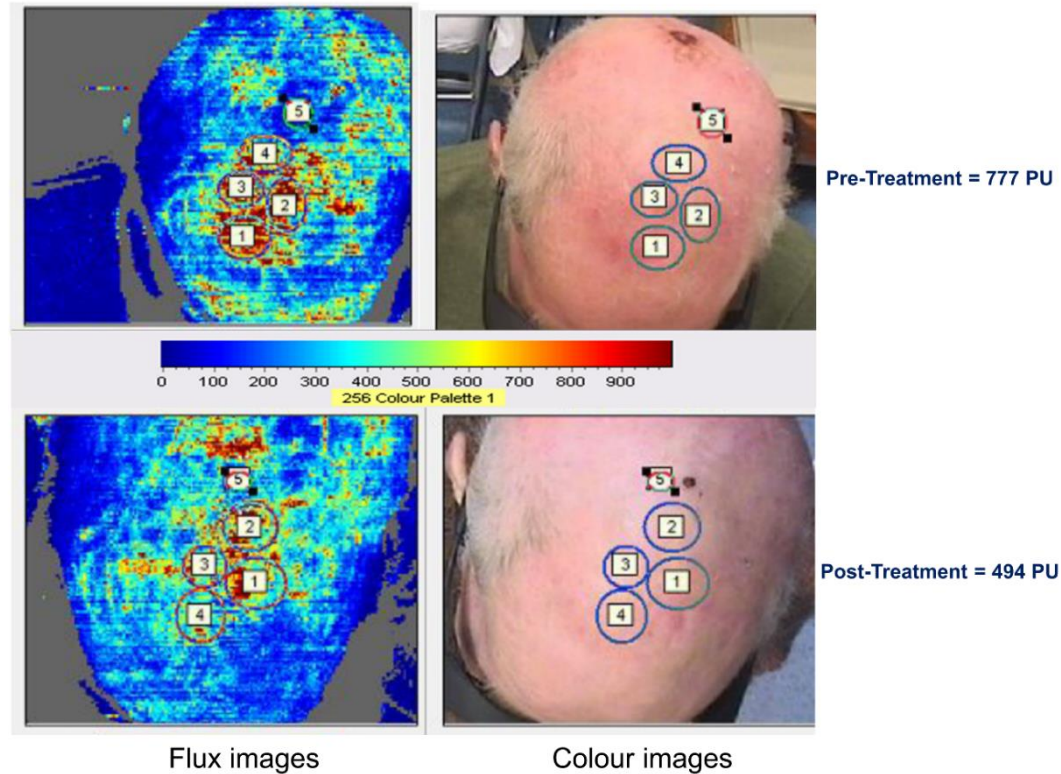
Figure 7-6 Images from laser doppler imaging of Patient 25 who had a partial reduction in ML-SADDLE score post-treatment

This patient had a partial reduction in ML-SADDLE score from 5 at baseline to 2 at week 12. This partial improvement as rated by clinical assessment was supported by LDI. The blue circles drawn outside the numbers 1-5 represent regions of interest (ROI) in the analyses. The ROI with the highest score was chosen. ML-SADDLE: Modified Limited Score of Activity and Damage in Discoid Lupus Erythematosus; PU: perfusion unit

7.3.6. Secondary endpoints: Safety

Incidence of AEs, ARs, SAEs, SARs and SUSARs

There were 53 AEs recorded in this study as described in Table 7-6. Of these, 29/53 were treatment-emergent recorded in 14 patients. Most of the AEs were due to lower respiratory tract infection (LRTI); n=6. Injection-site reaction or infection cases were recorded in 4 patients. Grade 3 or higher systemic toxicity AEs were recorded in 4 patients (LRTI = 1, Presumed infection, source unidentified = 1, Heart failure = 1, Worsening of chilblains = 1). One patient became pregnant after receiving one dose of intra-dermal injection of etanercept and she had to be withdrawn from the study. The outcomes for both mother and baby were uneventful. Withdrawals due to AEs were recorded in 2 patients.

Table 7-6 Adverse events recorded in TARGET-DLE trial

Characteristic	Values
All Adverse Events (AEs), n	51
Treatment-emergent AEs, n	29
Infection	
Lower respiratory tract infection	6
Presumed infection	1
Urinary tract infection	2
Injection related-skin infection	1
Pharyngitis	1
Otitis externa	1
Skin	
Pruritus	1
Injection related swelling/oedema	3
Worsening of subacute cutaneous lupus	1
Nervous system	
Headache	4
Dizziness	1
Respiratory	
Upper respiratory tract infection	1
Cough	1
Pleuritic chest pain	1

Characteristic	Values
Cardiovascular	
Heart failure	1
Gastrointestinal	
Vomiting	1
Faecal incontinence	1
General	
Fatigue	1
Grade 3 or higher AE, n	4
AE of special interest: Pregnancy, n	1
All Serious Adverse Events (SAEs), n	4
Presumed Infection – source/organism unidentified, n	1
Lower respiratory tract infection, n	1
Heart failure, n	1
Worsening of chilblains lupus, n	1
AE leading to discontinuation of study, n	2
AR, SAR and SUSAR, n	0
Deaths, n	0

AE: adverse event; AR: adverse reaction; SAE: serious adverse event; SAR; serious adverse reaction; SUSAR: suspect unexpected serious adverse reaction

Immunological parameters

No patient had new development of ANA or clinically significant worsening of autoantibodies titres (anti-dsDNA, anti-ENAs and ACA) from Baseline to Week 15.

One patient (4%) had Anti-B2 glycoprotein antibody positivity detected at Week 7; 21.00 U/mL from 14.70 U/mL at Baseline (normal <19.99 U/mL). There was no history of venous or arterial thrombosis observed. Her ANA remained negative. At Week 15, the Anti-B2 glycoprotein antibody reverted back to normal.

The one patient with low baseline complement levels had their levels normalised at Early Withdrawal visit (week 7). Two patients (8%) had changes in complement levels to <LLN at Week 7 but only one (4%) had persistently low levels at Week 15.

SLE disease activity

Of 6 patients with concurrent SLE, only 4 completed the study. Those who withdrew early did not have deterioration in either BILAG-2004 or SLEDAI-2K scores.

Of 4 patients who completed the study, only 1 patient had increased in SLEDAI-2K score from 8 to 10 points due to worsening of complement levels at week 7 and week 15. However, her BILAG-2004 activities improved at week 15. Details are as below:

- Her Baseline BILAG Activities were: (i) Grade B Mucocutaneous (Mild skin eruption – worse; mild and severe alopecia – worse), and (ii) Grade B Musculoskeletal (Mild and moderate arthritis – same). Her SLEDAI-2K score was 8 points (rash, alopecia and arthritis).

- At week 7, she had (i) Grade B Mucocutaneous (mild rash – same; mild and severe alopecia – same), and (ii) Grade B Musculoskeletal (mild and moderate arthritis – same). Her SLEDAI-2K score had increased at Week 7 to 10 points (rash, alopecia, arthritis and new low complement)

- At week 15, her BILAG activities improved; (i) Grade C Mucocutaneous (Mild skin eruption – improving, mild and severe alopecia – improving)

and (ii) Grade B Musculoskeletal (Mild and Moderate arthritis – same). Her SLEDAI-2K score remained at 10 points (arthritis, rash, alopecia, low complement).

Serum etanercept level

Trough serum etanercept levels were detected in 6/23 (26%) of the patients.

Exploratory analysis

Predictive values of the three ML-SADDLE response criteria were compared against responsiveness to therapy as measured by LDI. There were 12 responders as defined by ≥20% reduction in the relative difference in mean perfusion between active and non-active CLE lesions using LDI whilst 5 were non-responders. Of the three ML-SADDLE criteria, ML-SADDLE 20 was most strongly associated with improvement in LDI. Of those who were LDI responders, 11/13 (85%) had met ML-SADDLE 20 response versus 1/4 (25%) who had not; p=0.053. Moreover, ML-SADDLE 20 had higher sensitivity and similar specificity to ML-SADDLE 50 criteria in predicting responders as defined by LDI. The predictive values of the three ML-SADDLE response criteria are summarised in Table 7-7.

Table 7-7 Predictive values of ML-SADDLE criteria in terms of association with responsiveness to therapy

Response criteria	Sensitivity % (95% CI)	Specificity % (95% CI)	PPV % (95% CI)	NPV % (95% CI)
ML-SADDLE 20	91.7 (61.5 - 99.8)	60.0 (14.7 – 94.7)	84.6 (65.0 – 94.2)	75.0 (28.7 – 95.7)
ML-SADDLE 50	83.3 (51.6 – 97.9)	60.0 (14.7 – 94.7)	83.3 (62.4 – 93.8)	60.0 (26.0 – 86.5)
ML-SADDLE 70	33.3 (9.9 – 65.1)	80.0 (28.4 – 99.5)	80.0 (36.8 – 96.5)	33.3 (21.6 – 47.5)

ML-SADDLE: Modified Limited Score of Activity and Damage in Discoid Lupus Erythematosus

7.4. Discussion

This report presented the results from a phase II open label trial, which was the first to evaluate the efficacy and safety of an existing drug, etanercept, licensed for other indications but using a novel route of administration using intra-dermal injection for remission induction in DLE. The primary endpoint as assessed using ML-SADDLE 20 response rate was achieved and the therapy was well tolerated.

In this study, just over half of the patients responded to intra-dermal injection of etanercept. This response rate was particularly notable because of the inclusion of cohort, which comprised patients who were refractory to various systemic therapies as well as median disease duration of about a decade. A Cochrane review in 2017 only identified a small number of formal studies that had been undertaken in this field including topical therapies (n=4) and one study compared hydroxychloroquine and acitretin (198). The response rates reported by the authors based on variable outcome measures with these therapies ranged from 10% to 68% (427-431). However, none of these trials were of high quality when they were assessed using the Grading of Recommendation, Assessment, Development and Evaluation (GRADE) (432). In contrast, TARGET-DLE was a well-designed trial and this treatment exhibited efficacy across multiple endpoints including patient-reported outcomes and objective outcome measures.

This study offers insights into the pathogenesis of DLE and help direct future therapies. Deposition of immune complexes containing IgM, IgG and complement C3 at the dermo-epidermal junction is pathognomonic in DLE. However, this direct immunofluorescence test can also be detected in non-lesional biopsies (70). This observation suggests that although autoantibodies are involved in the formation of skin lesions, additional mediators are needed for DLE lesions to develop. This may also explain the failure of treatment with B-cell depleting agent in this particular subtype of CLE (17). TNF is a major pro-inflammatory cytokine that is overexpressed in the kidney and skin lesions from patients with SLE (433). Research in animals studies showed that intradermal injection of lupus serum into the skin of TNF-deficient mice failed to induce an inflammatory response (434), suggesting the importance of this

cytokine in the development of skin lesions. Thus translating findings from bench to bedside, this study shows that TNF-blockade is effective in inducing remission of active DLE. Analysis of skin biopsy samples for TNF and expression of other cytokines are in progress and can help stratify those who will respond to this therapy.

There were no major safety signals from administration of TNF-blockade therapy using an intra-dermal injection in this study. Although the number of AEs reported were high, only 29 were treatment-emergent. This could be attributed to frequency of visits and over 3/4 of the patients were either current or previous smokers. None of the patients had progression or worsening of lupus from immunological and disease activity perspectives. In addition, the frequency of injection-site reaction was very low. Compliance to treatment was also satisfactory with patients receiving the treatment on average 80% of the time. However, just over a quarter of the patients withdrew early in this study with 4/25 (16%) of them discontinued due to reasons other than adverse events or pregnancy. Thus, a more refined drug delivery of TNF-blockade either using topical or microneedles (435) would help resolve these issues. With regards to cost, treatment with intra-dermal injection is cost-saving. A 12-week course of treatment up to 10mg of etanercept weekly costs 5 times cheaper than systemic etanercept administration as well as without inducing systemic autoantibody production.

This study has some limitations. First, this was an open label trial. Hence, our results could be influenced by reporting bias from both the participants and investigators. However, evidences of efficacy were also supported by objective measures including LDI and thermography. Second, the lack of a control group made the results difficult to interpret. This was overcome by applying the Simon's 2-stage minimax design, which estimated the number of patients needed to treat based on an observed response rate over other experimental therapies i.e. after failure of an anti-malarial. Next, 19/25 (76%) were on concomitant csDMARDs or anti-malarials, thus efficacy could not be contributed to etanercept alone. Lastly, this study was designed for remission induction using a short course regimen. Although the ML-SADDLE response rate was maintained at week 15, longitudinal follow-up is needed.

7.5. Conclusion

To conclude, a low dose intradermal injection of etanercept up to 10mg substantially reduced clinical activity, met its primary and most secondary endpoints including patient-reported outcomes and objective measures. This therapy was tolerable in DLE patients who were refractory to anti-malarials and other systemic therapies. The results support further development of therapy in multi-centre trials. Analyses of other imaging and histological biomarkers are ongoing and can help stratifying patients for response.

7.6. Key messages

i. Administration of etanercept (potentially harmful in SLE) using a novel route, intra-dermal injection is effective for remission induction in refractory DLE.

ii. No major safety signals were observed including induction of systemic autoantibody production.

iii. The results from this trial will be used to power a phase III trial.

iv. This trial also confirms the role of TNF in the pathogenesis of DLE.

Chapter 8. Discussion

8.1. General discussion of results

The data presented in this thesis highlighted challenges in the management and the need to tailor therapies to the individual patient with SLE. Given the heterogeneity in clinical phenotype and response to therapy, it is likely that there is no one-size-fits-all therapy for the treatment of SLE. This programme of research was designed to formulate a number of ways where personalised therapy based on immunopathogenesis could be offered in order to improve the outcomes of At-Risk and established SLE patients. These were focussed on two key clinical areas: (i) the pre-clinical stage and (ii) refining the use of rituximab in patients with moderate to severe SLE. By identifying predictors of progression to clinical autoimmunity, response and non-response to B-cell depletion therapy and utilising new therapies to overcome deficiency of rituximab, the unifying hypothesis of this thesis had been addressed.

Disease prevention should be a focus of research and management of ARDs. However, this cannot not be undertaken if immunopathogenesis of the disease is poorly understood and prognostic biomarkers are not identified. IFN-Is are important mediators of autoimmunity but their role in AI-CTD initiation is unclear. This could be contributed to variability in the methods for quantifying IFN activities. A previous study retrospectively analysed IFN activity in sera from 55 At-Risk individuals for at least 5 years prior to SLE classification. They reported that enhancement of the IFN-II pathway led to accumulation of autoantibodies and subsequent elevations in IFN-I activity preceding the classification of SLE (340). However, these data need to be interpreted with caution since measurement of IFNs activity in serum are unreliable as proteins are unstable in serum. Although they showed that both types of IFNs were important in the pathogenesis of SLE, their use as prognostic biomarkers were lacking. In order to overcome the deficiency in quantifying IFN activity, our group, has published on two continuous ISG expression scores (IFN-Score-A and IFN-Score-B), that in combination better identified clinically meaningful differences in IFN status between, and within

ARDs (320). By undertaking the largest prospective study of At-Risk individuals to date with longitudinal follow-up until progression to AI-CTD (meeting classification criteria), I showed that IFN-Score-B and a family history of ARDs were independently predictive (in a multivariable analysis) of progression to AI-CTD at 12 months. These results confirmed the importance of IFNs in disease initiation; a cut-off level of IFN-Score-B with a moderate diagnostic accuracy was also defined (336). This important finding will contribute to the design of future prevention studies. Once these biomarkers are validated, the predictive value of IFN scores may allow us to identify patients with imminent AI-CTD for earlier intervention, which is aimed at preventing disease and avoiding irreversible organ damage.

My programme of research then looked into ways to improve or modify the use of rituximab in SLE by undertaking a prospective study of the largest cohort study reported to date. A high degree of response to the first cycle of rituximab (MCR + PCR = 82%; MCR = 50%) at 6 months was observed in our cohort (246). This response rate was similar to another large study from London (MCR + PCR = 67%) (341). However, the response rate in this cohort was higher compared to the BILAG-BR registry (MCR + PCR = 49%) (232). The discrepancy between this cohort and the BILAG registry could be attributed to inclusion of more non-Caucasian patients (with more severe end-organ involvement and poorer prognosis); 40% in the latter versus 32% in the former and more ANA negative patients; 13% in the latter versus 0% in the former. Additionally, the efficacy could be under-reported in the latter since only 71% of the patients (compared to 94% in the former) had complete BILAG response data at 6 months. There was also limited data on predictors of response to rituximab. I showed in a multivariable analysis that B-cell depletion post-rituximab (as measured using HSFC) was the only consistent predictor of any BILAG response and MCR to rituximab (246). This biomarker was validated in a second cohort in this thesis. Therefore, treatment with anti-CD20 mAb should aim of achieving complete B-cell depletion and this finding also provided a rationale for B-cell monitoring before and after therapy with rituximab. With regards to factors predicting B-cell depletion with rituximab, lower plasmablasts and normal complement levels were independently predictive. Therefore, for those with higher plasmablasts pre-rituximab,

treatment modification could be employed to improve depletion either by increasing the dose or adding an extra infusion, as our group previously showed in RA (236). For those with low complement levels pre-rituximab, experience in CLL showed that combining rituximab with complement correction therapy i.e. fresh frozen plasma could enhance depletion and response (436, 437). However, only one case report had reported on efficacy of similar strategy in SLE (438). Thus, more evidence are needed before we extrapolate findings between SLE and B-cell malignancies.

Most SLE patients experienced a relapse following the first cycle of rituximab. I had validated in a second cohort in this thesis that repopulation of plasmablasts of $>0.0008 \times 10^9$/L (measured at 6 months post-rituximab) increased the risk of clinical relapse within the next 6 months (246). Therefore, these patients could be considered for early retreatment in order to reduce the higher burden of B-cell numbers and enhanced depletion in the subsequent cycle. Importantly, for those with plasmablasts of $\leq 0.0008 \times 10^9$/L at 6 months, monitoring for clinical relapse would appear an acceptable strategy.

What about the efficacy of repeat cycles with rituximab? Repeat cycles with rituximab was effective with 85% of the patients continued to respond. However, we observed 12% cases of 2NDNR. This phenomenon was associated with anti-rituximab antibodies and could be predicted by higher pre-rituximab plasmablasts and non-use of concomitant therapy with DMARDs (excluding anti-malarials only). This 2NDNR was overcome by switching therapy from rituximab to humanised anti-CD20 agents which improved depletion and response subsequently.

Since the importance of achieving complete B-cell depletion and keeping lower B-cell numbers during rituximab therapy had been established in this thesis, a concern was that this strategy could lead to increased risk of an infection. By undertaking a large retrospective real-world cohort of SLE and other ARD patients who were treated with rituximab, I showed that serious infections within the 12 months of first cycle rituximab could be predicted by a previous history of cancer, chronic lung disease, previous severe infection and low IgG. The association of low IgG with serious infection was similar to the one reported by the French registry (262). However, the strengths of my study

compared to this registry were longer duration of follow-up, methods of dealing with missing data were better defined and majority of data for immunoglobulin levels were available (95% versus 49% in registry). Thus, patients with these characteristics should be counselled regarding the risk of serious infection prior to commencing rituximab. With regards to repeat cycles with rituximab, this was the first study to identify predictors of serious infections for up to the first five cycles using a complex mixed effect logistic regression analysis. Risk factors that might inform cessation of therapy or modify using half-dose regimen from safety perspective were baseline comorbidities including previous cancer, chronic lung disease and previous history of severe infection as well as pre-rituximab variables in any cycle such as higher corticosteroid dose, longer time-to-rituximab retreatment and larger percentage of change in IgM. Thus, my results provided a rationale for immunoglobulin monitoring before first cycle rituximab, at least 4-6 monthly post-treatment and before any retreatment particularly in those who demonstrated low baseline Ig levels. Importantly, achieving B-cell depletion and maintaining low B-cell subsets numbers during rituximab treatment appeared to be relatively safe from the perspective of infection.

It is important to note that not all SLE manifestations respond to rituximab. DLE appeared to be B-cell independent from our experience in treating this condition with B-cell depleting agent (17). Thus, targeting other molecules should be more appropriate than using rituximab. TNF is implicated in the pathogenesis of DLE (400-402). Although some case series had shown that the use of short-term systemic TNF-blockade therapies in SLE were effective and without major safety signals (281, 285, 439), two large RCTs that evaluated the efficacy and safety of infliximab and etanercept in SLE were terminated prematurely (281) due to poor recruitment because of a potential concern in the induction of pathogenic autoantibodies that might render SLE worse. Hence, a stronger level of evidence is needed for use of TNF-blockers in lupus. In the TARGET-DLE trial, I showed that a low dose intradermal injection of etanercept (up to 10mg) substantially reduced clinical activity, met its primary (ML-SADDLE 20 response rate of 52%) and most secondary endpoints including the patient-reported outcomes. Neither major safety signals nor evidence of induction of systemic autoimmunity were observed

with this therapy. Thus, these results supported further development of this therapy in multi-centre trials. Although this study was an open label trial, reporting bias was overcome by evidence for efficacy using objective outcome measures including LDI and thermography.

8.2. Impact of research

This programme of research is of importance both nationally and globally in improving the treatment of SLE. By optimising the way the current therapy with rituximab is used and providing the proof-of-concept for a novel therapy, unnecessary toxicity, cost and patient inconvenience could be avoided.

To undertake this research, I successfully secured two prestigious personal funding awards worth £0.5 million from the National Institute for Health Research (NIHR) and £15,000 worth of drugs supply from an industry partner, Pfizer.

Since the publication of my results, monitoring for B-cell biomarkers have been adopted routinely in Leeds to make treatment decisions regarding rituximab treatment that are cost-effective and safe. I am currently leading a project to establish a treatment algorithm on the use of rituximab based on the biomarkers and results presented in this thesis, to be used in the West Yorkshire region. Moreover, the outcomes of my long-term data particularly those with 2NDNR have provided a strategy for the most resistant patients and led to a proposal to NHS England on behalf of the UK lupus community. Furthermore, we have a formal collaboration with Roche to use B-cell biomarkers as one of the trial endpoints of a global phase II RCT in lupus nephritis, which is currently recruiting patients. Roche is also about to grant us the use of obinutuzumab in a small number of patients with 2NDNR to rituximab as a compassionate use.

As the outcome the clinical trial of intra-dermal injection of etanercept for remission induction in DLE is positive, these results will be used to power a large multicentre phase III trial which I plan to lead during my next stage of career as a post-doctoral researcher. If effective, this would be the only targeted therapy shown to work for DLE.

With regards to collaboration arising from my PhD training, I have worked with Prof Ian Bruce at the Lupus Unit, Manchester during my visiting placement. I am currently analysing the dataset pertaining to variability in mucocutaneous response to rituximab and belimumab using the BILAG-BR registry. This may confirm our previous finding that response to B-cell therapy depends on skin subtypes and will help in stratification of biological therapies. I am also appointed as the BILAG Renal Sub-Committee Working Group member for which I am leading a project to revise the glossary and scoring for the renal domain.

In terms of dissemination, I am the first author in three original research articles which have been published in a 3-star specialty-leading journal; Annals of the Rheumatic Diseases (Impact factor:12.8), which makes me Research Exercise Framework (REF)-returnable to the university for the 2021 assessment. I have delivered 7 oral and 6 poster presentations at international conferences, an oral presentation as an invited speaker at the British Society of Rheumatology Conference in 2017, awarded the Runner-up Prize in the University of Leeds Post-Graduate Researcher of the Year 2017 and won two poster prizes. My findings have also been disseminated widely using various medium of communications including twitter and research commentary published in press and newsletters. Lastly, I have organised a Lupus Awareness Event in 2016 where the coverage of our research was broadcast by BBC Look North.

8.3. Future perspectives

The available data clearly demonstrate that rituximab-based B-cell depletion therapy improves outcomes for the majority of patients with SLE. Moreover, the licensing of rituximab biosimilars also has increased its cost-effectiveness profile. However, some patients respond poorly, likely due to various rituximab-resistance mechanisms or heterogeneity in disease subtypes. Although several humanised anti-CD20 mAbs have now entered clinical trials for use in RA and SLE, it is still too early to predict whether they will be more effective as currently there is no data comparing these mAbs in head-to-head trials with rituximab. Additionally, whether these agents will also be effective

in patients with incomplete B-cell depletion with rituximab is also unknown. Presumably, this may depend upon the reason for resistance in the first place. If development of anti-rituximab antibody is responsible, then alternative humanised mAbs should be effective as demonstrated in this thesis. Alternatively, if a patient-intrinsic mechanistic defect is responsible, then unless this is overcome by the new therapies, then no improvement in response would be expected. Therefore, my next research agenda is to assess this mechanistic defect by undertaking laboratory works to assess the effect of FCGR polymorphism, expression of fc gamma receptor IIIa on natural killer (NK) cells, degranulation activity of NK cells in the presence of rituximab-coated patient's B-cells, complement function and BAFF/APRIL on the level of B-cell depletion to allow for treatment stratification as well as appropriate modification to therapy to be employed to improve depletion.

A significant minority of patients with SLE responded less well to B-cell depletion therapy despite complete B-cell depletion. This might be attributed to higher BAFF levels (248) and/or IFN-Is (440), which might promote B-cell differentiation and/or plasma cell activity. Therefore, sequential anti-BAFF or anti-IFN agents after B-cell depletion therapy may target these potential alternative resistance mechanisms. Alternatively, some patients with SLE who had persistently high anti-dsDNA antibodies despite B-cell depletion therapy might respond poorly owing to high plasma cell activity (441). Thus, targeting plasma cells using proteasome inhibitors may benefit this subgroup of patients although vigilance is needed in terms of safety from prolonged B-cell depletion (442).

Thus, optimising B-cell depletion therapy with agents that potentially overcome disease-related immune defects and mechanism of resistance that operate beyond B-cell depletion would be important to explore in clinical trials, if we are to improve overall clinical response to B-cell targeted therapies in SLE.

With regards to optimising the treatment for B-cell independent manifestation such as DLE, although compliance to intra-dermal injection of etanercept was satisfactory in the TARGET-DLE trial, a more refined drug delivery of TNF-blockade would help in improving patient's compliance and convenience. My

next research agenda is to evaluate whether a TNF-blocker can be effectively administered through microneedles (435) or use an alternative molecule containing a TNF receptor fragment that is currently under investigation for ocular indication. Should the outcome of this be positive, then I will embark on a large multi-centre RCT of intra-lesional TNF-blocker for remission induction in DLE.

Lastly, in terms of disease prevention, validation study of the prognostic ability of the IFN scores that we discovered is currently in progress. Once this is validated, a prevention study will be undertaken where patients with imminent AI-CTD are assigned earlier intervention using therapies that block IFNs or conventional immunosuppressants to avoid irreversible organ damage and glucocorticoid exposure.

8.4. Conclusions

IFN status that preceded clinically overt disease in AI-CTD was described through a comprehensive evaluation using clinical data, skin biopsy, blood interferons and imaging in a unique cohort of At-Risk (ANA-positive with limited symptoms) individuals. Using a novel two-score system for interferon status that we recently reported, higher IFN-Score-B and a family history of ARD were predictive of progression to AI-CTD (mostly SLE) within the following 12 months.

B-cell depletion (as measured using HSFC post-rituximab) was associated with better clinical response in SLE. Additionally, another B-cell biomarker i.e. repopulation of plasmablasts at 6 months post-rituximab was associated with imminent relapse over the next 6 months. Thus, from a treatment stratification perspective, B-cells should be monitored in clinical practice.

Despite a high degree of efficacy of rituximab demonstrated in the initial and subsequent repeat cycles, some patients lost response in subsequent cycles due to anti-rituximab antibodies. This was overcome by switching therapy to humanised anti-CD20 agents.

Finally, a novel therapy using intra-dermal injection of etanercept met its primary endpoint and most secondary endpoints for remission induction of a

B-cell independent manifestation, DLE, when investigated in a phase II open label trial. There was no major safety signal or induction of systemic autoimmunity observed during the study.

Chapter 9. References

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Chapter 10. Appendices

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On 26 October 2018 at 08:39, Yuzaiful Md Yusof <Y.Yusof@leeds.ac.uk> wrote:

To Whom It May Concern,

I am a Final Year PhD student at the University of Leeds. I am also the first author of two of the manuscript that have been published on Annals of the Rheumatic Diseases. Both these articles were published as open access in accordance with the

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2. Md Yusof MY, Shaw D, El-Sherbiny YM, Dunn E, Rawstron AC, Emery P, Vital EM. Predicting and managing primary and secondary non-response to rituximab using B-cell biomarkers in systemic lupus erythematosus. Ann Rheum Dis. 2017 Nov;76(11):1829-1836. doi: 10.1136/annrheumdis-2017-211191. PMID: 28684557 - 2 Figures and 2 Tables

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Thank you for your cooperation and assistance.

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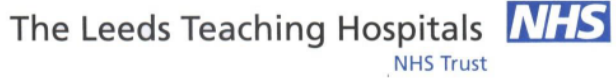
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10.2. Consent Form TARGET-DLE

The Leeds Teaching Hospitals **NHS**
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PATIENT CONSENT FORM

A single arm, phase II open label trial to investigate the efficacy and safety of TARGeted therapy using intradermal injection of ETanercept for remission induction in Discoid Lupus Erythematosus (TARGET-DLE)

Patient ID: ....   Initials: ....   Date of Birth: ....

Please initial in the box

1. I confirm that I have read and understand the information sheet dated 08 July 2015 (version _____) for the above study and have had the opportunity to ask questions.

2. I understand that my participation is voluntary and that I am free to withdraw at any time without my medical care or legal rights being affected.

3. I understand that my medical records may be looked at by authorised individuals from the University of Leeds, the UK Regulatory Authority or the Independent Ethics Committee in order to check that the study is being carried out correctly. I give permission, provided that strict confidentiality is maintained, for these bodies to have access to my medical records for the above study and any further research that may be conducted in relation to it.

4. I give permission to be tested for chronic infections such as tuberculosis, HIV and hepatitis to determine my eligibility for the study. I understand that if any of the tests is positive, the results will be explained to me by the doctor and I may be referred to the appropriate specialist for subsequent management.

5. I agree for photographs of my discoid rash to be taken and that my identity will be kept anonymous should these photographs are included in the study reports and publication.

6. I understand that even if I withdraw from the above study, the data, the photographs of my discoid rash, blood and skin samples collected from me will be used in analysing the results of the trial. In some cases, further information about any unwanted effects of my treatment may need to be collected by the study team, unless I specifically withdraw consent for this. I understand that my identity will remain anonymous.

7. I agree to allow any information or results arising from this study to be used for healthcare and/or further medical research upon the understanding that my identity will remain anonymous wherever possible.

8. I understand that the research nurse/study doctor will keep secure records at the hospital which will allow me to be followed up in hospital and at home (including name, date of birth, NHS number, hospital number, address and telephone number).

9. I agree for my blood and tissue samples to be stored and used for additional research investigations that form part of this study. I understand that strict confidentiality will be maintained at all times and that my name and individual details will not be stored with my samples (i.e. they will be anonymised). However, a unique reference number will be allocated to the samples which may allow them to be linked back to me in future for research purposes.

The following test is OPTIONAL. Even if you agree to take part in this study, you do not have to agree to this section.

Please initial

10. I agree to undergo the following procedure as a part of the study to develop new tests to measure inflammation to the skin:

    Yes   No

  (i) Punch skin biopsy

---

10. I understand that my blood and tissue samples are a 'gift' that may be used in future research that receives ethical approval. I understand that my samples and data collected from it may be shared on a collaborative basis with researchers in the UK and potentially, centres abroad, including outside the European Economic Area.

11. I consent to the storage including electronic, of personal information for the purposes of this study. I understand that any information that could identify me will be kept strictly confidential and that no personal information will be included in the study report or other publication.

12. I agree that my GP, or any other doctor treating me, will be notified of my participation in this study.

13. I agree to take part in the study.

_____	_____	_____
Name of the patient	Patient's signature	Date
_____	_____	_____
Name of the Investigator (taking the written consent)	Investigator's signature	Date

(Original to be retained and filed in the site file. 1 copy to patient and 1 copy to be filed in patient's notes)