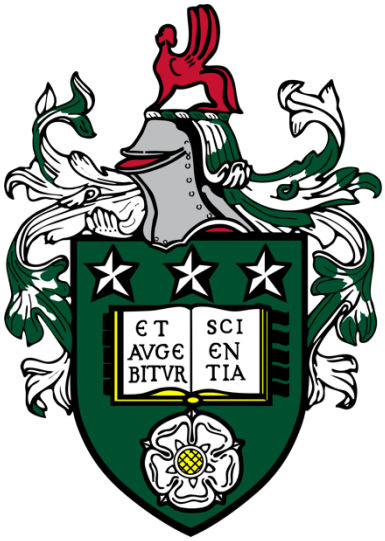
Regulation of type I interferons in health and autoimmune disease

Antonios Psarras



Submitted in accordance with the requirements for the degree of Doctor of Philosophy (PhD)

University of Leeds

Leeds Institute of Rheumatic and Musculoskeletal Medicine

September 2018

Intellectual property and publication statements

The candidate confirms that the work submitted is his own, except where work which has formed part of jointly-authored publications has been included. The contribution of the candidate and the other authors to this work has been explicitly indicated below. The candidate confirms that appropriate credit has been given within the thesis where reference has been made to the work of others.

Chapter 1 includes data from a jointly-authored publication:

Psarras A, Emery P, Vital EM. Type I interferon-mediated autoimmune diseases: pathogenesis, diagnosis and targeted therapy. Rheumatology (Oxford). 2017;56(10):1662-75.

Psarras A performed the review of literature, critically appraised scientific evidences of the relevant topics and led the writing of the manuscripts. Emery revised the manuscripts for important intellectual content and final approval of the manuscript.

Chapter 3 includes data from two jointly-authored publications:

El-Sherbiny YM*, **Psarras A***, Yusof MYM, Hensor EMA, Tooze R, Doody G, et al. A novel two-score system for interferon status segregates autoimmune diseases and correlates with clinical features. Sci Rep. 2018;8(1):5793.

*joint first author

El-Sherbiny YM, Emery P, and Vital EM performed conception and design of research, data interpretation and writing the manuscript; El-Sherbiny YM, Md Yusof MY, Wittmann M, and Vital EM designed the research and recruited patients and clinical

interpretation of the study; Tooze R and Doody G performed B cell design, analysis and interpretation, and writing the manuscript; El-Sherbiny YM, Md Yusof MY, Mohamed AAA, and Psarras A performed flow cytometry analysis and interpretation; El-Sherbiny YM and Psarras A performed gene expression analysis; Hensor EMA performed statistical and factor analysis method development and nanoparticle characterization; Psarras A, McGonagle D, and Vital EM performed data interpretation and writing the manuscript.

Md Yusof MY*, **Psarras A***, El-Sherbiny YM, Hensor EMA, Dutton K, Ul-Hassan S, 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;77(10):1432-9.

*joint first author

Md Yusof MY, Psarras A and Vital EM: substantial contributions to the conception or design of the work, or the acquisition, analysis or interpretation of data, drafting the work or revising it critically for important intellectual content, final approval of the version published and agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. El-Sherbiny YM, Hensor EMA, Dutton K, Ul-Hassan S, Shalbaf M, Alase AA, Wittmann M and Emery P: substantial contributions to the conception or design of the work, or the acquisition, analysis or interpretation of data, drafting the work or revising it critically for important intellectual content and final approval of the version published.

Acknowledgements

I would like to thank Dr Edward Vital, Prof Paul Emery and Dr Miriam Wittmann, my supervisors at Leeds Institute of Rheumatic and Musculoskeletal Medicine, for their guidance and endless support for my PhD thesis and my personal career development. I would also like to express my gratitude to Prof George Tsokos at Beth Israel Deaconess Medical Center, who kindly supervised the work I carried out in his lab at Harvard Medical School in 2017 for my PhD thesis.

I am especially indebted to the consultants Prof Paul Emery, Dr Edward Vital, Prof Maya Buch, Dr Shouvik Dass, Prof Mark Goodfield, Dr Emma Dunn, Dr John Bamford, specialist registrars Dr Md Yuzaiful Md Yusof, Dr Andrew Barr, Dr Lesley-Anne Bissell, clinical trial coordinator Huma Cassamoali and clinical trial assistant Sabina Khan as well as nurses and healthcare assistants for their services at the Leeds Connective Tissue Disease and Vasculitis Clinic. Registering patients to research cohorts and collecting biological samples on weekly basis would have not been made possible without their endless assistance and valuable contribution. I would also like to thank Diane Corscadden and Katie Mbara at Chapel Allerton Hospital for collecting and sending any patient samples to St. James's University Hospital.

I am grateful to Dr Elizabeth Hensor, who helped with statistical analysis performing the factor analysis, Dr Mohammad Shalbaf, who performed the tissue sectioning of skin biopsies and Dr Adewonuola Alase, who helped with *in situ* hybridization of the skin and *in vitro* culturing of human skin cells. I would also like to mention Dr Yasser El-Sherbiny, post-doctoral fellow, and Zoe Wigston, research technician, for their valuable help in the lab. Special thanks to Adam Davison and Liz Straszynski from

Flow Cytometry and Imaging Facility in Wellcome Trust Brenner Building for providing induction courses in flow cytometry and confocal microscopy as well as facility's precious service in cell sorting. I am also indebted to Next Generation Sequencing Facility for performing and analysing the RNA-sequencing data from the samples given, especially Dr Ian Carr, lecturer in medical bioinformatics, Dr Agne Antanaviciute, bioinformatician, and Ummey Hany, technical specialist, for their major contribution to the project.

In addition to all contributors above, I would like to express my special thanks and respect to all researchers at University of Leeds and Harvard Medical School I have shared my data and scientific thoughts with, but more importantly, to all the patients that without their actual contribution I would not have been able to perform my experiments and investigate basic mechanisms of human autoimmunity.

I would like to express my gratitude to the University of Leeds for enabling me to undertake the current PhD by supporting me with University of Leeds 110 Anniversary Research Scholarship in the research theme "Immunology, Inflammation & Infection". Moreover, I would like to special mention the Erdheim Travel Scholarship awarded by the School of Medicine at Leeds and the Summer Placement Award Scheme awarded by the British Society for Immunology, which provided financial support during my research visit at Harvard Medical School in Boston, USA.

Last but not least, I would like to thank my beloved family and friends for their endless support, love and patience throughout all these years of hard work and scientific challenges that enabled me to submit this PhD thesis.

List of publications and presentations arising from this thesis

Original articles:

El-Sherbiny YM*, **Psarras A***, et al. A novel two-score system for interferon status segregates autoimmune diseases and correlates with clinical features. Sci Rep. 2018;8(1):5793.

*joint first author

Md Yusof MY*, **Psarras A***, 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;77(10):1432-9.

*joint first author

Review articles:

Psarras A, et al. Type I interferon-mediated autoimmune diseases: pathogenesis, diagnosis and targeted therapy. Rheumatology (Oxford). 2017;56(10):1662-75.

Oral presentations:

Psarras A, et al. The role of skin tissue in initiation of Systemic Lupus Erythematosus. Annual Northern and Yorkshire Rheumatology Meeting, York (UK), September 2018.

Psarras A, et al. Type I interferon is produced by non-haematopoietic tissue cells but not pDCs in preclinical autoimmunity and SLE. Annual European League Against Rheumatism (EULAR) Congress, Madrid (Spain), June 2018.

Psarras A, et al. Prediction of connective tissue disease in at-risk cohort using a novel interferon-stimulated gene expression score. Annual Congress of Japanese College of Rheumatology (JCR), Tokyo (Japan), April 2018.

Psarras A, et al. Type I interferon regulation in preclinical and established autoimmunity. Annual Northern and Yorkshire Rheumatology Meeting, York (UK), September 2017.

Psarras A, et al. Towards prevention of Systemic Lupus Erythematosus: predicting disease and identification of therapeutic targets. 7th NIHR Infrastructure Doctoral Research Training Camp, Ashridge Business School (UK), July 2016.

Psarras A, et al. Distinct subsets of interferon-stimulated genes are associated with incomplete and established systemic lupus erythematosus. 36th European Workshop for Rheumatology Research, York (UK), February 2016.

Poster presentations:

Psarras A, et al. TNF-α regulates plasmacytoid dendritic cells by suppressing IFN-α production and enhancing Th1 and Th17 cell differentiation. Annual European League Against Rheumatism (EULAR) Congress, Madrid (Spain), June 2018.

Psarras A, et al. TNF-α is a major regulator of human plasmacytoid dendritic cells by promoting a functional drift to antigen presentation. Annual Congress of British Society for Immunology, Brighton (UK), December 2017.

Abstract

Type I interferons (IFN) have a crucial role in the pathogenesis of a range of autoimmune diseases including systemic lupus erythematosus (SLE). Increased IFN activity is observed at preclinical stages and associated with disease progression, but the cause of this dysregulation remains unclear. Plasmacytoid dendritic cells (pDCs) produce large amounts of IFNs in viral infection, however their precise role in autoimmunity is still elusive.

Peripheral blood and skin biopsies from different patient groups were used for gene expression assays, immunophenotyping, *in vitro* functional assays, transcriptomics and other assays to investigate the dysregulated IFN axis and the role of pDCs in preclinical autoimmunity and SLE.

In preclinical autoimmunity and SLE, pDCs were found to exhibit an exhausted phenotype with: (i) loss of TLR-mediated IFN-α production; (ii) failure to induce T cell activation; (iii) transcriptional profile of cellular senescence; (iv) increased telomere erosion. In contrast, diffuse expression of type I IFNs was observed in the epidermis but not in leucocyte-infiltrating areas of patients with SLE as well as in non-lesional skin of individuals with preclinical autoimmunity. Additionally, keratinocytes isolated from non-lesional skin of patients with SLE and individuals with preclinical autoimmunity showed a significantly enhanced type I IFN expression in response to UV light and nucleic acids. Lastly, TNF-α regulates the function of pDCs by suppressing IFN-α production but enhancing a functional drift to antigen presentation and T cell activation.

These findings revise our understanding of immune regulation in human autoimmunity. Non-haematopoietic tissue cells can perpetuate IFN responses; meanwhile the professional IFN-producing pDCs have lost their immunogenic properties. In patients with SLE, these insights may indicate potential therapeutic targets outside the conventional immune system, while knowledge of how IFN dysregulation initiates could allow disease prevention.

List of abbreviations

ACR	American College of Rheumatology
AI-CTD	Autoimmune connective tissue disease
ANA	Anti-nuclear antibody
ANCA	Anti-neutrophil cytoplasmic antibody
ANOVA	Analysis of variance
APRIL	A PRoliferation-Inducing Ligand
AZA	Azathioprine
BAFF	B-cell Activating Factor of the tumour ncrosis factor of the ligand Family
BCR	B-cell receptor
bDMARDs	Biological disease modifying anti-rheumatic drugs
BILAG	British Isles Lupus Asessment Group
BlyS	B-lymphocyte stimulator
Bregs	Regulatory B-cells
BSA	Bovine serum albumin
CCLE	Chronic cutaneous lupus erythematosus
CI	Confidence interval
CNS	Central nervous system
CRP	C-reactive protein
csDMARDs	Conventional synthetic disease modifying anti-rheumatic drugs
CTLA4	Cytotoxic T-lymphocyte antigen 4
CyC	Cyclophosphamide
DAPI	4',6-diamidino-2-phenylindole
DC	Dendritic cell
DLE	Discoid lupus erythematosus
DMEM	Dulbecco's Modified Eagle Medium
dsDNA	Double stranded deoxyribonucleic acid
EBV	Epstein-Barr virus
eGFR	Estimated glomerular filtration rate
ELISA	Enzyme-linked immunosorbent assay
ENA	Extract nuclear antigen
ESR	Erythrocyte sedimentation rate
EULAR	European League Against Rheumatism
FA	Factor Analysis
FACS	Fluorescence-activated cell sorting
FcγR	Fc gamma receptor

FD	Fold difference
FDA	Food and Drug Administration
GCSF	Granulocyte-colony stimulating factor
GM-CSF	Granulocyte macrophage colony stimulating factor
GWAS	Genome-wide association studies
HC	Healthy control
HCQ	Hydroxychloroquine
HERV	Human endogenous retrovirus
HIV	Human immunodeficiency virus
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
Ig	Immunoglobulin
IL	Interleukin
ILE	Incomplete Lupus Erythematosus
IRF	Interferon regulating factor
ISG	Interferon-stimulated gene
JAK	Janus kinase
LFA	Lymphocyte function-associated antigen
LN	Lupus nephritis
mAb	Monoclonal antibody
MHC	Major histocompability
miRNA	Micro ribonucleic acid
MMF	Mycophenolate mofetil
MTX	Methotrexate
NF-κB	Nuclear factor kappa B
NHS	National Health Service
NICE	National Institute for healthcare and Clinical Excellence
OR	Odds ratio
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
pDC	Plasmacytoid dendritic cell
PPIA	Peptidylprolyl isomerase A

pSS	Primary Sjogren's Syndrome
qPCR	Quantative Polymerase Chain Reaction
RA	Rheumatoid arthritis
REC	Research Ethics Committee
RF	Rheumatoid factor
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute Medium
RT-PCR	Reverse transcription Polymerase Chain Reaction
RTX	Rituximab
SCLE	Subacute cutaneous lupus erythematosus
SD	Standard deviation
SLE	Systemic Lupus Erythematosus
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
TACI	Tumour necrosis factor receptor superfamily member 13b
Tfh	T follicular helper cell
TGF-β	Tissue growth factor beta
TLR	Toll-like receptor
TNF	Tumour necrosis factor
T_{reg}	T regulatory cell
UK	United Kingdom
US	United States
UV	Ultraviolet

Table of contents

List of tables

List of figures

CHAPTER 1.

REVIEW OF THE LITERATURE

1.1 Systemic Lupus Erythematosus (SLE)

Systemic Lupus Erythematosus (SLE) is a multisystemic autoimmune disorder characterised by a general breakdown of immune tolerance. The pathogenesis of SLE is quite complex and the immune mechanisms contributing to disease development still remain unclear. SLE is a heterogeneous disease and it often comes along with a wide spectrum of clinical manifestations affecting virtually any organ or tissue and typically running a relapsing and remitting course. The severity of symptoms can range from mild to severe and may vary significantly between patients.

1.2 Epidemiology of SLE

SLE can affect all individuals of every gender, race or age. However, it predominantly affects women between 15 and 45 years old, approximately 9 times more frequently than men (1). While the prevalence of SLE is notably higher among women of childbearing age, men tend to develop more severe disease with multiple organ complications often requiring more intensive treatment. On the other hand, postmenopausal women generally present milder disease in comparison with younger premenopausal women. The majority of patients (65%) have a disease onset between 15 and 55 years old, while only 20% of patients present clinical manifestations before the age 16. Interestingly, children and adolescents with SLE present more severe disease and higher morbidity, especially in lupus nephritis (2).

The incidence of SLE has nearly tripled in the last 40 years mainly due to improved recognition of symptoms and diagnosis of mild disease (3). Incidence rates of SLE in North America, South America and Europe are estimated between 1 to 23 per 100 000 per year (4). The prevalence of SLE in the USA population is estimated to be 51 cases per 100,000 people, while certain ethnic groups like women of Afro-Caribbean origin tend to present lupus more frequently (up to 9 times) and with greater severity than populations of Caucasian origin (5). 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 (6).

1.3 Classification criteria for SLE

Classification criteria for SLE were initially developed by the American College of Rheumatology (ACR) in 1971 (7), and subsequently revised in 1982 (8) and 1997 (9). Based on 1997 criteria, the classification of SLE was typically based on the presence of at least 4 out of 11 criteria (**Table 1.1**). These criteria presented high sensitivity (>85%) and specificity (>95%) and included both clinical and serological parameters; all features contributed equally to the diagnosis without any weight on any particular feature. However, these criteria were developed and validated in patients with longstanding disease and might have excluded those with early or limited disease. Apart from that, some systems were over-represented (for instance, mucocutaneous manifestations), whilst there was a significant problem to classify patients with organ-threatening manifestation such as lupus nephritis. Lastly, there were

individuals presenting only immunological abnormalities or individuals fulfilling four clinical criteria but they were negative for ANA.

Criteria	Definition
Malar Rash	Fixed erythema, flat or raised, over the malar eminences, tending to spare the nasolabial folds
Discoid Rash	Erythematous raised patches with adherent keratotic scaling and follicular plugging; atrophic scarring occurs in older lesions
Photosensitivity	Skin rash as a result of unusual reaction to sunlight, by patient history or physician observation
Oral Ulcers	Oral or nasopharyngeal ulceration, usually painless, observed by a physician
Arthritis	Non-erosive arthritis involving two or more peripheral joints, characterised by tenderness, swelling or effusion
Serositis	a. Pleuritis: convincing history of pleuritic pain or rub heard by a physician or evidence of pleural effusion or b. Pericarditis: documented by ECG or rub or evidence of pericardial effusion
Renal Disorder	a. Persistent proteinuria >0.5 g per day or >3+ if quantitation is not performed or b. Cellular casts: may be red cell, haemoglobin, granular tubular, or mixed
Neurological Disorder	a. Seizures: in the absence of off ending drugs or known metabolic derangements (eg, uraemia, acidosis, or electrolyte imbalance) or b. Psychosis: in the absence of off ending drugs or known metabolic derangements (eg, uraemia, acidosis, or electrolyte imbalance)
Haematological Disorder	a. Haemolytic anaemia with reticulocytosis, or b. Leucopenia: <4000/mm3, or c. Lymphopenia: <1500/mm3, or d. Thrombocytopenia: <100 000/mm3 in the absence of off ending drugs
Immunological Disorder	a. Anti-DNA: antibody to native DNA in abnormal titer, or b. Anti-Sm: presence of antibody to Sm nuclear antigen, or c. Positive finding of antiphospholipid antibodies based on: (1) an abnormal serum concentration of IgG or IgM anticardiolipin antibodies, (2) a positive test result for lupus anticoagulant using a standard method, or (3) a false positive serologic test for syphilis known to be positive for at least 6 months and confirmed by Treponema pallidum immobilisation or fluorescent treponemal antibody absorption test
Antinuclear Antibody	An abnormal titre of antinuclear antibody by immunofluorescence or an equivalent assay at any point in time and in the absence of drugs known to be associated with ‘drug-induced lupus’ syndrome

Table 1.1 The American College of Rheumatology revised classification criteria for SLE.

To solve the problem of individuals fulfilling clinical but not immunological criteria, a revision of the criteria was suggested in 2012 by the Systemic Lupus International Collaborating Clinics (SLICC), so each patient has to have at least one clinical and one laboratory criteria in the total 4 for the diagnosis of SLE. This demonstrated the significance of both clinical and immunological features for diagnosis and disease evaluation (10). However, the SLICC 2012 criteria were validated again for SLE patients with longstanding disease history, therefore patients at early stages of disease might be excluded. The advantages of the 2012 SLICC classification criteria over the ACR criteria included a greater sensitivity but similar specificity for classifying patients with SLE as well as a reduction in overlapping clinical features, for instance malar rash and photosensitivity. In addition, lupus nephritis in the presence of at least one of the immunologic variables was classified as a "stand alone" criterion (10). The 2012 SLICC classification criteria for SLE can be seen in **Table 1.2**. 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 with a classification threshold of 10 (11).

Criterion	Definition
Mucocutaneous	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. Inflammatory synovitis in ≥2 joints: a. Characterised by swelling or effusion, or b. Tenderness and ≥30 minutes of morning stiffness 6. Any of a. Typical pleurisy lasting >1 day, or pleural effusions or pleural rub 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. 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: a. Seizures b. Psychosis c. Mononeuritis multiplex d. Myelitis e. Peripheral or cranial neuropathy f. Cerebritis (acute confusional state)
Haematological	9. Haemolytic anaemia 10. Leukopenia (<4000/mm^3), or lymphopenia (<1000/mm^3) of at least once 11. Thrombocytopaenia (<100 000/mm^3) of at least once
Immunological	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

Criterion	Definition
Anti-nuclear antibody (ANA)	18. ANA above laboratory reference range
Rules for Classification	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

Table 1.2 The 2012 SLICC classification criteria for SLE.

1.4 Clinical manifestations of SLE

As presented in the clinical criteria above, SLE is characterised by significant heterogeneity in clinical manifestations and patients usually come along with several clinical symptoms varying in severity (12, 13). Most of the patients complain about general symptoms, such as fatigue, arthralgia, myalgia, fever and lymphadenopathy. Mucocutaneous manifestations are among the most frequent clinical findings and lupus-specific skin lesions can be classified as acute, subacute and chronic as described in the 2012 SLICC criteria. The "butterfly rash" is typical of acute cutaneous SLE, although it can be found only in 30% of patients, and it usually appears as an acute, painful and erythematous region with malar distribution. The malar rash is strongly related to exposure to sunlight. Other mucocutaneous manifestations include non-scarring alopecia and oral and/or nasal ulcers (14). Moreover, serositis – inflammation of pleural and pericardial cavities– can be persistent in patients with SLE and it can require high doses of corticosteroids to be treated (15).

Musculoskeletal manifestations are considered to be universal, as more than 90% of lupus patients will present a form of arthritis. In comparison with rheumatoid arthritis, lupus arthritis is less severe and not erosive. However, a deforming non-

erosive joint disorder known as Jaccoud's arthropathy can be seen in up to 5% of SLE patients (16-18).

Haematological manifestations such lymphopenia, neutropenia, thrombocytopenia, autoimmune haemolytic anaemia, thrombotic thrombocytopenic purpura and myelofibrosis are also known features of SLE (19). Coagulopathies are strongly correlated with the coexistence of antiphospholipid antibodies, which can promote activation of endothelial cells, platelets, and other cells of the immune system leading to venous, arterial, small-vessel thrombosis, and pregnancy loss (20). Apart from the thromboembolic events closely related to secondary antiphospholipid syndrome, the chronic inflammatory state of patients with SLE is linked to acceleration of the atherosclerotic process and in turn to an increased incidence of cardiovascular disease (myocardial infarction, stroke) (21). Premature coronary heart disease has emerged as a major cause of morbidity and mortality in SLE (22).

SLE can affect both the central and the peripheral nervous system, which is referred as neuropsychiatric SLE (NPSLE). NPSLE can often be severe with multiple symptoms imitating other neurological or psychiatric diseases (23). Although the underlying mechanisms are still unclear, several pathogenic pathways are related to antibody-mediated neurotoxicity, vasculopathy due to anti-phospholipid antibodies and cytokine-induced neurotoxicity (24).

Despite the wide spectrum of clinical features, renal involvement (“lupus nephritis”) still remains one of the most severe complications and important cause of morbidity and mortality amongst patients with SLE (25). Kidney disease in lupus is related to glomerular inflammation and the clinical symptoms of glomerulonephritis often include edema and weight gain, high blood pressure, nephritic or nephrotic

syndrome. Lupus nephritis usually requires close monitoring of 24-hour urine for total protein and intensive treatment with immunosuppressant drugs (26). Lupus nephritis can be characterised by low levels of serum albumin, whilst urinalysis demonstrates increased cellular casts, dysmorphic red blood cells, and the presence of haematuria or proteinuria (27). Although clinical features and laboratory investigations are important for the diagnosis of renal involvement, renal biopsy is the gold standard for the diagnosis and staging of lupus nephritis, since histopathological classification is essential for the management and treatment of patients (28). The initial classification of lupus nephritis was suggested in 1974 and it was lastly modified in 2003 by the International Society of Nephrology/Renal Pathology Society (ISN/RPS) (29, 30). The new classification of glomerulonephritis in SLE can be seen in **Table 1.3**.

Stage I	Minimal mesangial lupus nephritis
Stage II	Mesangial proliferative lupus nephritis
Stage III	Focal lupus nephritis[a]
Stage IV	Diffuse segmental (IV-S) or global (IV-G) lupus nephritis[b]
Stage V	Membranous lupus nephritis[c]
Stage VI	Advanced sclerosing lupus nephritis
[a] Indicate the proportion of glomeruli with active and with sclerotic lesions (<50%).	
[b] Indicate the proportion of glomeruli with fibrinoid necrosis and cellular crescents (>50%).	
[c] Class V may occur in combination with class III or IV in which case both will be diagnosed.	
Indicate and grade (mild, moderate, severe) tubular atrophy, interstitial inflammation and fibrosis, severity of arteriosclerosis or other vascular lesions.	

Table 1.3 Abbreviated International Society of Nephrology/Renal Pathology Society (ISN/RPS) classification of glomerulonephritis in SLE.

1.5 Management and treatment of SLE

Despite the fact that 5-year survival for patients with SLE has improved from 50% in the 1950s to more than 90% nowadays, lupus patients still have worse health-related quality of life in comparison with the general population or even patients with other chronic diseases (31). The major factors for this notorious decrease in mortality seen in patients with SLE are undoubtedly the introduction of steroids and other immunosuppressants. For patients with general symptoms and mild-to-moderate SLE, current guidelines recommend the use of antimalarial drugs (for instance, hydroxychloroquine), glucocorticosteroids, non-steroidal anti-inflammatory drugs and additional immunosuppressive therapy in case of persistently high disease activity (32). The management of severe SLE requires intensified immunosuppressive therapy and higher doses of glucocorticosteroids. Particularly, patients with class III and IV lupus nephritis require induction therapy with combined glucocorticosteroids, and other immunosuppressants such as azathioprine, cyclophosphamide, mycophenolic acid/sodium and mycophenolate mofetil (MMF), whilst for maintenance therapy, glucocorticosteroids, azathioprine and MMF are recommended (33).

Novel agents targeting specific immune cells and pathways are under clinical trials evaluating safety and effectiveness in treating patients with SLE (34). B cell clonal expansion is crucial in SLE pathogenesis and begins at preclinical stages (35). Rituximab is a chimeric anti-CD20 monoclonal antibody depleting naïve B cells and is currently used for treatment of patients with lupus nephritis who are not responsive to first-line therapy (36, 37). B-cell activating factor (BAFF) alongside a proliferation-inducing ligand (APRIL) has a crucial role in regulation of B cells promoting plasma

cell survival and naïve B-lymphocyte repertoire selection. Belimumab is a human monoclonal antibody targeting BAFF and is approved to treat adult patients with autoantibody-positive SLE with active disease despite receiving standard therapy (38). Apart from autoreactive B cells, T cells are also important in the development and progression of SLE. Abatacept is a CTLA-4-IgG1 fusion protein that inhibits T-cell activation. Although it failed to demonstrate any difference in complete response in patients with lupus compared to placebo, treatment with abatacept was associated with greater improvements from baseline in anti-double-stranded DNA antibody, C3, and C4 levels (39). Novel drugs focusing on type I IFN pathway will be analysed in detail in the relevant section below.

Assessment and monitoring of disease activity in patients with SLE is complicated, thus relevant indices have been developed and validated for use in clinical practice as well as clinical trials. For the purpose of this thesis, the revised British Isles Lupus Assessment Group Index (BILAG-2004) was used, which measures disease activity in different target organs/systems (domains) affected by SLE based on clinical assessments and laboratory results (40, 41). The BILAG-2004 index covers 97 items over nine domains (constitutional, mucocutaneous, neuropsychiatric, musculoskeletal, cardiorespiratory, gastrointestinal, ophthalmic, renal and haematological) recording disease activity that has occurred during the past 4 weeks. Disease activity in each domain is then categorised into five grades: A (severe disease), B (moderate active disease), C (mild stable disease), D (inactive disease) and E (never affected). The global BILAG-2004 score can be calculated by adding the total points from the nine BILAG grades (42). The grading system for BILAG-2004 Index can be seen in **Table 1.4**.

Grade	Definition
A	Severely active disease (sufficient to require disease-modifying treatment, i.e. >20mg/day prednisolone, immunosuppressant and cytotoxic drugs)
B	Moderately active disease (requires only symptomatic therapy, i.e. 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

Table 1.4 Grade and definition of BILAG-2004 Index for disease activity in SLE.

1.6 Pathogenesis of SLE

Individuals with specific genetic polymorphisms are at greater risk for developing SLE compared to the general population, whilst environmental triggers contribute to the initiation and perpetuation of the disease. Activation of the innate immune system leads to enhanced antigen presentation to T cells and aberrant production of proinflammatory cytokines and type I IFNs, which in turn results in activation of the adaptive immune system and the production of autoantibodies by autoreactive plasma cells. The onset of clinical manifestations is associated with systemic inflammation and tissue injury, which in turn amplifies the autoimmune responses and irreversible end-organ damage.

1.6.1 Genetics

The importance of genetics in the pathogenesis of SLE is supported by the high concordance rate (up to 57%) of SLE in monozygotic twins (43, 44). In addition, siblings of patients with SLE are 29 times more likely to develop SLE than the general population, whilst first-degree relatives of patients with SLE have a 17-fold increased risk to develop the disease compared with the general population (45, 46). The commonest genetic predisposition is found at the major histocompatibility locus

which contains genes for antigen-presenting molecules such class I HLA molecules (HLA-A, -B and -C) and class II HLA molecules (HLA-DR, -DQ and -DP) (47, 48). In more detail, HLA-DRB1*0301 and HLA-DRB1*1501 loci predispose to development of SLE, but HLADRB1*1401 reduces the risk of disease development (49). Genetic factors associated with deficiencies in the complement components (C1q, C2, C4A and C4B) and the presence of *TREX1* mutations consist of the highest risk for developing lupus-like disease (50-52).

Genome-wide association studies (GWAS) have identified over 50 gene loci with polymorphisms predisposing to SLE (53-55). Most of the SLE susceptibility genes are associated with pathways related to regulation apoptosis and clearance of apoptotic material, autophagy, the NF-κB pathway, other immune cell signalling pathways and cell migration as well as pathways related to activation type I IFN axis (56-58). Several single-nucleotide polymorphisms (SNPs) are associated with specific organ manifestations such as lupus nephritis (59). However, lupus is beyond genetics, as this accounts for only 20% of susceptibility to SLE suggesting a large component of epigenetic and environmental triggers (60).

1.6.2 Epigenetic and environmental factors

Epigenetic effects such as DNA methylation, post-translational histone modifications and micro ribonucleic acids (miRNAs) greatly influence the risk of SLE (61). The role of epigenetics in the pathogenesis of SLE is supported by the studies carried out in identical twins who are discordant for SLE (62). Abnormal methylation has been associated with development of SLE, whilst aberrantly expressed miRNAs in different cell types and tissues are thought to play an important role in the progression of SLE (63, 64).

Amongst the most important environmental triggers for lupus pathology is exposure to ultraviolet (UV) light culminating in aberrant apoptosis of keratinocytes (65). Accumulation of apoptotic cells due to defective phagocytosis leads to secondary necrosis and subsequently production of pro-inflammatory cytokines including IL-1, IL-3, IL-6, granulocyte macrophage colony-stimulating factor (GM-CSF), type I and II IFNs, which in turn activate B cells to differentiate into plasma cells secreting antibodies (66). Furthermore, a number of exogenous viruses, particularly Epstein-Barr virus (EBV), have been linked to the pathogenesis of SLE. High antibody titres to EBV alongside increased circulating EBV viral loads amongst patients with SLE have been suspected for the development of systemic autoimmunity via molecular mimicry of viral protein regions that are homologous to nuclear antigens (67). Apart from EBV, human endogenous retroviruses including HRES-1, HERV-3, HERV-E4-1, HERV-K10 and HERV-K18 have been linked to the development of SLE (68). Finally, drug-induced lupus is defined as a syndrome with clinical and serological features similar to SLE that is temporally related to continuous drug exposure and which resolves after discontinuation of this drug (69). More than 100 drugs have been associated with this entity in genetically predisposed individuals (70). Unlike SLE, clinical manifestations of drug-induced lupus subside when the drug is withdrawn.

1.6.3 Apoptosis and autoantibodies

A defect in both regulation of apoptosis and clearance of apoptotic material is a hallmark of the pathogenesis of SLE. As mentioned above, inherited complement deficiency of the classical pathway is one of the strongest associations with systemic autoimmunity. C1q knock-out murine models and humans with C1q deficiency, a rare monogenic disorder, develop autoantibodies and lupus-like disease due to impaired apoptotic cell debris clearance (71). Experimental lupus models presented an increased generation of apoptotic material and a decreased phagocytic function, so that usual clearance mechanisms are overwhelmed (72). The apoptotic cells can release nucleosomes, the main component of which is chromatin, into the cytoplasm and subsequently attach them to the plasma membrane (73, 74). Moreover, blebs of apoptotic keratinocytes in SLE contain autoantigens that can be found clustered on the cell surface (66). Additionally, lupus nephritis is correlated with nucleosome release within the glomeruli due to increased cell apoptosis locally in the kidney (75). On the other hand, anti-dsDNA antibodies can be found in approximately 70% of patients with SLE and this percentage is higher in patients with renal disease (76). Autoantibodies can bind to exposed nuclear antigens and form *in situ* immune complexes that can potentially cause tissue damage via binding to Fc receptors and subsequent activation of cellular immunity and/or via the activation of complement cascade. In murine models, certain monoclonal anti-dsDNA antibodies were able to bind to the glomeruli leading to proteinuria and histopathological lesions (77). A study using immune electron microscopy demonstrated that nephritogenic anti-dsDNA antibodies were able to target intraglomerular membrane-associated nucleosomes in lupus nephritis (78).

1.6.4 Cellular components

Innate and adaptive immune cell present a series of abnormalities contributing to autoreactivity and lupus pathology. Altered functional properties of neutrophils such as diminished phagocytic and lysosomal activity, upregulation of adhesion molecules and intravascular activation *in vivo* are prominent in SLE (79). Neutrophils undergo a particular cell death realising neutrophil extracellular traps (NETs) and this NET formation is linked to the exposure of nuclear autoantigen in SLE (80). Interestingly, mitochondrial reactive oxygen species (ROS) production can enhance NET formation in low-density granulocytes from patients with SLE without activation of functional NADPH, which is normally required in NET formation (81).

Professional antigen presenting cells (for instance, cDCs, macrophages, B cells) express MHC class II molecules along with co-stimulatory molecules and pattern recognition receptors, being very potent T cell stimulators. cDCs have been proposed to perform prolonged self-antigen presentation and produce pro-inflammatory cytokines production in autoimmune diseases, while they exhibit a defective tolerogenic function failing to contribute to self-tolerance (82). In addition, a reduction in numbers of macrophages alongside with their impaired function in uptake of apoptotic bodies leads to the accumulation of apoptotic cells near germinal centres (83).

$CD4^+$ T cells in SLE display an altered signalling phenotype exhibiting a rewiring of their T cell receptor (TCR); expression of the CD3ζ chain is decreased and replaced by the homologous Fcγ receptor chain, which recruits the downstream signalling Syk kinase instead of the normal CD3ζ partner Zap70 (84). This autoreactive profile is linked to defective gene transcription and altered cytokine production; a defect in IL-

2 production and an increased production of interleukin 17 (IL-17) having their effector and regulatory capacities significantly compromised (85). Although $CD4^+CD25^{hi}FoxP3^+$ regulatory T cell (T_{reg}) numbers appear to be reduced in patients with active SLE, it is not clear whereas they actively contribute to SLE pathogenesis (86, 87). $CD4^+CXCR5^+PD1^+OX40^+ICOS^+$ follicular T helper (T_{fh}) cells is a dynamic subset of $CD4^+$ T cells secreting IL-21 essential for B cell immunoglobulin production, isotype switching, and somatic hypermutation (88). In addition, extrafollicular helper T cells (eT_{fh}) represent a $CD4^+$ T cell subpopulation analogous to T_{fh} that can promote immunoglobulin production by B cells in extrafollicular compartments (89). Remarkably, eT_{fh} produce more IL-21 in SLE patients and eT_{fh} numbers are increased in the peripheral blood correlating with disease activity and other immunological features such as plasmablast numbers as well as with anti-dsDNA titres (90, 91).

Apart from T cell abnormalities, B cell phenotype seems to be notably affected in SLE. Reduced numbers of naïve B cells ($CD19^+CD27^-$) and IL-10-secreting B_{reg} ($CD19^+CD24^{hi}CD38^{hi}$) subsets as well as increased numbers of transitional B cells ($CD19^+CD24^{hi}CD38^{hi}$), switched memory B cells ($CD19^+CD27^+IgD^-$), double-negative B cells ($CD19^+CD27^-IgD^-$) and plasmablasts ($CD27^{hi}CD38^+CD19^{dim}sIg^{low}CD20^-CD138^+$) correlate with disease activity characterising active SLE (92-94).

Apart from differences in enumeration of B cell subsets, SLE patients also exhibit increased numbers of self-reactive B cells in emigrating and mature naïve B cell subsets (35). The $9G4^+$ antibody correlates with disease activity in SLE, whilst it represents a significant component of the anti-apoptotic cell repertoire in SLE comprising an important step in development of SLE (95). Furthermore, DNA-reactive B cells carry risk alleles such as *BANK1*, *BLK*, *CSK*, *FCGR2B* linked to increased

susceptibility to SLE and can promote hyper-responsiveness to B-cell receptor (BCR) engagement and enhanced B cell activation as well as *PTPN22* risk allele resulting in diminished tolerance in immature B cells. DNA-reactive B cells are more likely to mature, participate in germinal centre reactions, and eventually undergo plasma cell differentiation in lupus patients (96).

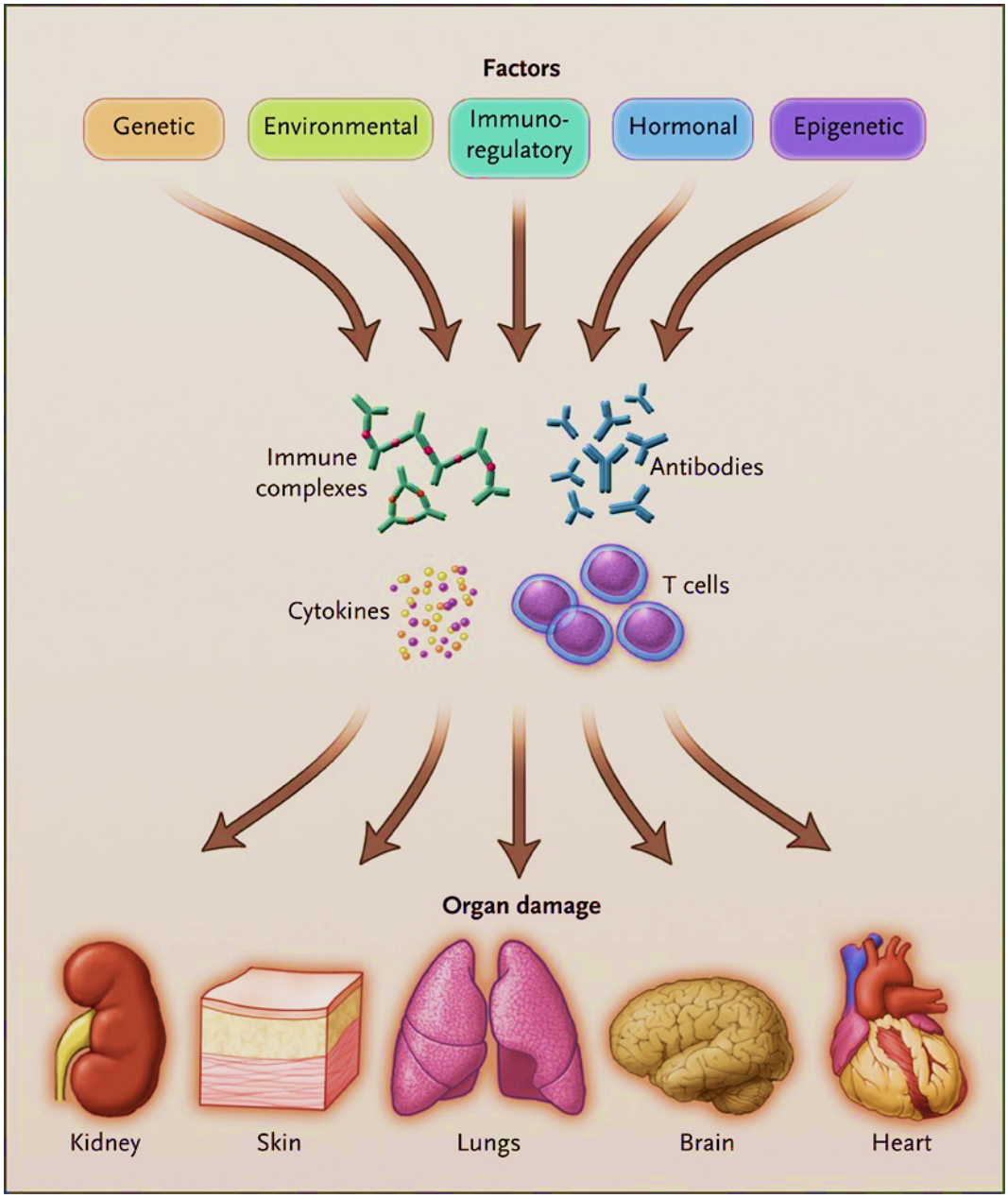


Figure 1.1 Overview of the pathogenesis and the main organs affected in SLE. Genetic, environmental, hormonal, epigenetic, and immunoregulatory factors act either sequentially or simultaneously on the immune system resulting in the generation of autoantibodies, immune complexes, autoreactive or inflammatory T cells, and inflammatory cytokines that may initiate and amplify inflammation and damage to various organs. *(97)*.

1.7 Preclinical autoimmunity and progression to SLE

Up to 25% of the general population present positive ANA, usually at low titre (1:40), but only a small percentage will progress to a clinically overt systemic autoimmune disease (98, 99). ANA positivity correlates with female gender and age, a feature that might be associated with higher prevalence of SLE among women. African Americans also present a greater titre of ANA in comparison with other populations in the United States (100).

The high prevalence of autoreactivity in the general population suggests that autoantibodies may be a major part of a healthy immune response critical to immune regulation. Healthy individuals usually have IgM autoantibodies, also known as natural autoantibodies. In mice, natural IgM antibodies can recognise apoptotic cells enhancing the phagocytic clearance of dead and dying cells and suppressing innate immune signaling pathways (101). In patients with SLE, natural IgM autoantibodies can bind to neo-epitopes on apoptotic cells and are present at higher levels in patients with lower disease activity and less severe organ damage (102). Natural IgM autoantibodies are predominantly produced by $CD5^+$ B cells, which consist of around 20% of peripheral blood B lymphocytes in adults, and are polyreactive presenting low affinity for a variety of autoantigens (103).

Many individuals who might have features suggestive of an autoimmune disease do not necessarily complete the criteria for SLE diagnosis. This condition has been described as incomplete lupus erythematosus (104, 105). A period of ANA positivity and other immune dysregulation can precede clinically overt disease (106). Approximately 15-20% of these individuals eventually progress to full SLE within 5-10 years (107). In some individuals, presumably with additional pre-existing genetic

and/or epigenetic susceptibility factors, these autoantibodies might promote activation of immune responses that culminate in progression from benign autoimmunity to clinical disease. The severity of disease might also increase as autoimmune responses escalate and tissue damage accumulates (108). Nevertheless, the factors determining the transition from this benign preclinical stage are still unclear, since the majority of people presenting ANA positivity will not develop any further clinical manifestations. A better understanding of early stages of SLE pathogenesis can improve on time diagnosis, early intervention and potentially more effective treatment options. A schematic presentation of the gradual progression from the stage of ANA positivity to clinical symptoms and established SLE can be seen in **Figure 1.2**.

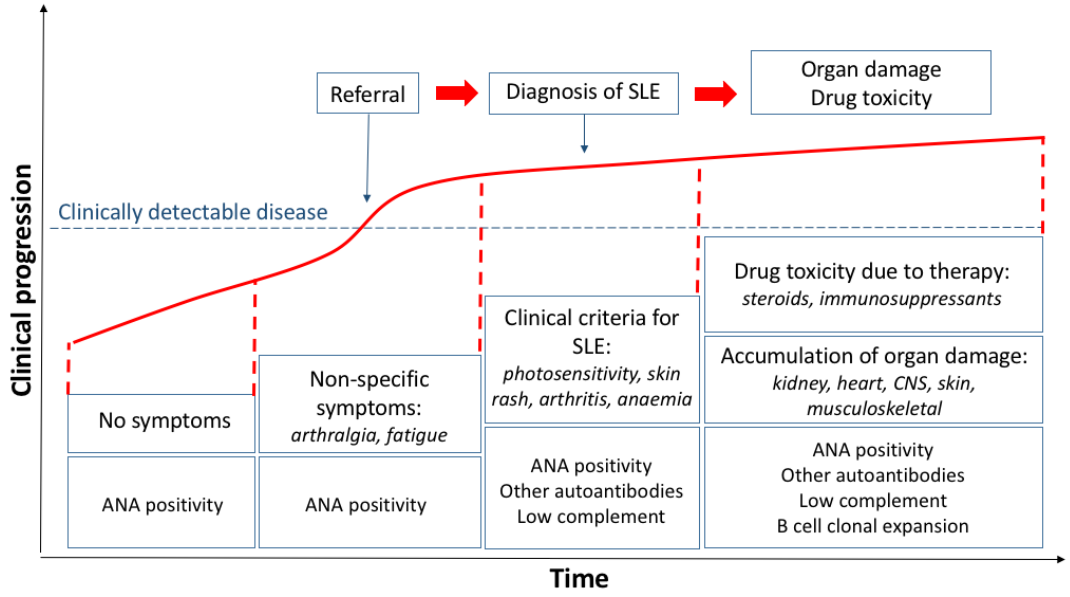


Figure 1.2 Schematic presentation of the gradual progression from the stage of ANA positivity to SLE. Immunological abnormalities and clinical symptoms accumulate before the diagnosis of SLE, while organ damage is escalated due to disease activity and drug toxicity.

1.8 Type I interferons (IFNs)

Autoimmune rheumatic diseases are characterised by a breakdown of immune tolerance leading to inflammation and irreversible end-organ tissue damage. Diverse cellular components and molecules contribute to the development of autoimmunity, and their roles vary between individuals as well as diseases. However, common features may be used to classify, diagnose and target therapy to groups or subsets of patients. The use of anti-TNF and B cell-depleting therapies has led to a rethinking of diagnosis and investigation in terms of ultimate therapy. Dysregulation of type I interferons (IFNs) is a common factor in multiple autoimmune rheumatic diseases and is of increased interest recently due to appreciation that it may define clinical phenotypes and therapy responses, as well as the potential to treat with direct type I IFN blockade (109, 110).

IFNs are generally classified into 3 families –IFN-I, IFN-II and IFN-III– which differ in their immunomodulatory properties, their structural homology and the group of cells they are secreted from (111, 112). Type I IFNs (IFN-α, -β, -ω, -ε, -κ) consist of the largest family and alongside IFN-III (IFN-λ) activate intracellular signalling pathways which mediate immune responses against viruses and tumours (111, 113, 114). Although most cells are capable of producing type I IFNs, in most situations the majority comes from dedicated danger-sensing cells called plasmacytoid dendritic cells (pDCs). Type I IFNs act on all nucleated cells during viral invasion to inhibit viral replication (112). They also have potent immunostimulatory properties, including inducing the maturation and activation of myeloid dendritic cells (DCs), favouring Th1 phenotype and promote B cell activation, antibody production and Ig class switching (115-117). These immunostimulatory properties underlie their roles in

autoimmunity. In contrast, although there is overlap in the gene sets whose expression they induce, IFN-II (IFN-γ) is functionally distinct. It is produced mainly by NK cells and certain T cell subsets, and regulates aspects of immune responses like phagocytosis and antigen presentation (118).

1.9 Plasmacytoid dendritic cells (pDCs)

1.9.1 Development of pDCs

Plasmacytoid dendritic cells (pDCs) have a distinct phenotype compared to conventional dendritic cells (cDCs) and play a crucial role mediating type I IFN production predominantly during acute viral infections (119). Both pDCs and cDCs derive from a common DC progenitor, which is characterised by lack of lineage markers and expression of Fms-like tyrosine kinase 3 (FLT3; CD135), macrophage colony-stimulating factor receptor (M-CSFR; CD115) and the receptor tyrosine kinase KIT (CD117) (120-122). Another progenitor characterised as $LIN^{-}KIT^{int/lo}FLT3^{+}IL\text{-}7R\alpha^{-}M\text{-}CSFR^{-}$ expresses high levels of E2-2, the main transcription factor defining pDC lineage, and it can be generated by the common DC progenitor under conditions that favour E2-2 upregulation such as exposure to thrombopoietin or M-CSF (123, 124). E2-2 can bind to a large fraction of pDC-enriched genes and its continuous expression is required to maintain cell fate (125). E2-2 targets transcriptional factors that encode proteins involved in the development, homeostasis and function of pDCs, for instance SPIB, BCL11A, IRF8, RUNX2 and CIITA, the pDC-related surface markers BDCA-2, ILT7, SIGLEC-H and the intracellular nucleic acid sensors TLR7, TLR9 and PACSIN1 (124, 125). Deletion of E2-2 in mature pDCs causes the loss of pDC-related markers and differentiation into cDC-like cells by upregulating MHC-II molecules and

therefore enhancing the ability to prime T cells (125). On the other hand, ID2 is the repressor of E2-2 and its expression is absent in pDCs but prominent in cDCs (126).

FLT3 and its ligand (FLT3L) are of paramount importance in pDC development inducing activation of signal transducer and activator of transcription 3 (STAT3)- and phosphoinositide 3-kinase (PI3K)-dependent activation of mammalian target of rapamycin (mTOR) (127-129). Intriguingly, pDCs can also derive from a lymphoid-primed multipotent progenitor characterised by lack of lineage markers and as $KIT^{+}SCA1^{+}CD34^{+}FLT3^{+}$, in which type I IFNs and FLT3L act synergistically to promote pDC development by inducing FTL3 expression (123, 130).

1.9.2 pDCs as IFN-α-producing cells

pDCs appear distinct from the cDCs, since instead of antigen presentation pDCs' primary role is the production of type I IFNs in response to danger signals (131, 132). Their main function is to produce type I IFNs, predominantly IFN-α, after sensing nucleic acids mainly via toll-like receptors (TLRs) -TLR7 and TLR9- located in endosomal compartments. TLR7 can sense RNA viruses, endogenous RNA and synthetic oligoribonucleotides, whereas TLR9 can detect DNA viruses containing unmethylated CpG-rich DNA sequences, synthetic CpG oligodeoxyribonucleotides, and endogenous DNA (133). Upon binding of TLRs to viral or self-nucleic acids, the myeloid differentiation primary response protein 88 (MyD88)-IRF7 and the MyD88-nuclear factor-κB (NF-κB) pathways are activated inducing the secretion of type I IFNs and other pro-inflammatory cytokines (134, 135).

The type of compartment in which TLR7 and TLR9 encounter their ligands is crucial for the production of type I IFNs and/or pro-inflammatory cytokines (136). Multimeric CpG-A oligonucleotides aggregate in early endosomes where they

activate the MyD88–IRF7 pathway that induces type I IFNs. On the other hand, monomeric CpG-B is transferred to an endo-lysosomal compartment, in which it induces the MyD88–NF-κB pathway upregulating costimulatory molecules and triggering the secretion of pro-inflammatory cytokines (137). Notably, IkappaB kinase-alpha (IKKα), osteopontin, and mTOR are key elements of the MyD88-IRF7 signalling pathway, while IRF5 is necessary for MyD88-NF-κB signalling pathway (136-141). Trafficking of TLR9 to the appropriate compartment for type I IFN production is dependent on adapter protein 3 (AP3) (142, 143). The peptide/histidine transporter 1 (PHT1), BLOC1 and BLOC2 Hermansky-Pudlak syndrome proteins are encoded by *Slc15a4* and are thought to be key mediators of TLR9 signalling in pDCs (143). Nonetheless, TLR9-mediated sensing of large DNA-containing immune complexes is independent of AP3 requiring phagocytic and autophagic pathways instead. TLR7-mediated sensing of single stranded RNA (ssRNA) also occurs in a distinct subcellular compartment requiring transport of viral nucleic acids from cytosol into the lysosome by autophagy (144).

TLR7 and TLR9 consist of the main receptor sensing nucleic acids leading to activation of pDCs and the production of type I IFNs and pro-inflammatory cytokines. However, TLR-independent pathways of sensing nucleic acids mediated via other transcription factors play important role as well (145). CpG-A oligonucleotides can bind to the DExD/H-box helicase 36 (DHX36) resulting in the translocation of IRF7 to the nucleus and type I IFN production (146). In contrast, CpG-B oligonucleotides can bind to DHX9 resulting in nuclear translocation of NF-κB and eventually the secretion of pro-inflammatory cytokines. Viral nucleic acids can be detected in a RIG-I-like helicase-dependent manner, while other major cytosolic sensors include cGAS and STING

(147, 148). The main intracellular pathways of TLR-mediated type I IFN and pro-inflammatory cytokine production can be seen in **Figure 2.3**.

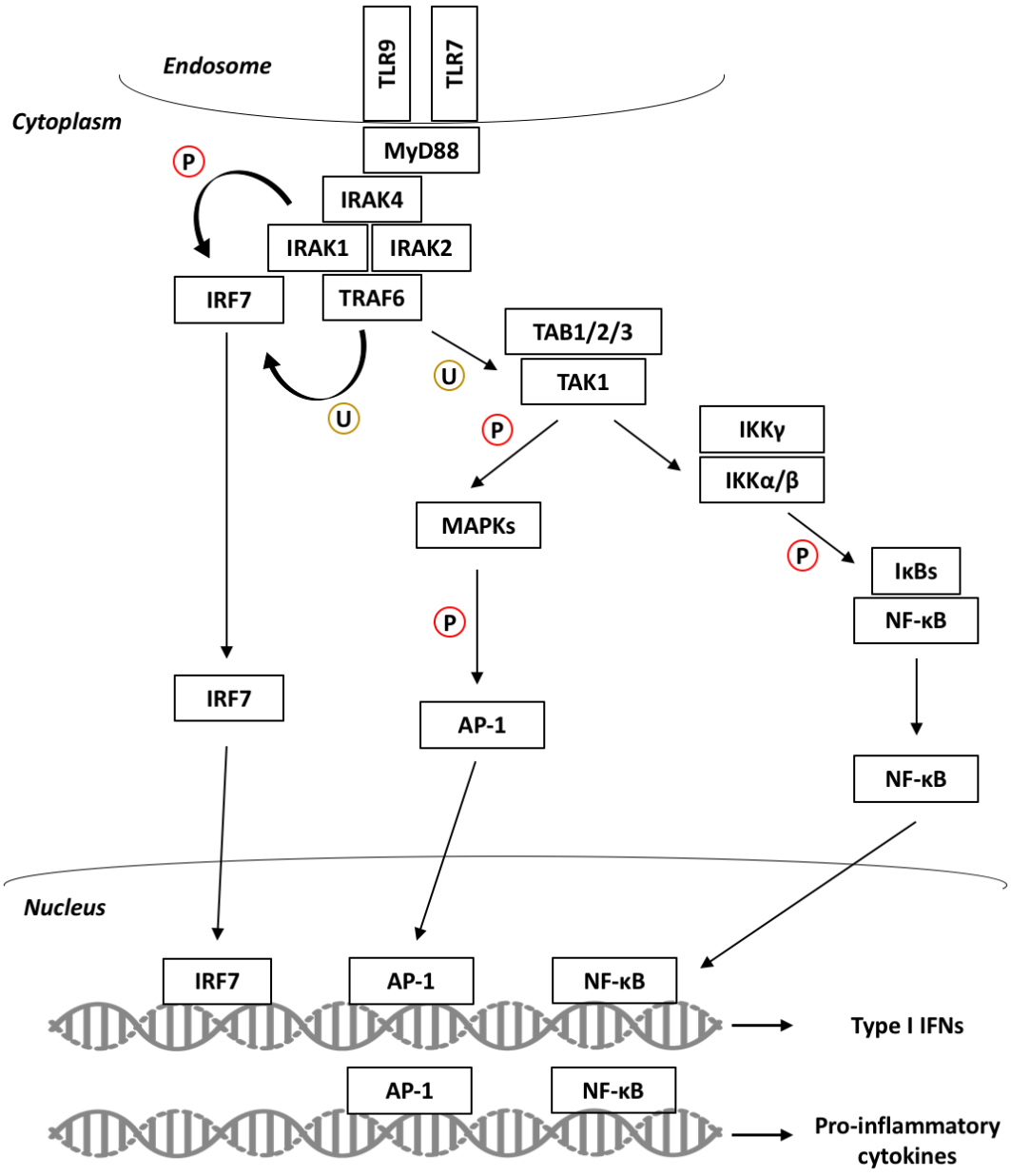


Figure 1.3 TLR7 and TLR9 intracellular signalling pathways in pDCs leading to type I IFN and pro-inflammatory cytokine production.

1.9.3 pDCs as antigen-presenting cells

Although not as efficient as cDCs, pDCs express MHC class II molecules and they are able to capture, process and present antigens to $CD4^+$ T cells inducing their activation (149, 150). TLR-activated pDCs have enhanced antigen-presenting function and can promote Th1 and Th17 differentiation (151-153). Combination of TLR stimulation and antigen delivery via BST2 (CD317, tetherin) can induce robust cellular and humoral immune responses against viral infection or tumour growth (154). Despite their weaker antigen-presenting properties, pDCs can also cross-present exogenous antigens to $CD8^+$ T cells and therefore induce antiviral and antitumor responses (155-157). Recycling endosomes are also important features of pDCs which allow peptides to be continuously loaded on to MHC-I molecules. This process facilitates the presentation of viral antigens to $CD8^+$ T cells contributing to antiviral immunity.

However, unstimulated or alternatively stimulated (e.g. HIV) pDCs are predominantly known to be involved in the induction of tolerogenic immune responses by expressing indoleamine-2,3-dioxygenase (IDO), ICOSL, OX40L, PD-L1 and by promoting $CD4^+$ T cell anergy and T_{reg} differentiation (158-162). pDCs that capture antigens in peripheral tissues use CCR9 to migrate to the thymus, where they can promote deletion of antigen-specific thymocytes, actively inducing immune tolerance (163). Interestingly, conjugation of antigens with BDCA-2 on pDC cell surface can promote immune tolerance by inhibiting antigen-specific $CD4^+$ T cell as well as antibody responses upon secondary exposure to antigen in the presence of adjuvant. This process involves increase in T_{reg} cells and decrease in effector $CD4^+$ T cells (164). Therefore, pDCs have strong tolerogenic properties apart from their main role to mediate type I IFN immune responses.

A schematic summary of the pleiotropic functions of pDCs on the immune system can be seen in **Figure 1.4**.

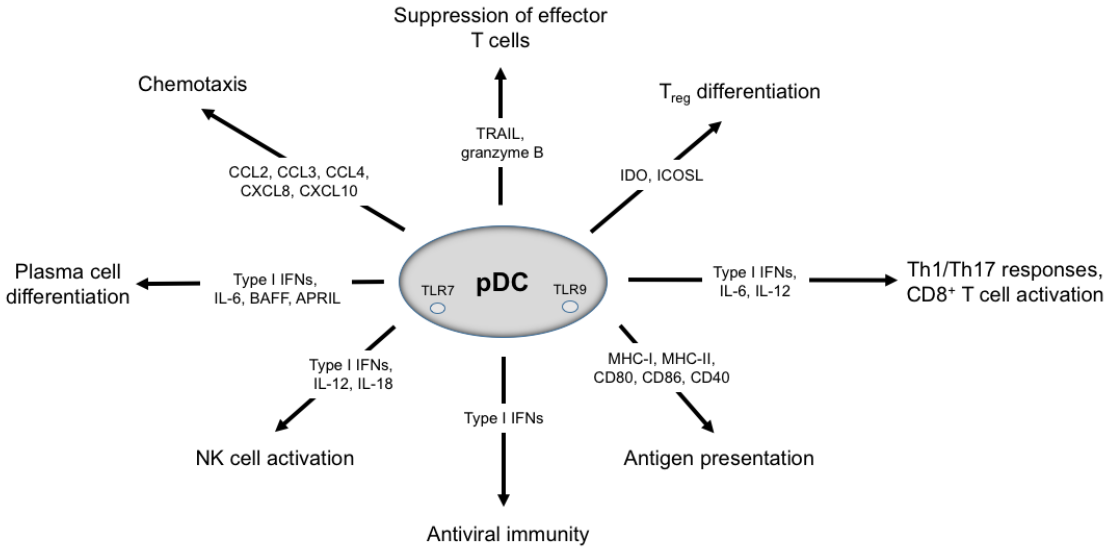


Figure 1.4 Pleiotropic functions of pDCs on the immune system.

1.10 Regulation of type I IFN production

The balance of immune responses induced by type I IFNs is regulated at multiple stages to limit the toxicity to the host by preventing tissue damage and autoimmunity (165). These include regulation of IFN production and response to target cells.

The interferon regulatory factor (IRF) family of transcription factors is crucial for the propagation of IFN production (166). IRFs have heterogeneous functions in the regulation of both innate and adaptive immunity and are associated with the recognition of PAMPs from TLRs (167). PDCs constitutively express IRF7, which – alongside IRF5– induces the transcription of IFN-α related genes (23, 24). A wide range of regulatory receptors including BDCA-2, ILT7, NCR2, CD32 (FcγRII), are expressed on the cell surface of human pDCs, which modulate the intracellular

signalling pathways in response to TLR ligands (119, 168, 169). In particular, the interaction between BST2 (CD317; tetherin) and ILT7 was proposed as a regulatory mechanism to control the continuous activation of TLR signalling in pDCs (170). Although cross-linking of ILT7 was confirmed to truly inhibit type I IFN production, another study showed that BST2-mediated ILT7 cross-linking failed to act a negative feedback for IFN production in TLR-activated pDCs (171).

Although pDCs are the main source of type I IFNs, other cells such as epithelial cells or fibroblasts can secrete these cytokines (172). IFN production by neutrophils may be important in autoimmunity (173). NK cells can induce secretion of IFN-α by pDCs stimulated by RNA-containing immune complexes, while monocytes play an inhibitory role (174). Furthermore, microRNAs such as miR-155 and miR-146a seem to have an immunomodulatory effect on activation signalling pathways (175, 176). Oestrogen might favour type I IFN production through activation of TLR7 signalling pathway, consistent with female predominance of these diseases (177).

1.11 Effects of type I IFNs on target cells

Outcomes of type I IFN signalling may be as diverse as promotion of cell survival and promotion or prevention of apoptosis (178-180). Interestingly, although all type I IFN ligands signal through the same receptor (IFNAR), they result in different biological outcomes (181). This is important for therapy as either ligands or receptors may be targeted. The IFNAR2 subunit of the receptor has a surface-bound (IFNAR2b) and a soluble form, both with regulatory activity (182). In contrast, type II IFN (IFN-γ) signals via the IFNGR receptor. Type III IFN signals via a receptor that combines a unique subunit (IFNLR1) with one also used by IL-10 family cytokines and its expression is

much more restricted to cells of epithelial origin and dendritic cells (183). Interestingly, our group found that type III IFN signalling could also vary between cells: skin fibroblasts respond to type III IFNs (not only keratinocytes as previously thought) but they do so via MAPK instead of STAT1 (184). Each receptor type is associated with different pathways of STAT signalling and gene promoters. However, there is considerable overlap between the genes whose expression is induced by these pathways. This makes measurement of activity using gene expression, as in an interferon signature, complex. Effect of type II and III IFNs, variations in circulating immune cells, for example lymphopenia seen in lupus patients, and changes in other immune functions could all influence results.

Early evidence about the link of type I IFNs to autoimmunity was given in patients receiving immunotherapy with IFNs for chronic viral infections or malignant carcinoid tumours (185, 186). Interestingly, the presence of autoantibodies prior to IFN therapy considerably increased the risk for autoimmune phenomena that often characterise SLE, RA, polymyositis, suggesting that type IFNs might contribute to the development of clinical manifestations from a preclinical stage. Nevertheless, autoimmunity may remit after cessation of treatment, implying that regulatory factors control autoimmune responses and the transition to clinically overt disease is much more complicated (187).

Whilst the mechanisms behind the dysregulation of the IFN system are complex and remain unclear, advances have been made in understanding their role in systemic autoimmune diseases.

1.12 Systemic Lupus Erythematosus and type I IFNs

SLE is a prototypic type I interferon-mediated autoimmune disease whose clinical manifestations are diverse in organs affected, severity, and response to targeted and non-targeted therapies (1). Its pathogenesis is similarly complex, but a defining feature is an immune response against endogenous nuclear antigens, with anti-nuclear antibodies (ANA) being central to diagnosis, activity and tissue inflammation (188). ANA positivity may precede clinical symptoms by years, and only a proportion of such individuals develop organ inflammation, suggesting that autoantibodies are an incomplete explanation for pathology (108). ANA production by B cells may arise secondary to innate immune abnormalities in the sensing of nuclear antigens; type I IFNs are crucial mediator by which innate immune cells stimulate B cells.

Increased levels of serum IFN-α were described in patients with SLE over 30 years ago and were associated with disease activity and specific clinical manifestations such as fever, arthralgia, rash, and leukopenia (189, 190). High dose IFN-α treatment can induce a variety of neuropsychiatric adverse effects, while similar symptoms in neuropsychiatric SLE are linked to IFN-α production. Higher levels of IFN-α were detected in cerebrospinal fluid but decrease, when the manifestations of lupus psychosis subsided (191, 192). Type I IFNs also contribute to lupus nephritis (193). In murine lupus models, it exacerbated glomerulonephritis by increasing immune complex deposition in the kidneys (194). Patients with SLE have reduced numbers of pDCs in blood, but increased numbers of BDCA-2^+ cells intraglomerularly (195). In cutaneous lupus erythematosus, there is a unique IFN environment in the skin. Keratinocytes produce type III IFNs. Both type I and III IFNs mediate immune

responses, and the latter stimulates further type I IFN production (196). Patients with active CLE also have detectable serum levels of IFN-λ1 (197).

Genes in the IFN-pathway and regulation of innate immune responses are prominent in SLE susceptibility. These include variants in HLA and Fcγ receptor genes, *IRF5*, *STAT4*, *PTPN22*, *TNFAIP3*, *BLK*, *BANK1*, *TNFSF4* and *ITGAM* (198). Intriguingly, high type I IFN activity seems to be a heritable risk factor being clustered in specific families in both SLE patients and their healthy first-degree relatives (199). The risk haplotypes in the interferon regulatory factors IRF5 and IRF7 are associated with increased type I IFN activity and risk is dependent on particular autoantibodies (58, 200-204). The risk haplotype of IRF5 is also associated with risk of progression to clinical disease in ANA positive individuals (205). Gene variants in *IFIH1* (a cytoplasmic dsRNA sensor that activates IFN-α signalling) correlate to anti-dsDNA antibodies and increased sensitivity to IFN-α (206). In addition, IRF8 is strongly related to increased cardiovascular risk in mouse models as well as SLE patients (207, 208).

What is the environmental trigger for induction of type I IFN production? It has been proposed that nucleic acids from common viruses like Epstein-Barr virus (EBV) could initiate the IFN-α production via activation of intracellular TLR7 and TLR9 leading to disease in genetically predisposed individuals (209). An alternative theory suggests that self-derived nucleic acids comprise the major inducer of IFN-α secretion in SLE via the intracellular receptors responsible for antiviral immunity (210). Apoptosis and clearance of apoptotic material are defective in SLE allowing the maintenance of DNA (or RNA)-containing immune complexes (211). These nucleic acid-autoantibody complexes can be internalised by Fc receptors and recognised by endosomal TLR7 and TLR9 inducing aberrant IFN-α production by pDCs (168, 212). Degradation of

nucleic acids or inhibition of FcγRIIa can negate IFN-α production (172). Autoantibodies against RNA-associated proteins such as snRNP, Ro (SSA), La (SSB) can also augment immune responses (213, 214). The RNA binding protein Ro60 has been recently shown to regulate IFN-stimulated gene expression (215).

Expansion of plasmablasts/plasma cells is a hallmark of SLE positively correlated to disease activity and type I IFN enhances the differentiation of B cells to plasmablasts (216, 217). Hence, there may be positive feedback between B cells and pDCs via autoantibodies and IFN-α respectively. Type I IFNs can promote differentiation of plasma cells *in vitro* and can also confer a unique phenotype; type I IFNs can stimulate plasma cells, including those derived from SLE patients, secrete ISG15, via which they have pro-inflammatory effects independently of antibody secretion (218).

In mice TLR9-MyD88 signalling is crucial for switching of autoreactive of IgM anti-self B cells to the pathogenic IgG2a and 2b subclasses (219). T cells are directly affected by IFN-α promoting the generation of effector and memory $CD8^+$ T cells (220). Therefore, innate immunity may moderate adaptive immune responses against self-antigens.

Although self-nucleic acid containing immune complexes have been proposed as trigger of pDCs to produce IFN-α, other cells could an equally important role. For instance, there is increasing interest in the role of neutrophils in autoimmunity. The presence of neutrophils in inflamed kidney tissue was reported long ago in both experimental models and patients with autoimmune conditions affecting the kidneys (221, 222). Neutrophils seem to be key players in inducing type I IFN production by pDCs in a DNA- and TLR9-dependent manner (223). As mentioned above, neutrophils undergo special type of cellular death (NETosis), in which they release web-like

structures known as neutrophil extracellular traps (NETs) composed of chromatin and granule proteins that can bind and kill microorganisms (224). NETs also contain nuclear material, DNA and histones, and antimicrobial agents (LL37, HMGB1) that prevent nucleic acids from degradation. Thus, they can potentially facilitate the internalisation of endogenous DNA and immune complexes by pDCs and subsequently IFN-α production (223, 225). Many cytokines, including IFN-α, can actually act as priming factors on mature neutrophils, allowing the formation of NETs upon subsequent stimulation with complement factor 5a (226). As a consequence, neutrophils could be in the centre of another positive feedback loop between induction and maintenance of type I IFNs perpetuating immune responses.

1.13 Sjögren's Syndrome

Although dysregulation of type I IFN system has been mostly studied in SLE, there is evidence of increased type I IFN activity in many other rheumatic and inflammatory disorders, potentially sharing common molecular pathways (227, 228). Primary Sjögren's Syndrome (pSS) is an autoimmune disorder primarily affecting the salivary and lacrimal glands. It is characterised by autoantibodies against ribonucleoproteins, Ro (SSA) and La (SSB) (229). ISG expression is upregulated in both humans and mouse models, especially in those with detectable autoantibodies, and many studies tried to correlate these findings with disease pathogenesis (230). As in SLE, autoantigens of apoptotic origin provide the immunogenic stimulus for the initiation of pathogenic responses (231). RNA-containing immune complexes can activate pDCs in salivary glands and enhance the production of IFN-α, while IFN-α itself can upregulate the expression of ISGs in the target organs (232, 233). Immune responses being

developed in affected tissues appear mainly TLR-dependent (234, 235). Early studies clearly identified an IFN signature in salivary glands from patients with pSS; IRF7, IRF8, and IRF9 were significantly upregulated (236, 237). PBMCs also expressed an IFN signature and closely correlated with anti-Ro(SSA) and anti-La(SSB) titres (238, 239). A subgroup of pSS patients with monocyte IFN signature also presented higher disease activity alongside higher BAFF mRNA expression (240).

1.14 Inflammatory Myositis

In myositis, pDCs infiltrate tissues and secrete aberrant amounts of type I IFNs and ISGs are significantly upregulated in both inflamed muscles and PBMCs (241-243). Serum IFN-α is correlated to serum muscle enzyme levels in untreated disease among patients with juvenile dermatomyositis and inversely correlated to the duration of untreated disease (244). Additionally, anti-Jo1 and anti-Ro(SSA) autoantibodies were associated with higher expression of ISGs in PBMCs and higher disease activity in patients with dermatomyositis (245).

1.15 Other Systemic Autoimmune Diseases

Other connective tissue diseases associated with anti-nuclear antibodies also have some evidence for involvement of type I IFNs, at least in subsets of patients. An interferon signature similar to SLE and myositis was identified in patients with scleroderma (243). Antiphospholipid syndrome was reported as a side effect in patients receiving interferon-alpha therapy for unrelated diseases (246, 247). Patients with early incomplete forms of connective tissue diseases, of whom a proportion progressed to SLE or other diseases, had increased interferon activity

(248). Furthermore, a subgroup of patients with established undifferentiated connective tissue diseases of more than 12 months duration also had increased interferon activity (249).

1.16 Rheumatoid Arthritis

IFN signature was studied in RA as a biomarker for disease activity and response to therapy. In preclinical RA individuals with arthralgia and elevated type I IFN signature were at greater risk to develop arthritis (250). Type I IFN also predicted therapy response, and interestingly had opposite predictive value for two targeted therapies. Patients with high type I IFN signature had a poor response to rituximab (251, 252). Although RA patients with high IFN signature presented higher disease activity, in a recent study higher IFN score in neutrophils correlated with a good response to anti-TNF treatment (253, 254). Type I IFN status may predict complications of RA. Increased IFN-regulated transcripts, including *IFIT*, *IFIT2*, and *IRF7*, in a subset of RA patients were associated with upregulated pathways related to coagulation, complement activation and fatty acid metabolism (255).

1.17 Outside systemic autoimmunity: roles for type I IFNs in other diseases

Type I IFN axis influences host immune response to cancer as well as response to radiotherapy (256). Intratumorally, type I IFNs can enhance antitumor immunity as well as having beneficial anti-angiogenic effects (257). Type I IFNs have complex roles in chronic infection. They are mediators of antiviral defence and evasion of type I IFN effects significantly influences the pathogenicity of HIV and CMV infection, although unhelpful immunosuppressive effects of type I IFNs have also been described (258-

261). Type I IFNs may mediate atherosclerosis, which is of particular interest given the prevalence of this complication in autoimmune rheumatic diseases (262).

1.18 Interferonopathies

"Interferonopathies" are a heterogeneous group of disorders mainly presenting an autosomal recessive inheritance pattern, which are characterised by constitutive upregulation of type I IFNs. Aicardi-Goutieres syndrome (AGS), the most well studied interferonopathy, usually presents an early onset during childhood with lupus-like symptoms (263). IFN signature in peripheral blood has been reported to be universal in AGS patients with mutations in *TREX1*, *IFIH1*, *RNASEH2A*, *RNASEH2C*, *ADAR1*, while each mutation in these genes has been correlated with different clinical manifestations (264-266). These monogenic diseases culminating in the dysregulation of IFN-related responses strongly support the linkage between type I IFNs and autoimmunity.

1.19 Therapeutic targeting of type I IFN pathway

Given its pleotropic roles diverse diseases, blockade of type I IFN axis has potential to become a versatile treatment throughout in rheumatology and beyond. The most direct approach, with greatest use in human clinical trials, is the monoclonal antibody blockers of IFN-alpha or its receptor. However, the traditional lupus therapy hydroxychloroquine has relatively selective effects on type I IFNs. It blocks TLR7 and TLR9 activation by engaging TLR-binding epitopes and can efficiently impair the ability of pDCs to produce IFN-α upon stimulation, also suggesting that TLR-7 and TLR-9 antagonists might be potential treatment options in the future for many

autoimmune diseases (267-269). A number of small molecules or oligonucleotide inhibitors of TLRs for potential use in SLE or other autoimmune diseases are in preclinical or Phase I development (270). IFN signalling may also affect the efficacy of glucocorticoids. Glucocorticoids present decreased activity to inhibit the IFN pathway in pDCs activated via TLR-dependent pathways in SLE patients and lupus-mouse models (271, 272).

New therapeutic approaches targeting directly IFN-α by neutralizing monoclonal antibodies (sifalimumab, rontalizumab, AGS-009) have shown encouraging results. Phase I clinical trials confirmed their safety, tolerability and their ability to partially inhibit the overexpression of ISGs (273-275). The inhibition of IFN-α/β-inducible genes in whole blood was dose-dependent and the expression of genes for BAFF, IL-10, IL-1β, GM-CSF were also suppressed (276). In a phase IIb, randomised, double-blind, placebo-controlled study, sifalimumab achieved its primary endpoint by reducing disease activity in patients with SLE with acceptable safety profile in both IFN signature positive and negative subjects. However, immunological parameters such as complement levels and anti-dsDNA antibodies remained unchanged (277). In a recent phase II study, rontalizumab proved superiority in comparison with the control only in the group of patients with low IFN signature, who presented higher SLE response index and had lower use of steroids (278). Given the multiple forms of type I IFNs, targeting the shared IFNAR1 receptor may more effectively block type I IFN signalling (279, 280). Anifrolumab, an anti-IFNAR1 monoclonal antibody, met its primary endpoints of reduction in global disease activity score in patients with SLE and the level suppression of IFN signature was clearly associated with increased anifrolumab concentrations (281). Inhibition of IFNAR1 reduced ISG expression more

than sifalimumab with better efficacy in the IFN signature high subset and is now in phase III clinical trials.

Other strategies have directly targeted pDCs. Early, transient depletion of pDCs in BXSB lupus-prone mice before disease initiation led to reduced expansion of T and B cells, reduced production of autoantibodies an amelioration of glomerulonephritis (282). In NZB/NZW lupus-prone mice, inhibition of Bcl-2, a necessary molecule for pDC survival, resulted in selectively depletion of pDCs and reduction of IFN-α production (283). Furthermore, proteasome inhibitors (carfilzomib, bortezomib) managed to suppress the IFN-α production by TLR-activated pDCs by inhibiting pDC survival and function in lupus mice models (284). More recently, the pDC inhibitory receptor BDCA-2 (CD303) has been used to block type I IFN production in preclinical studies (285).

Finally, the immunization of SLE patients presenting mild to moderate disease with IFN-α-kinoid (IFN-K), a drug composed of inactivated IFN-α coupled to a carrier protein, induced anti-IFN-α antibodies and significantly improved disease biomarkers in all patients (286). Interestingly, a higher titre of anti-IFN-α antibodies were found in IFN signature positive patients, which were also linked to the reduction of IFN score. A summary of the main pharmaceutical agents targeting the type I IFN pathway can be seen in **Table 1.5**.

Pharmaceutical agent	Manufacturer	Definition	Therapeutic target
Sifalimumab	MedImmune, Inc.	Fully human mAb	IFN-α
Rontalizumab	Genetech	Recombinant humanized mAb	IFN-α
AGS-009	Argos Therapeutics	Humanized IgG4 mAb	IFN-α
Anifrolumab	MedImmune, Inc.	Fully human mAb	IFN-α/β receptor
IFN-α-kinoid	Neovacs	Vaccine	IFN-α
IMO-3100	Idera Pharmaceuticals	Oligonucleotide antagonist	TLR7/9 inhibition
DV1179	Dyvanax	Oligonucleotide antagonist	TLR7/9 inhibition

Table 1.5 Main pharmaceutical agents targeting type I IFN pathway.

1.20 Measuring Interferon Activity in Patients

While type I IFNs are known to mediate clinical manifestations of SLE, assays for IFN activity have not yet become routinely used in the care of SLE patients in the same way as B cell biomarkers such as autoantibody titres and complement levels. Type I IFN activity is commonly measured in patients using presence or absence of expression of interferon stimulated genes (ISGs), referred to as an interferon signature, or level of expression, referred to as an interferon score.

In research cohorts, 60-80% of lupus patients exhibit an increased expression of ISGs in PBMCs, known as interferon signature. In childhood-onset SLE the IFN signature is almost universally observed (287). Interferon scores are similar but are generally used to refer to a continuous parameter derived from qPCR rather than absence or presence of increased expression. Interferon signatures and scores consistently have increased B cell biomarkers of activity such as titres of anti-dsDNA, anti-Ro, anti-U1RNP, anti-Sm autoantibodies and lower complement (C3) levels (288). Type I IFN

assays showed association with disease activity in cross-sectional studies (287, 289, 290). However, these were inconsistent with other studies failing to demonstrate any association (291, 292). Longitudinal analyses of ISG expression in SLE patients have also given more complex results. Although patients with higher IFN scores had greater disease activity, scores of individual patients could not predict flares (293). This discrepancy might be due to the choice of ISGs or methods used to derive unidimensional interferon scores from genome-wide micro-array data (294). Some studies have suggested that higher ISG expression is associated with particular organ involvement in SLE. For instance, five type I IFN-inducible genes (*LY6E*, *OAS1*, *OASL*, *MX1*, *ISG15*) were highly expressed in patients with active renal or neurological disease but not in other manifestations (291). However, this is complex to analyse; variations in methodology for measurement of type I IFN activity comparing activity between different organ domains is complex. That study used a categorical measurement for each organ.

Given the pleotropic effects of type I IFNs on all cells, the varying transcriptional response of individual circulating populations may also be important. Although high-density oligonucleotide microarray has proven to be valuable to investigate the genetic mechanism of pathogenesis of SLE, most of these studies used unseparated leukocytes or whole blood (295). A recent study investigated the ISG expression in multiple sorted cell types, including monocytes, dendritic cells, NK cells, B and T lymphocytes, from SLE patients and showed distinct profiles in different cell types (296). A distinct gene expression profile has been recently identified even in classical and non-classical monocytes from SLE patients (297). Genome-wide DNA methylation analyses of $CD4^+$ T cells from SLE patients revealed a persistent

hypomethylation of certain ISGs, for example *IFIT1*, *IFIT3*, *MX1*, *STAT1*, *IFI44L*, *USP18*, *TRIM22*, *BST2*, suggesting that epigenetic modifications could influence the responsiveness of autoreactive T cells (248, 294, 298-300).

IFN signature might contribute to the early stages of the disease development, as the expression of certain genes has been linked to certain autoantibody profiles in patients with incomplete lupus erythematosus, suggesting that IFN signature might be used as a biomarker for individuals with higher risk for disease progression (301). The results confirmed a different IFN signature in peripheral B cells, T cells and myeloid cells leading to the upregulation of distinct transcriptional factors, which favour a pro-inflammatory phenotype. Interestingly, cytosolic nucleic acid sensing pathways were mostly upregulated in myeloid cells.

1.21 Hypothesis

Type I interferon axis is critically implicated in the pathogenesis of the SLE. However, the source of this dysregulation still remains unclear. As pDCs are the main type I IFN producing cells during viral infection, I have hypothesised: (1) pDCs are overactive in SLE producing aberrant amounts of type I IFNs and potentially contributing to disease progression from preclinical benign autoimmunity to clinically overt disease; (2) pDCs are compromised for their function to produce type I IFNs by immune regulatory mechanisms and the source of type I IFN dysregulation is located outside of the immune system, for example in non-haematopoietic tissues.

1.22 Aims

To address the above hypothesis, the current PhD thesis aims:

- To investigate whether pDCs from patients with SLE as well as individuals with preclinical autoimmunity present distinct immunological abnormalities compared to pDCs of healthy individuals.
- To investigate whether certain defects in immune function of pDCs are associated with clinical and immunological features in patients with SLE and individuals with preclinical autoimmunity.
- To investigate whether non-haematopoietic tissue resident cells contribute to type I IFN dysregulation observed in patients with SLE and individuals with preclinical autoimmunity
- To investigate immune mechanisms regulating the function of human pDCs, particularly the effect of TNF-α on IFN- α production.

CHAPTER 2.

MATERIALS AND METHODS

2.1 Patients and controls

Peripheral blood and skin biopsies were obtained from healthy individuals and patients from different disease groups:

1. Systemic Lupus Erythematosus (SLE)
2. Primary Sjögren's Syndrome (pSS)
3. At-Risk individuals (At-Risk)

Patients were recruited based on 2012 SLICC classification criteria for SLE, 2016 ACR/EULAR classification criteria for pSS, while At-Risk individuals were classified as ANA positive, $\leq$1 SLE clinical criterion, symptom duration <12 months and being treatment-naïve. **Table 2.1** summarises the characteristics and treatment of SLE patients.

2.2 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, while blood samples from healthy individuals 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.

Age, median (range) years	44 (18 -76)
Female patients (%)	94
Ethnicity (%)	
Caucasian	74
South Asian	16
East Asian	5
African/Caribbean	5
Clinical symptoms: BILAG Score A/B (%)	
Mucocutaneous	23
Musculoskeletal	23
Haematological	3
Renal	16
Neurological	3
Cardiorespiratory	2
Gastrointestinal	3
Opthalmic	0
General	0
Hydroxychloroquine (%)	83
Other immunosuppressants (%)	
Methotrexate	14
Azathioprine	18
Mycophenolate mofetil	27
Cyclophosphamide	1
Oral steroids (%)	47

Table 2.1 Clinical characteristics and treatment of SLE patients recruited in this study.

2.3 Clinical immunology assessment

ANA was tested using indirect immunofluorescence. 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 β2-Glycoprotein IgGs) was assessed 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.

2.4 Isolation of human peripheral blood mononuclear cells

Human peripheral blood mononuclear cells (PBMCs) were separated from the whole blood by a density gradient centrifugation method using 50 mL LeucoSep tubes (Greiner Bio-One; Cat. No.: 227290P). Fresh human venous blood was collected in EDTA-containing vials. After collection, the tubes were mixed well by gently inverting several times. The blood was gently layered on the top of the porous barrier using an auto-pipette. Then the tubes were centrifuged (without any delay) for 20 min at 800 x g at 20°C in a swing-out bucket. The whitish buffy coat (about 1 ml) of PBMCs formed in the interphase between porous barrier and medium was aspirated and washed with 50 mL of sterile PBS followed by centrifugation in 500 x g for 10 min at 20°C. After centrifugation, the supernatant was decanted carefully and the pellet of PBMCs was washed with 50 mL of sterile filtrated RBC lysis buffer (8.99 g NH_4Cl, 1 g

$KHCO_3$, 2 mL EDTA, 1,000 mL ddH_2O) to lyse any remaining RBCs followed by centrifugation in 500 x g for 10 min at 20°C. Finally, PBMCs were washed with 50 mL of sterile PBS and centrifuged in 500 x g for 10 min at 20 °C.

2.5 Evaluating IFN activity in peripheral blood

2.5.1 RNA isolation from PBMCs

RNA was extracted from freshly isolated PBMCs by using an Animal Tissue RNA Purification Kit (Norgen Biotec; Cat. No.: 25700) according to the manufacturer's instructions. RNA was eluted into 50 µL of the provided buffer; the final concentration of purified RNA was then quantified by using NanoDrop Lite (Labtech International). The purified RNA samples were stored at -80^{o}C.

2.5.2 Gene probe selection

To evaluate IFN activity, interferon-stimulated genes (ISGs) from each one of IFN-annotated modules (M1.2, M3.4, M5.12) of a previous microarray study reported by Chiche *et al.* (294) were selected; additional common ISGs, for instance IFI27 and IFI6, were also included. Therefore, a total of 31 ISGs were evaluated.

2.5.3 Gene quantification

Purified RNA from each sample was reverse-transcribed to cDNA using the Fluidigm Reverse Transcription Master Mix buffer 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

BioMarkTM HD System with appropriate cycling protocols for the 96.96 chip. Data were normalised using *PP1A* as the reference gene to calculate ΔCt.

2.5.4 Factor analysis

Factor analysis is a statistical method that was used to describe the variability among observed, correlated variables in terms of a potentially smaller number of unobserved (latent) continues variables. In this case, the observed variables were the gene expression values (ΔCt) of multiple interferon-stimulated genes (ISGs), which were clustered into only two variables (called factors) explaining the majority of the variability in the data. The factor analysis for all samples collected was kindly performed by Dr Elizabeth Hensor, biostatistician at Leeds Institute of Rheumatic and Musculoskeletal Medicine and it is described in detail in my relevant published work (302). The two factors derived by the factor analysis explained 84% of the variance in the data with limited cross-loading among the ISGs. **Table 2.2** shows the ISGs that contributed to each factor; these factors were named IFN Score A, which comprised 12 co-clustered genes (*ISG15, IFI44, IFI27, CXCL10, RSAD2, IFIT1, IFI44L, CCL8, XAF1, GBP1, IRF7, CEACAM1*), and IFN Score B, which comprised 14 co-clustered genes (*LAMP3, IFIH1, PHF11, SERPING1, IFI16, BST2, SP100, NT5C3B, SOCS1, TRIM38, UNC93B1, UBE2L6, STAT1, TAP1*).

Genes	Modules from previous study using microarray	Rotated Factor Loading	
		IFN Score A	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

Table 2.2 Interferon-stimulated genes used for developing IFN scores to measure IFN activity. Gene expression was measured in PBMCs by TaqMan assays and analysed by factor analysis to reduce the variability in the data acquired. Two factors explained 84% of the variance of the data with limited cross-loading among the ISGs. These factors were named IFN Score A and IFN Score B.

2.6 Evaluating IFN activity in skin biopsies

Skin biopsies (4 mm) were obtained from non-lesional, non-sun-exposed areas (upper back or upper arms) of At-Risk individuals (n=10) and healthy individuals (n=6) as well as 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 was extracted using RNeasy mini kit (Qiagen) according to manufacturer's instructions. The RNA quantity was measured and assessed for quality using NanoDrop spectrophotometer (ND-1000). Gene expression analysis by TaqMan assays and calculation of IFN scores by factor analysis were performed as described above for evaluating IFN activity in peripheral blood.

2.7 Isolation of human plasmacytoid dendritic cells

pDCs were purified from freshly isolated PBMCs by negative selection using the Diamond Plasmacytoid Dendritic Cell Isolation Kit II (Miltenyi Biotec). Briefly, after determining the PBMC number, the cell suspension was centrifuged at 300 x g for 10 minutes before the supernatant was aspirated completely and the cell pellet was resuspended in 400 µL of MACS buffer [PBS, pH 7.2, 0.5% bovine serum albumin (BSA), and 2 mM EDTA] and 100 µL of Non-PDC Biotin-Antibody Cocktail II per 10^8 total cells. The suspension was mixed well and incubated at 4^{o}C for 10 minutes. After incubation, the cells were washed by adding 10 ml of MACS buffer and centrifuged at 300 x g for 10 minutes. The supernatant was then aspirated completely and the cell pellet was resuspended in 400 µL of MACS buffer and 100 µL of Non-PDC Microbead Cocktail II per 10^8 total cells. The suspension was mixed well and

incubated at 4°C for 15 minutes. After incubation, the cells were washed by adding 10 ml of MACS buffer, centrifuged at 300 x g for 10 minutes and resuspended in a final volume of 500 µL of MACS buffer per 10^8 total cells. For depletion of non-PDCs by magnetic separation, LD columns (Cat. No. 130-042-901, Miltenyi Biotec) were placed in the magnetic field of a suitable MACS Separator. The columns were prepared by rinsing 2 mL of MACS buffer and the cell suspension was applied onto the column. The unlabelled cells passing through were collected and the columns were washed with 2 x 1 mL of MACS buffer. Finally, the number of pre-enriched pDCs was counted using an automated cell counter (Beckmann Coulter).

2.8 Isolation of human naïve $CD4^+$ T cells

Naïve $CD4^+$ T cells were purified by negative selection using the Naïve $CD4^+$ T Cell Isolation Kit II (Miltenyi Biotec). Briefly, Briefly, after determining the PBMC number, the cell suspension was centrifuged at 300 x g for 10 minutes before the supernatant was aspirated completely and the cell pellet was resuspended in 40 µL of MACS buffer and 10 µL of Naïve $CD4^+$ T Cell Biotin-Antibody Cocktail II per 10^7 total cells. The suspension was mixed well and incubated at 4°C for 5 minutes before 30 µL of MACS buffer and 20 µL of Naïve $CD4^+$ T Cell MicroBead Cocktail II were added per 10^7 total cells. For magnetic cell separation, LS columns (Cat. No. 130-042-401, Miltenyi Biotec) were placed in the magnetic field of a suitable MACS Separator. The columns were prepared by rinsing 3 mL of MACS buffer and the cell suspension was applied onto the column. The unlabelled cells passing through were collected and the columns were washed with 3 x 1 mL of MACS buffer. Finally, the number of enriched naïve $CD4^+$ T cells was counted using an automated cell counter (Beckmann Coulter).

2.9 Sorting of plasmacytoid dendritic cells

Pre-enriched pDCs were initially purified by negative selection as described above and were finally sorted using an antibody to BDCA-4 (Miltenyi Biotec). Cell sorting was carried out at the SCIF Flow Cytometry and Imaging Facility of the Wellcome Trust Brenner Building, University of Leeds, with a BD Influx 6 Way Cell Sorter (BD Biosciences). Representative sorting images of unstained cells and BDCA-4^{+} pDCs can be seen in **Figure 2.1** and **Figure 2.2** respectively.

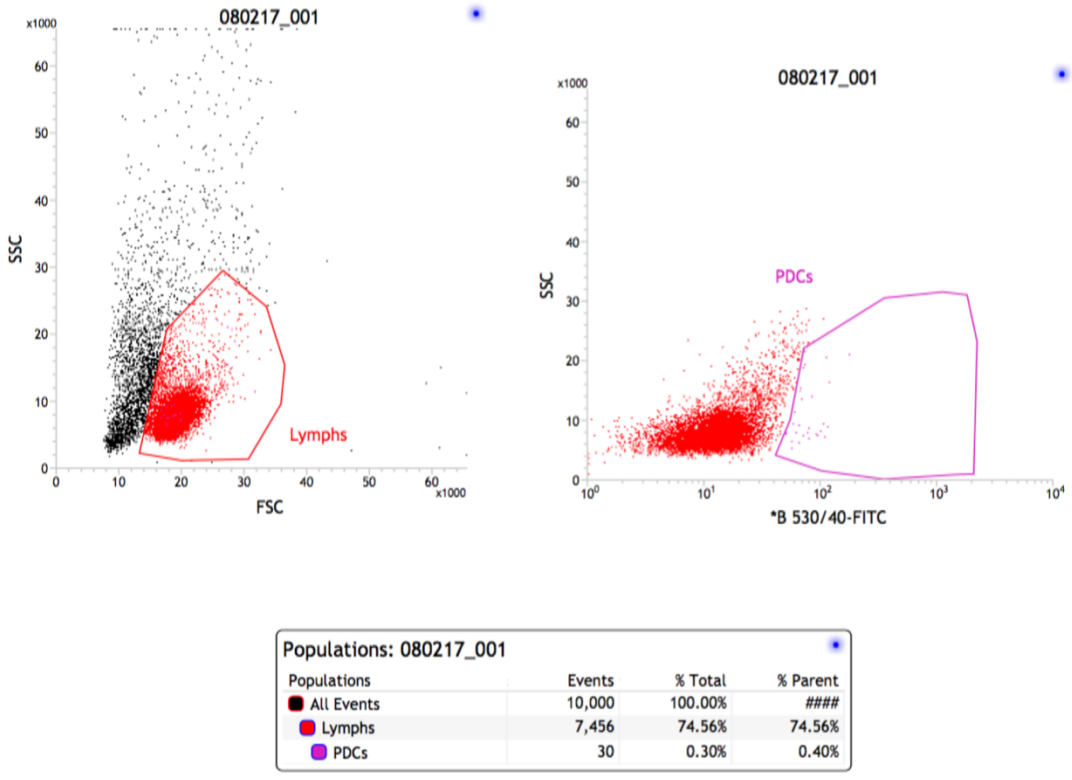


Figure 2.1 Sorting of BDCA-4^{+} cells from pre-enriched pDCs previously purified by negative selection. Representative picture of unstained cells used as a gating control.

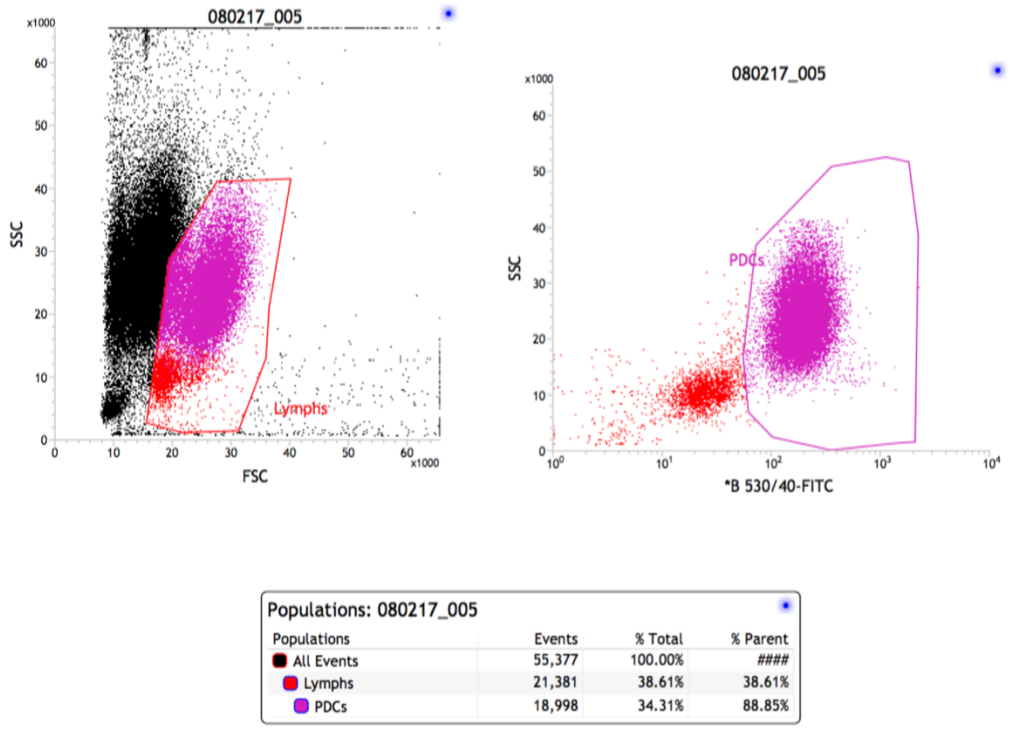


Figure 2.2 Sorting of BDCA-4$^+$ cells from pre-enriched pDCs previously purified by negative selection. Representative picture of a sample.

2.10 Culture and stimulation of plasmacytoid dendritic cells *in vitro*

After isolation of PBMCs, cells were resuspended in RPMI medium 1640 with GlutaMAX supplement (ThermoFisher Scientific) containing 10% (vol/vol) FBS and 100 U/ml penicillin/streptomycin. The cells were then seeded into 96-well polystyrene round bottom plates (2 x 10^6 cells per well, 100 µL per well). TLR9 (ODN2216; Miltenyi Biotec) or TLR7 (ORN R-2336; Miltenyi Biotec) agonists were used at concentration of 2 µM to stimulate pDCs within the context of PBMCs. The stimulated cells were incubated for 2 hours at 37°C with 5% CO_2 before GolgiPlug (BD Biosciences) at concentration of 10 µg/mL was added to inhibit cytokine secretion. The cells were incubated for additional 4 hours at 37°C with 5% CO_2.

2.11 Co-culture of pDCs and T cells *in vitro*

For pDC/T-cell co-culture, pDCs (1 x 10^4) purified by negative selection were cultured for 5 days with autologous or allogeneic naïve $CD4^+$ T cells (5 x 10^4) purified by negative selection in the absence or presence of anti-CD3/CD28 beads (T cell activation/expansion kit; Miltenyi Biotec). Briefly, Anti-Biotin MACSiBead Particles were resuspended thoroughly by vortexing to obtain a homogenous suspension. Then 500 µL of Anti-Biotin MACSiBead Particles (1 x 10^8) were mixed with 100 µL of CD3-Biotin and 100 µL of CD28-Biotin and 300 µL of buffer [PBS pH 7.2, supplemented with 0.5% human serum albumin (HSA) and 2 mM EDTA] to adjust to a total volume of 1 mL. The mixture was incubated for 2 hours at 4°C under constant, gentle rotation at approximately 4 rpm. The desired amount of Anti-Biotin MACSiBead Particles was added to the cell culture to achieve a bead-to-cell ratio of 1:2.

On the last day of culture, cells were re-stimulated with PMA (500 ng/mL) and Ionomycin (1 µl/mL) for 6 hours in the presence of GolgiPlug (BD Biosciences) in the last 4 hours at concentration of 10 µg/mL to prevent cytokine secretion. Cytokine production was measured by intracellular staining following the protocol described in the flow cytometry analysis.

2.12 T cell proliferation

Cell proliferation was measured using the CellTrace Violet Cell Proliferation kit (ThermoFisher Scientific) according to the manufacturer's instructions. Briefly, CellTrace Violet stock solution was prepared immediately prior to use by adding 20 µL of DMSO to one vial of CellTrace Violet reagent and mixing well. Then 1 µL of

CellTrace Violet stock solution in DMSO was added to 1 mL of cell suspension in PBS for a final working solution, which was incubated for 20 minutes at 37°C protected from light. After incubation, 5 mL of RPMI medium 1640 with GlutaMAX supplement (ThermoFisher Scientific) containing 10% (vol/vol) FBS and 100 U/ml penicillin/streptomycin was added to the cells and incubated for further 5 minutes to remove any free dye remaining in the solution. The suspension was then centrifuged at 300 x g for 10 minutes, the supernatant was decanted carefully and the cell pellet was resuspended in fresh pre-warmed culture medium. The cells were incubated for 5 days before they were analysed for their proliferation using flow cytometry.

2.13 Human TNF-α neutralisation

Pre-enriched pDCs were stimulated with ODN 2216 (1 ng/mL) or ORN R-2336 (1 ng/mL) in the presence or absence of human TNF-α antibody (R&D Systems). After 24 hours, the plates were centrifuged to collect the supernatants and the cells were washed twice before they were stimulated again with ODN 2216 (1 ng/mL) or ORN R-2336 (1 ng/mL) for additional 24 hours. Supernatants collected at 24 and 48 hours were analysed by Human IFN-alpha Platinum ELISA Kit (eBioscience) according to the manufacturer's protocol.

2.14 Flow cytometry analysis

2.14.1 Surface staining

Following 6 hours of incubation, the cells were washed with sterile PBS and centrifuged at 500 x g at 20°C for 5 minutes. The supernatant was then decanted, the

pellet was resuspended in 50 µL of blocking buffer (20% mouse serum) and incubated for 15 minutes at 4°C. After incubation with blocking buffer, 5 µL of each monoclonal antibody for surface proteins were added in a total volume of 100 µL per well. The cells were incubated for 30 minutes at 4°C and were then washed with 200 µL of PBS and centrifuged at 500 x g at 20°C for 5 minutes. The washing step was repeated twice. Finally, the cells were resuspended in 300 µL of FACS buffer (x1 PBS, 1% BSA, 1% Sodium Azide Solution) and analysed by flow cytometry.

2.14.2 Intracellular staining

After following the protocol for surface staining, an Intracellular Fixation & Permeabilization Buffer Set (Affymetrix eBioscience; Cat. No.: 88-8824-00) was used. The kit included both fixation and permeabilization solution (containing formaldehyde) and 10x permeabilization/wash buffer (containing saponin). Each well containing 2 x 10^6 cells was resuspended in 200 µL of fixation and permeabilization solution according to manufacturer's instructions and incubated for 30 minutes at 4°C. The cells were then washed with permeabilization/wash buffer, centrifuged (300 x g at 4°C) for 10 minutes, and resuspended in a total volume of 100 µL containing permeabilization/wash buffer and antibodies for detection of intracellular proteins as well as appropriate isotype controls. The cells were incubated for 30 minutes at 4°C before they were washed with 200 µL of permeabilization/wash buffer twice (centrifugation at 300 x g for 10 minutes at 4°C). Finally, the cells were resuspended in 300 µL of FACS buffer (x1 PBS, 1% BSA, 1% Sodium Azide Solution) and analysed by flow cytometry.

For FoxP3 intracellular staining, cells were first stained for surface markers and then fixed and permeabilised using the FoxP3 Staining Buffer Set (Miltenyi Biotec)

according to manufacturer's instructions. Briefly, after surface staining up to 10^6 cells were resuspended in 1 mL of cold, freshly prepared Fixation/Permeabilization solution and mixed well. After incubation of 30 minutes in the dark at 4°C, the cells were washed with the appropriate buffer and centrifuged at 300 x g for 5 minutes at 4°C. The supernatant was aspirated completely and the cells were washed by adding 1 mL of cold 1x Permeabilization Buffer and centrifuged again at 300 x g for 5 minutes at 4°C. The supernatant was aspirated completely and the cells were resuspended in 80 µL of cold 1x Permeabilization Buffer and 20 µL of FcR Blocking Reagent; after incubation of 5 minutes, 10 µL of Anti-FoxP3 antibody were added and the cells were further incubated for 30 minutes in the dark at 4°C. The cells were then washed by adding 1 mL of cold 1x Permeabilization Buffer and centrifuged at 300 x g for 5 minutes at 4°C. After aspirating the supernatant completely, the cell pellet was resuspended in 300 µL of FACS buffer and analysed by flow cytometry.

2.14.3 Data acquisition and analysis

Flow cytometry data acquisition was performed on LSRII (BD Biosciences) or Cytoflex S (Beckman Coulter) and the data were further analysed using FACS DiVA (BD Biosciences) or CytExpert (Beckman Coulter) software.

2.15 RNA-sequencing data generation

RNA from sorted pDCs was extracted using PicoPure RNA Isolation Kit (ThermoFisher Scientific) according to manufacturer's instructions and quantified using Qubit RNA HS Assay Kit (Thermo Fisher Scientific). RNA libraries were made by using SMART-Seq V4 ultra low Input RNA Kit (Takara Bio USA) and Nextera XT DNA Library Preparation Kit (Illumina) for NGS sequencing. Indexed sequencing libraries were pooled and

sequenced on a single lane on HiSeq 3000 instrument as 151bp paired-end reads. Pooled sequence data was then demultiplexed using Illumina bcl2fastq software allowing no mismatches in the read index sequences.

2.16 RNA-sequencing data processing and analysis

Raw paired-end sequence data in Fastq format was initially analysed using FastQC software in order to identify potential issues with data quality. Cutadapt software was then used to remove poor quality bases (Phred quality score <20) and contaminating technical sequences from raw sequenced reads. Contaminating technical sequences identified at the initial QC stage were as follows:

CTGTCTCTTATA – Next Era Transposase Sequence

GTATCAACGCAGAGTACT– SmartSeq Oligonucleotide Sequence

dT30 – SmartSeq 3’ CDS Primer II sequence

Reads trimmed to fewer than 30 nucleotides and orphaned mate-pair reads were discarded to minimise alignment errors downstream.

Reads were aligned to human hg38 analysis set reference sequences, obtained from UCSC database (303) using splicing-aware STAR aligner (304) for RNA-Sequencing data. STAR aligner was run in 2-pass mode, with known splice junctions supplied in GTF file format, obtained from hg38 RefSeq gene annotation table from UCSC database using Table Browser tool (305). The resulting alignments in BAM file format were checked for quality using QualiMap software (306) and Picard tools (307). Picard tools were used to mark PCR/Optical duplicate alignments. Custom code was used to filter out contaminating ribosomal RNA alignments, using ribosomal RNA coordinates for hg38 analysis set reference obtained using UCSC Table Browser tool.

The final alignment files were sorted and indexed using Samtools software (308) and visualised using IGV browser (309).

Bioconductor R package RSubread (310) was used to extract raw sequenced fragment counts per transcript using RefSeq hg38 transcript annotation set, as before. Paired-end reads were counted as a single fragment and multi-mapping read pairs were counted as a fraction of all equivalent alignments. Raw count data was normalised for library size differences using median ratio method (311), as implemented in DESeq2 R Bioconductor package (312). DESeq2 was also used to perform additional data QC steps and differential expression analyses. Differentially expressed gene expression was visualised as clustered heatmaps using Pheatmap R package (313) using log-transformed normalised gene expression values as input. Gene functional and pathway enrichment analyses were performed using R Bioconductor packages clusterProfiler (314) and ReactomePA (315). Additionally, KEGG (316) pathways were visualised using Pathview package (317).

2.17 Measurement of relative telomere length

Purification of human pDCs from freshly isolated PBMCs was carried out following the protocol for negative selection (Miltenyi Biotec) described in detail above. To achieve maximum purity (> 95%) for pDCs, an additional step of positive selection was added to the protocol. Briefly, the cell suspension of pre-enriched fraction of pDCs collected by negative selection was centrifuged at 300 x g for 10 minutes at 4^{o}C before the supernatant was aspirated completely and the cell pellet was resuspended directly in 100 µL of CD304 (BDCA-4/Neuropilin-1) Diamond MicroBeads. The suspension was mixed well and incubated for 15 minutes in the dark

at 4^{o}C. After incubation, the cells were washed by adding 1 mL of cold MACS buffer and centrifuged at 300 x g for 10 minutes at 4^{o}C. The supernatant was aspirated completely and the cell pellet was resuspended in 500 µL of MACS buffer. In the meantime, MS columns (Miltenyi Biotec) were placed in the magnetic field of a suitable MACS Separator. The columns were prepared by rinsing 2 mL of MACS buffer and the cell suspension was applied onto the column. The flow-through containing the unlabelled cells was collected and the columns were washed with 2 x 1 mL of MACS buffer. Then the column was removed from the separator and it was placed immediately on a suitable collection tube. The column reservoir was added with 1 mL of MACS buffer and the magnetically labelled cells were flushed out by firmly pushing the plunger into the column. Finally, the number of BDCA-4^{+} pDCs was counted using an automated cell counter (Beckmann Coulter).

Relative telomere length was measured using Telomere PNA Kit/FITC for Flow Cytometry (Agilent) according to manufacturer's protocol. Briefly, on a single cell suspension consisting of purified pDCs and control cells (1301 cell line; Sigma-Aldrich), the sample DNA was denatured for 10 minutes at 82^{o}C either in the presence of hybridization solution without probe or in hybridization solution containing fluorescein-conjugated PNA telomere probe. Then hybridization took place in the dark at room temperature overnight. The sample was then resuspended in appropriate buffer for further flow cytometric analysis. The data obtained were used for determination of the relative telomere length (RTL) as the ratio between the telomere signal of each sample (pDCs) and the control cell (1301 cell line) with correction for the DNA index (which is to be 1 for known diploid cells such as human pDCs and 2 for the tetraploid cell line used as a control) of $G_{0/1}$ cells:

$$\text{RTL} = \frac{(\text{mean FL1 sample cells with probe} - \text{mean FL1 sample cells withour probe}) \text{x DNA index of control cells x100}}{(\text{mean FL1 control cells with probe} - \text{mean FL1 control cells withour probe}) \text{x DNA index of sample cells}}$$

2.18 Oxidative stress assay

Freshly isolated PBMCs from healthy donors were exposed to H_2O_2 (0 – 500 µM) for 15 minutes. After exposure, cells were washed thoroughly and resuspended at 1 x 10^6 in culture medium at 37^{o}C before they were stimulated with 2µM ODN 2216 (Miltenyi Biotech) for 6 hours. GolgiPlug (BD Biosciences) at concentration of 10 µg/mL was added in the last 4 hours of culture to inhibit cytokine secretion. The production of IFN-α in viable pDCs was measured by intracellular staining as described above in flow cytometry analysis. The viability of the cells was assessed using 7-AAD (7-amino-actinomycin D; Miltenyi Biotec), which is excluded from viable cells, but can penetrate cell membranes of dead or dying cells

2.19 UV provocation

UV provocation was performed based on a published protocol designed for use in clinical trials (318, 319). Briefly, a solar simulator was used in routine clinical practice, which replicated the protocol of UV-A and UV-B provocation in a single exposure. On day 1, four 1.5 cm^2 areas of skin were exposed to solar simulated radiation depending on skin type; 4, 8, 12, 16 J/cm^2 for skin types I and II, and 6, 12, 18, 24 J/cm^2 for skin types III-VI. On day 2, the minimal erythema dose was then determined. A 10 cm^2 non-sun exposed area of skin was exposed to minimal erythema dose x 1.5 on three consecutive days. A biopsy of the pre-exposed and exposed area of skin was obtained

when a reaction was seen clinically (mean time to a positive reaction to provocation was 7 (±6) days, and rarely more than 14 days).

2.20 Tissue section

Skin biopsies were obtained from healthy individuals and patients, then snap frozen in liquid nitrogen within 5 minutes, embedded in OCT and stored in -80^{o}C freezer. Fresh frozen skin biopsies were cryosectioned to 10-20 µM, placed on superfrost plus slides (Thermo Scientific) and used for *in situ* hybridization.

2.21 *In situ* hybridization and fluorescence microscopy

In situ hybridization of type I IFNs transcripts in skin samples was performed using RNAscope Multiplex Fluorescent Reagent Kit v2 (Advanced Cell Diagnostics) according to manufacturer's instructions. Before hybridization of the tissue, cryosections were fixed and dehydrated before they were exposed to hydrogen peroxide and protease treatment. Negative and positive controls for hybridization were provided by the manufacturer. A schematic procedure of the RNAscope assay can be seen in **Figure 2.3**.

In brief, materials were prepared by warming 50X Wash Buffer for 10–20 minutes to remove any precipitation and preparing 3 L of 1X Wash Buffer by adding 2.94 L distilled water and 1 bottle (60 mL) of 50X Wash Buffer to a large carboy. Probes were warmed up for 10 minutes at 40°C, then cooled down to room temperature, before they were mixed and added to the tissue section. To hybridize the probes, excess liquid was initially removed from the tissue slides, which were placed in the HybEZ Slide Rack, and then 4–6 drops of the probe mix were added to entirely cover each

slide. The rack containing the slides was inserted into the HybEZ Oven for 2 hours at 40°C and then the slides were washed twice with 1X Wash Buffer for 2 minutes at room temperature. The slides were then stored overnight at room temperature in saline-sodium citrate buffer (5x; 0.75M NaCl, 0.075M sodium citrate).

After the overnight incubation, hybridize amplification 1 was applied by adding 4-6 of Amp 1 and by further incubating in the HybEZ Oven for 30 minutes at 40°C before the slides were washed twice in 1X Wash Buffer for 2 MIN at room temperature. The same amplification step was applied for hybridize amplification 2. The reconstitution of TSA® Plus fluorescein and TSA® Plus Cyanine 3 stocks following Perkin Elmer's TSA® Plus System instructions; the TSA® Plus fluorophore stocks were diluted in the TSA buffer provided in the RNAscope® Multiplex Fluorescent Kit V2 (1:1,500).

The next step was the development of HRP-C1 and HRP-C2 signal. The excess liquid was removed from slides, which were placed in the HybEZ Slide Rack and 4–6 drops of RNAscope® Multiplex FL V2 HRP-C1 were added to entirely cover each slide. The slides were incubated in the HybEZ Oven for 15 minutes at 40°C before they were washed twice in 1X Wash Buffer for 2 minutes at room temperature. The excess liquid was removed from slides again, they were placed in the HybEZ Slide Rackand 4-6 drops of RNAscope® Multiplex FL V2 HRP blocker were added to entirely cover each slide. Then the slides into the HybEZ Oven for 15 minutes at 40°C and washed twice in 1X Wash Buffer for 2 minutes at room temperature. The exactly same step was applied for RNAscope® Multiplex FL V2 HRP-C2.

Finally, the excess liquid was removed from slides and ~4 drops of DAPI were added to each slide before they were incubated for 1-2 minutes at room temperature. The DAPI was removed and 1–2 drops of Prolong Gold antifade mounting medium was

immediately added on the slide. A 24 mm x 50 mm glass coverslip was carefully placed over each tissue section avoiding trapping air bubbles. Then the slides were dried overnight in the dark before they were stored in the dark at 2–8°C.

Images were acquired on a Nikon A1R confocal laser scanning microscope system at 20-40x magnification. Images were analysed in Nikon NIS Elements software. Representative images of negative and positive controls can be seen in **Figure 2.4**

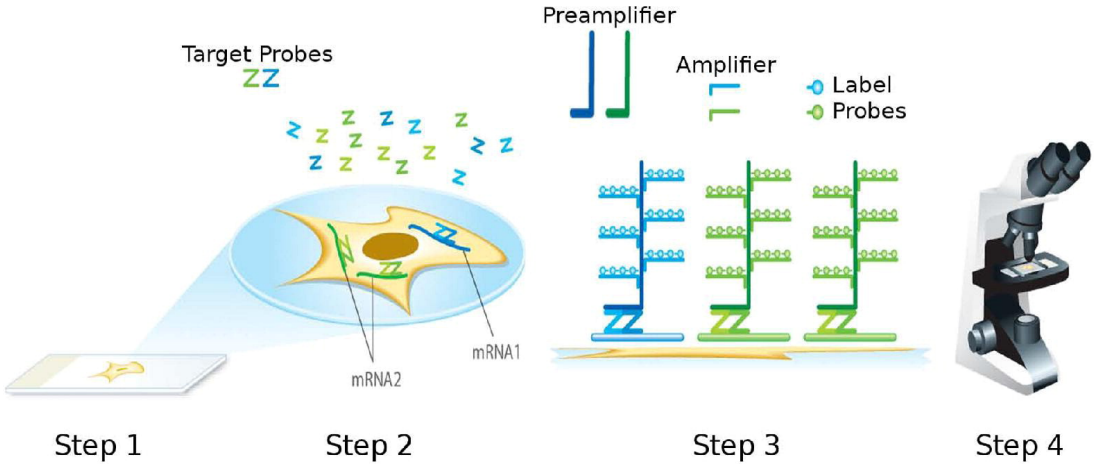


Figure 2.3 Schematic procedure of the RNAscope assay. In step 1, tissues are fixed and permeabilised to allow for target probe access. In step 2, target RNA-specific oligonucleotide probes (Z) are hybridized in pairs (ZZ) to multiple RNA targets. In step 3, multiple signal amplification molecules are hybridized, each recognising a specific target probe, and each unique label probe is conjugated to a different fluorophore or enzyme. In step 4, signals are detected using a fluorescent microscope. Picture and text adapted from (320).

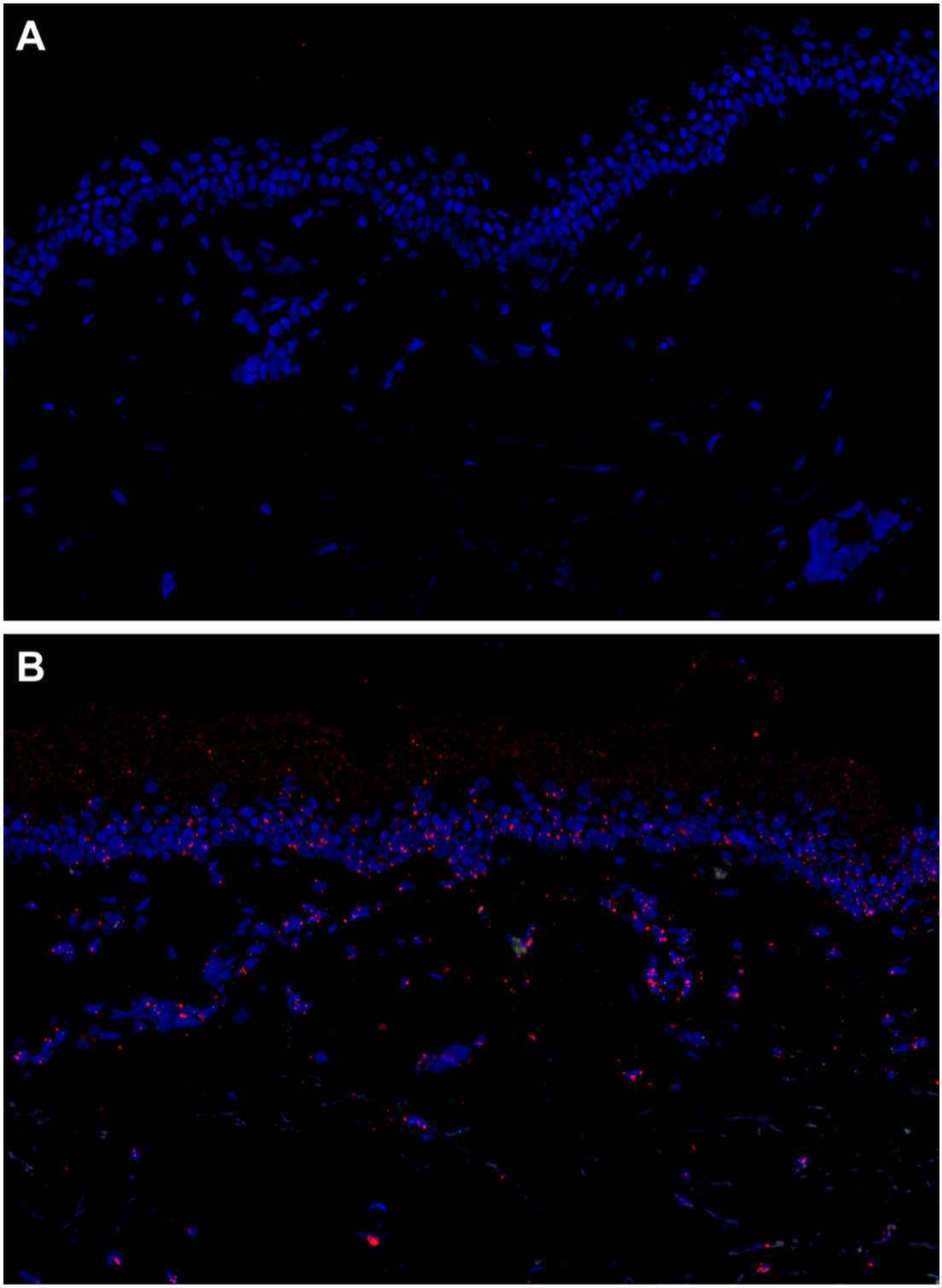


Figure 2.4 Representative pictures of *in situ* hybridization using RNAscope Multiplex Fluorescent Reagent Kit v2: (A) negative control; (B) positive control (unknown transcript; provided by the manufacturer).

2.22 Culture of human keratinocytes and dermal fibroblasts

Human keratinocytes and dermal fibroblasts were isolated from 3 mm punch skin biopsies. For keratinocytes, the epidermal component of the biopsy was placed in a T75 flask and cultured at 37^{o}C in low glucose DMEM (Fischer Scientific) containing 10% (vol/vol) FBS (Fischer Scientific) and 1% penicillin/streptomycin. Keratinocytes were passaged and sub-cultured into keratinocyte growth medium (PromoCell) for continuous culture. For dermal fibroblasts, the dermal compartment of the biopsy was placed in a T25 flask and cultured at 37^{o}C in low glucose DMEM. Dermal fibroblasts were then passaged and sub-cultured. Both keratinocytes and dermal fibroblasts were passaged and plated in 24-well plates for subsequent stimulation. At 90% confluence, cells were either untreated or treated with 1 µg/ml Poly I:C (InvivoGen) or 100 ng/ml Poly dA:dT (InvivoGen) for 6 or 24 hours.

2.23 Quantitative RT-PCR for keratinocytes and dermal fibroblasts

RNA was extracted from keratinocytes and dermal fibroblasts using Quick-RNA MiniPrep kit (Zymo Research) according to manufacturer's instructions. Extracted RNA was reverse transcribed using First Strand cDNA Synthesis kit (ThermoFisher). The cDNA was then used in qRT-PCR assay using QuantiFast SYBR Green PCR kit (Qiagen). For the assay, the following quantitech primers were used: *IFNK* (QT00197512; Qiagen), *IFNB1* (QT00203763; Qiagen), *IFNL1* (QT00222495; Qiagen), *IFNA2* (QT00212527; Qiagen), *U6snRNA* (forward—5'-CTCGCTTCGGCAGCACA-3'; reverse—5'-AACGCTTCACGAATTTGC-3'; Sigma-Aldrich). For gene expression analysis, ddCt method was used and all samples were normalised to the housekeeping gene (*U6snRNA*).

2.24 Statistical analysis

Statistical analyses were carried out with Prism software (GraphPad). Continuous variables were compared using either Student's T test or ANOVA followed by pairwise Tukey tests. Pearson's correlation was used for associations. A p value of ≤ 0.05 was considered significant (ns, not significant; *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001). In all figures, error bars indicate SEM.

2.25 Key resources table

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-human CD3 clone BW264/56, VioBlue	Miltenyi Biotec	Cat# 130-094-363
Anti-human CD3 clone BW264/56, VioGreen	Miltenyi Biotec	Cat# 130-096-910
Anti-human CD4 clone M-T466, APC-Vio770	Miltenyi Biotec	Cat# 130-100-457
Anti-human CD19 clone LT19, VioBlue	Miltenyi Biotec	Cat# 130-098-598
Anti-human CD14 clone TÜK4, VioBlue	Miltenyi Biotec	Cat# 130-094-364
Anti-human CD56 clone B159, BV450	BD Biosciences	Cat# 560360
Anti-human CD11c clone MJ4-27G12, VioBlue	Miltenyi Biotec	Cat# 130-097-328
Anti-human HLA-DR clone AC122, APC-Vio770	Miltenyi Biotec	Cat# 130-104-200
Anti-human CD123 clone AC145, PerCP-Vio700	Miltenyi Biotec	Cat# 130-103-802
Anti-human CD303 (BDCA-2) clone AC144, FITC	Miltenyi Biotec	Cat# 130-090-510
Anti-human CD304 (BDCA-4) clone AD5-17F6, VioBright FITC	Miltenyi Biotec	Cat# 130-104-272
Anti-human CD85g clone REA100, PE-Vio770	Miltenyi Biotec	Cat# 130-099-009
Anti-human CD85j clone GHI/75, PE-Vio770	Miltenyi Biotec	Cat# 130-101-552
Anti-human CD69 clone FN50, FITC	Miltenyi Biotec	Cat# 130-092-166
Anti-human CD25 clone 4E3, PE	Miltenyi Biotec	Cat# 130-091-024
Anti-human CD317 clone RS38E, PE	BioLegend	Cat# 348406
Anti-human IFN-α clone LT27:295, APC	Miltenyi Biotec	Cat# 130-092-602
Anti-human TNF-α cA2, PE-Vio770	Miltenyi Biotec	Cat# 130-096-755
Anti-human IL-6 clone MQ2-13A5, PE	Miltenyi Biotec	Cat# 130-096-086

Anti-human IFN-γ clone 45-15, APC	Miltenyi Biotec	Cat# 130-091-640
Anti-human IL-10 clone JES3-9D7, PE	Miltenyi Biotec	Cat# 130-096-043
Anti-human TNF-α clone Mab11, APC/Cy7	BioLegend	Cat# 502944
Anti-human IFN-γ clone 4S.B3, PE/Cy7	BioLegend	Cat# 502528
Anti-human IL-17A clone BL168, APC	BioLegend	Cat# 512334
Anti-human TLR9 clone eB72-1665, APC	BD Biosciences	Cat# 560428
Anti-human TLR7 clone 533707, PE	R&D	Cat# IC5875P
Anti-human FoxP3 clone 3G3, APC	Miltenyi Biotec	Cat# 130-093-013
Human TNF alpha antibody clone 1825	R&D Systems	Cat# MAB210-SP
Chemicals, Peptides, and Recombinant Proteins		
ODN 2216	Miltenyi Biotec	Cat# 130-100-243
ORN R-2336	Miltenyi Biotec	Cat# 130-104-431
Human IL-3, premium grade	Miltenyi Biotec	Cat# 130-095-071
Human TNF-α, premium grade	Miltenyi Biotec	Cat# 130-094-014
Hydrogen peroxide solution [30% (w/w) in H_2O_2]	Sigma-Aldrich	Cat# H1009
Prolong Gold Antifade Mountant	ThermoFischer Scientific	Cat# P36930
Poly(I:C) LMW	InvivoGen	Cat# 31852-29-6
Poly(dA:dT)	InvivoGen	Cat# 86828-69-5
TaqMan Universal PCR master Mix	ThermoFischer Scientific	Cat# 4304437
SYBR Green PCR Master Mix	ThermoFischer Scientific	Cat# 4309155
T cell activation/expansion kit	Miltenyi Biotec	Cat# 130-091-441
7-AAD Staining Solution	Miltenyi Biotec	Cat# 130-111-568
Critical Commercial Assays		
Diamond Plasmacytoid Dendritic Cell Isolation Kit II	Miltenyi Biotec	Cat# 130-097-240
Naive $CD4^+$ T Cell Isolation Kit II	Miltenyi Biotec	Cat# 130-094-131
Intracellular Fixation & Permeabilization Buffer Set	eBioscience	Cat# 88-8824-00
FoxP3 Staining Buffer Set	Miltenyi Biotec	Cat# 130-093-142
CellTrace Violet Cell Proliferation Kit	ThermoFischer Scientific	Cat# C34571
Total RNA Purification Kit	Norgen Biotek	Cat# 17200

PicoPure RNA Isolation Kit	ThermoFischer Scientific	Cat# KIT0204
Qubit RNA HS Assay Kit	ThermoFisher Scientific	Cat# Q32852
SMART-Seq V4 ultra low Input RNA Kit	Takara Bio	Cat# 634888
Nextera XT DNA Library Preparation Kit	Illumina	Cat# FC-131-1024
Telomere PNA Kit/FITC for Flow Cytometry	Agilent	Cat# K532711-8
Superfrost plus slides	ThermoFisher Scientific	Cat# J1800AMNT
RNAscope Multiplex Fluorescent Reagent Kit v2	Advanced Cell Diagnostics	Cat# 323100
TSA Cy 3, Cy 5, TMR, Fluorescein Evaluation Kit	Perkin Elmer	Cat# NEL760001KT
RevertAid First Strand cDNA Synthesis Kit	ThermoFisher Scientific	Cat# K1622
QuantiFast SYBR Green PCR Kit	Qiagen	Cat# 204054
Quick-RNA MiniPrep Kit	Zymo Research	Cat# R1055A
Human IFN alpha Platinum ELISA	Invitrogen	Cat# BMS216
Experimental Models: Cell Lines		
Human: 1301 cell line	Sigma-Aldrich	Cat# 0105161
Oligonucleotides		
U6snRNA Primer: Forward 5'-CTCGCTTCGGCAGCACA-3'Reverse 5'-AACGCTTCACGAATTTGC-3'	Sigma-Aldrich	N/A
IFNK Primer	Qiagen	Cat# QT00197512
IFNA Primer	Qiagen	Cat# QT00212527
IFNB1 Primer	Qiagen	Cat# QT00203763
IFNL1 Primer	Qiagen	Cat# QT00222495
Software and Algorithms		
Prism 7	Graphpad Software, Inc.	https://www.graphpad.com/
FACS DiVA	BD Biosciences	http://www.bdbiosciences.com/
CytExpert 2.0	Beckman Coulter	https://www.beckman.com/
Fluidigm Real Time PCR Analysis	Fluidigm	https://www.fluidigm.com/software

FastQC	BaseSpace Labs	https://www.illumina.com/
Cutadapt	Cutadapt	http://cutadapt.readthedocs.io/en/stable/index.html/
Table Browser	UCSC Genome Browser	https://genome.ucsc.edu/
QualiMap 2.0	QualiMap	http://qualimap.bioinfo.cipf.es/
Picard tools	Broad Institute	https://broadinstitute.github.io/picard/
ReactomePA	Bioconductor	https://www.bioconductor.org/
KEGG	Kyoto University	https://www.genome.jp/kegg/
Nikon NIS Elements	Nikon Instruments	https://www.nikoninstruments.com/
Other		
50mL Leucosep Tubes	Greiner Bio-One	Cat# 89048-938

Table 2.3 Key reagents and resources used for experiments and data analysis.

CHAPTER 3.

TYPE I INTERFERON REGULATION IN PRECLINICAL AUTOIMMUNITY AND SYSTEMIC LUPUS ERYTHEMATOSUS

3.1 Introduction

Systemic lupus erythematosus (SLE) and related conditions represent a group of autoimmune connective tissue diseases (CTDs) characterised by the breakdown of immune tolerance and systemic inflammation (97). Clinical symptoms and severity may significantly vary among patients with SLE, but an immune response against endogenous nuclear antigens as well as other immune dysregulation are central in disease pathogenesis (188). Although the presence of anti-nuclear antibodies (ANA) may precede clinical symptoms by years, only a minority of individuals at this stage of preclinical benign autoimmunity will eventually progress to clinically overt disease and develop irreversible end-organ tissue damage (108).

Alongside ANA positivity, the dysregulation of type I IFN axis has been recognised as a common feature in multiple autoimmune rheumatic diseases, predominantly SLE (172, 321). A lot of lupus susceptibility genes are related to IFN pathway, while the risk haplotypes in *IRF5* and *IRF7* are associated with increased IFN activity and specific autoantibodies (198, 200, 203, 204). Increased levels of serum IFN-α were described in patients with SLE more than 30 years ago, while 60 – 80% of SLE patients can exhibit increased expression of interferon-stimulated genes (ISGs) in their peripheral blood, as described by IFN signatures or scores (189, 287, 290, 322). Overall, a higher

IFN signature or score was often associated with higher disease activity, whilst some studies suggested an association with particular organ involvement in SLE (288, 291, 294, 322). Notably, increased type I IFN activity was observed in individuals with preclinical autoimmunity, particularly in those who progressed to clinical disease, suggesting that early activation of IFN pathways could be of pivotal importance in disease initiation (301, 323).

Despite the increased interest in the role of type I IFNs in the pathogenesis of SLE, the source of this dysregulation still remains elusive. The majority of both haematopoietic and non-haematopoietic cells are capable of producing type I IFNs (IFN-α, -β, -κ, -ω, -ε) as first line of defence against viral infections. Plasmacytoid dendritic cells (pDCs) are thought to be the professional IFN-α-producing cells upon recognition of nucleic acids via intracellular toll-like receptors, particularly TLR7 and TLR9 (119, 132). Engagement of TLRs within endosomal compartments with appropriate ligands can lead to activation of IRF7 and NFκB pathways and eventually production of IFN-α and pro-inflammatory cytokines (TNF-α, IL-6) (135). Apart from the secretory function, pDCs exhibit antigen-presentation properties potentially leading to T cell activation (149). TLR-activated pDCs can promote Th1 and Th17 differentiation (151, 153). On the other hand, unstimulated or HIV-stimulated pDCs can induce tolerogenic immune responses promoting T_{reg} differentiation (159, 161, 162). In the context of autoimmunity, nucleic acids from common viruses or alternatively self-DNA or self-RNA forming immune complexes with autoantibodies were proposed as possible stimuli for pDC activation (134, 168, 174, 324). However, more recent data suggested that pDCs might not contribute to type I IFN activity seen in SLE as previously thought (325). Experimental work on interferon-mediated

autoimmune disorders (interferonopathies) suggested that type I IFN responses could initially emerge in epithelial tissues, which in turn could promote the development of autoreactive T cell and B cell clones and systemic inflammation (326).

3.2 Results

3.2.1 High IFN activity in peripheral blood characterises preclinical autoimmunity and SLE

The type I IFN activity was evaluated based on expression of multiple ISGs measured by TaqMan assays and further analysed by factor analysis as described in detail in Materials and Methods. Factor analysis indicated two distinct sets of genes (IFN Score A and IFN Score B), which explained > 80% of the variability in the data and associated with different features in patient groups. At baseline, IFN Score A differed among healthy controls, At-Risk individuals and SLE patients ($P < 0.001$). IFN Score A was found to be significantly higher in both At-Risk individuals [n = 105; FD (95% CI) 2.21 (1.22, 4.00), $P = 0.005$] and SLE patients [n = 114; 7.81 (4.33, 14.04), $P < 0.001$] compared to healthy controls (n=49); it was also increased in SLE patients relative to At- Risk individuals [3.54 (2.22, 5.63), $P < 0.001$]. Regarding IFN Score B, although it differed among groups overall (F = 63.35; $P < 0.001$), it did not show any difference in expression level between At-Risk individuals and healthy controls [0.98 (0.66, 1.46), $P = 0.993$]. However, IFN Score B was increased in SLE patients compared to both healthy controls [3.85 (2.60, 5.72), $P < 0.001$] and At-Risk individuals [3.93 (2.87, 5.37), $P < 0.001$]. The summary of the results can be seen in **Figure 3.1**.

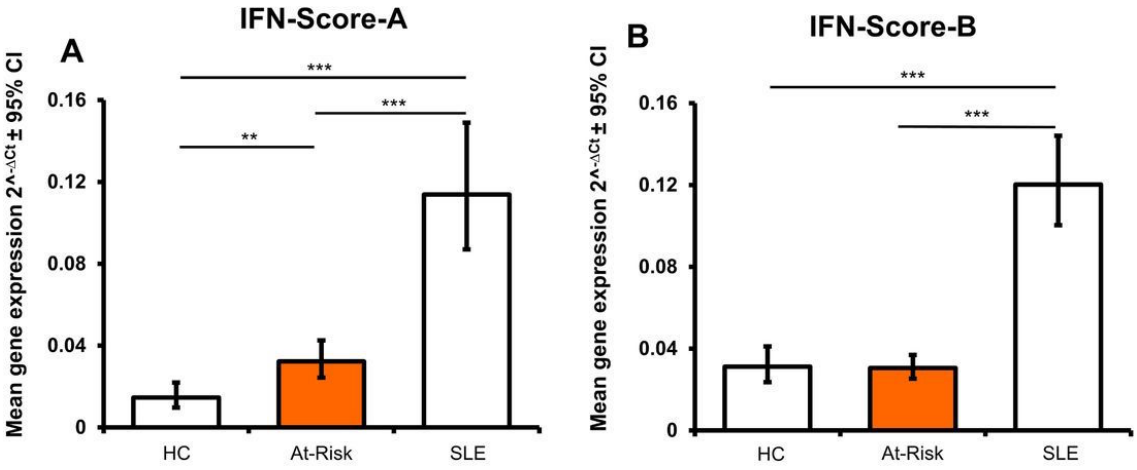


Figure 3.1 Expression of IFN scores at baseline in At-Risk individuals and patients with established SLE. (A) Baseline expression of IFN Score A was higher in patients with SLE and At-Risk individuals compared with healthy controls. (B) For IFN Score B, only patients with SLE had an increased expression; At-Risk individuals showed no statistically significant difference compared to healthy controls. $^{**}P < 0.01$; $^{***}P < 0.001$.

After 12 months of follow up, At-Risk individuals were divided into two groups according to their status of progression to an autoimmune connective tissue disease. Both IFN Score A and B differed among the groups overall ($P < 0.001$) with both scores to be significantly higher in At-Risk individuals who eventually progressed (progressors; n = 19) versus in those who did not progress (non-progressors; n = 86). Nevertheless, IFN Score B was found to be increased at a greater extent [FD 3.22 (1.74, 5. 95), $P < 0.001$] in comparison with IFN Score A [2.94 (1.14, 7.54), $P = 0.018$]. The level of expression of both scores did not show any statistical difference between non-progressors and healthy controls (IFN Score A, p=0.096; IFN Score B, $P = 0.520$). Interestingly, neither IFN Score A nor IFN Score B differed between At-Risk progressors and SLE patients ($P > 0.1$). The summary of the results can be seen in **Figure 3.2**.

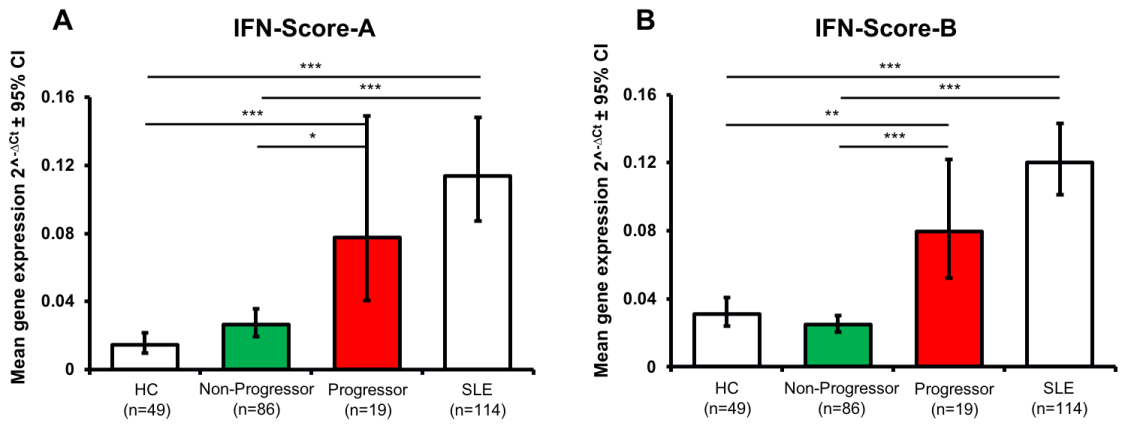


Figure 3.2 Baseline expression IFN scores in peripheral bloods as prognostic marker for disease progression. (A–B) Baseline expression of both IFN Score A and IFN Score B were higher in At-Risk individuals who progressed to an autoimmune connective tissue disease compared with the non-progressors, but to a greater fold difference in IFN Score B.

3.2.2 Phenotyping pDCs in peripheral blood

Peripheral blood pDCs were enumerated and immunophenotyped from freshly isolated PBMCs using flow cytometry in At-Risk individuals (n = 64), patients with SLE (n = 81) and pSS (n = 21) as well as age- and sex-matched healthy controls (n = 37). pDCs are characterised by the lack of lineage markers CD3 (T cells), CD19 (B cells), CD56 (NK cells), CD14 (monocytes) and CD11c (conventional DCs), intermediate to high expression of HLA-DR (MHC-II), high expression of CD123 (IL-3R) and other markers such as CD303 (BDCA-2) and CD304 (BDCA-4). pDCs were gated as lineage$^-$ HLA-DR$^+$CD123$^+$CD303$^+$ cells (**Figure 3.3**).

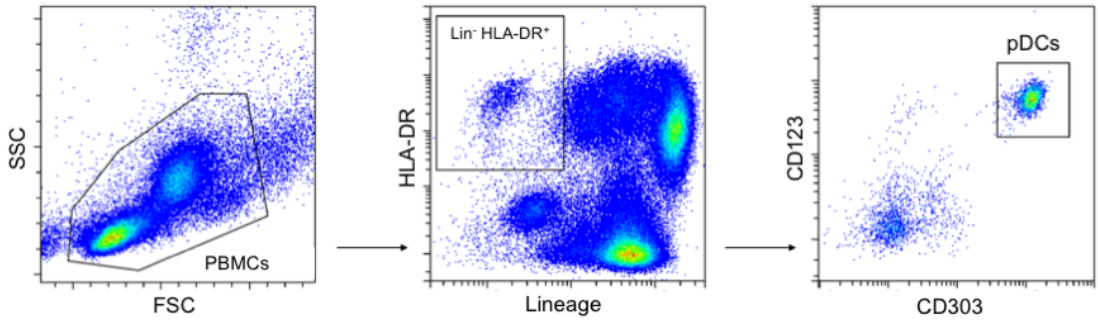


Figure 3.3 Gating strategy to identify the pDC population within PBMCs. pDCs are characterised by the lack of expression of lineage markers (CD3, CD19, CD56, CD14, CD11c), intermediate to high expression of HLA-DR, high expression of CD123 (IL-3R) and CD303 (BDCA-2).

The cells were studied for the surface expression of multiple molecules known to be important in regulating immune functions of pDCs. In more detail, pDCs in SLE patients showed no statistically significant difference in the expression of HLA-DR or BDCA-2 (CD303), which are known to be involved in antigen presentation and negative regulation of IFN-α production respectively. On the other hand, CD123 (IL-3R) and ILT2 (CD85j), molecules involved in regulation of immune responses, were found to be upregulated on pDCs of SLE patients compared to healthy controls ($P < 0.001$). Interestingly, CD317 (BST2; tetherin), a molecule known to be induced by type I IFNs, also presented higher expression on pDCs of SLE patients ($P < 0.05$); however, its ligand ILT7 (CD85g) appeared to be downregulated on pDCs of SLE patients ($P < 0.05$). The expression levels of pDC surface molecules in healthy controls, At-Risk individuals and SLE patients are summarised in **Figure 3.4**.

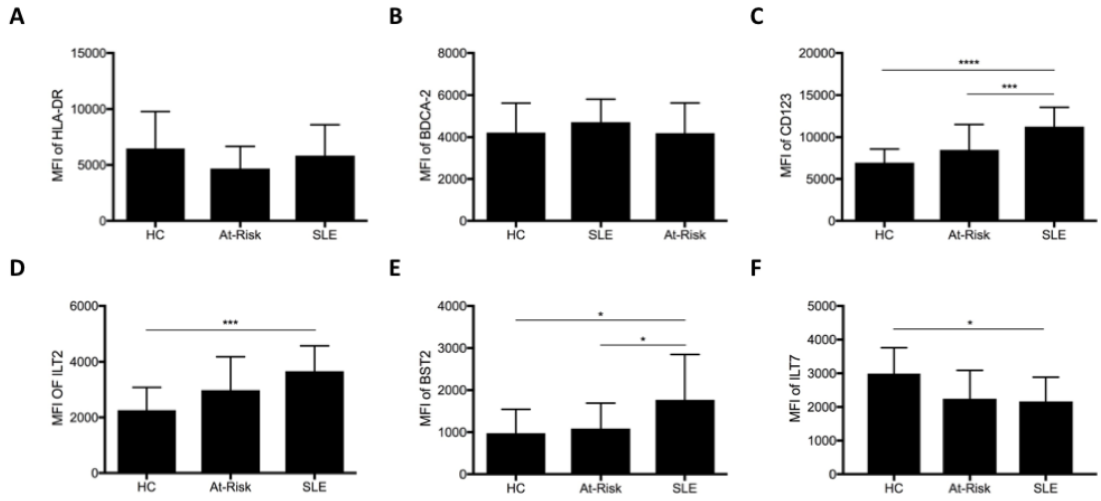


Figure 3.4 Phenotyping of peripheral blood pDCs in At-Risk individuals and SLE patients. Surface expression of: (A) HLA-DR (MHC-II), (B) BDCA-2 (CD303), (C) CD123 (IL-3R), (D) ILT2 (CD85j), (E) BST2 (CD317), (F) ILT7 (CD85g). **P* < 0.05; ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001.

3.2.3 Circulating pDCs are decreased in preclinical autoimmunity and SLE

The literature has contradictory data about the number of circulating pDCs in patients with SLE, whilst the data about patients with pSS and are more limited. Here, I investigated whether or not there were any discrepancies in the number of pDCs in peripheral blood not only in patients with established autoimmune diseases such as SLE and pSS, but also in individuals with preclinical autoimmunity (At-Risk). The average percentage of pDCs in PBMCs was found significantly decreased in patients with SLE (n = 81; *P* < 0.0001) and pSS (n = 21; *P* < 0.0001) compared to healthy controls (n = 37). Intriguingly, this reduction in circulating pDCs was also observed in treatment-naïve At-Risk individuals (n = 64; *P* < 0.0001), the majority of whom (83%) remained at the stage of preclinical benign autoimmunity after 12 months of follow up (**Figure 3.5**).

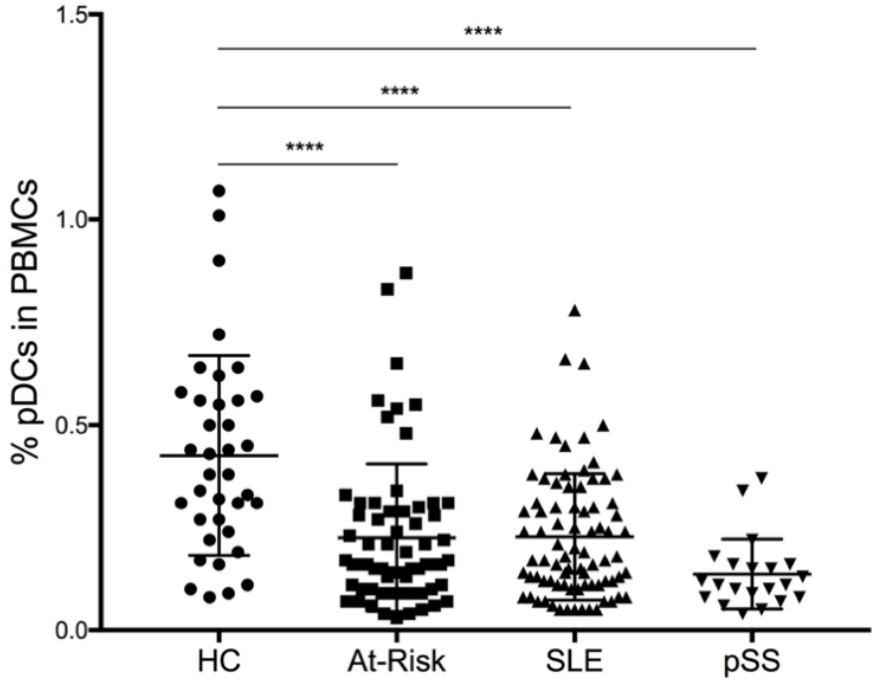


Figure 3.5 Enumeration of pDCs in peripheral blood. Average percentage of pDCs in PBMCs of At-Risk individuals (At-Risk; n = 64), patients with systemic lupus erythematosus (SLE; n = 81) and primary Sjögren's Syndrome (pSS; n = 21) in comparison with age- and sex-matched healthy controls (HC; n = 37). ****$P < 0.0001$.

Next, to determine whether this reduction in numbers of circulating pDCs was associated with other immunological biomarkers such as type I IFN activity and autoantibody profile, I evaluated the expression of IFN Score A in PBMCs from the patients (SLE and pSS), At-Risk individuals and healthy controls using TaqMan assays as described in Materials and Methods. Overall, IFN Score A was notably higher in patients with both SLE and pSS as well as At-Risk individuals. Nevertheless, no association was found between the level of expression of IFN Score A and the percentage of circulating pDCs (**Figure 3.6A-D**). Although IFN Score A was associated with increased number of autoantibodies (ENA count), no association was found

between serology and the percentage of pDCs in any of the sample groups (**Figure 3.6E-F**).

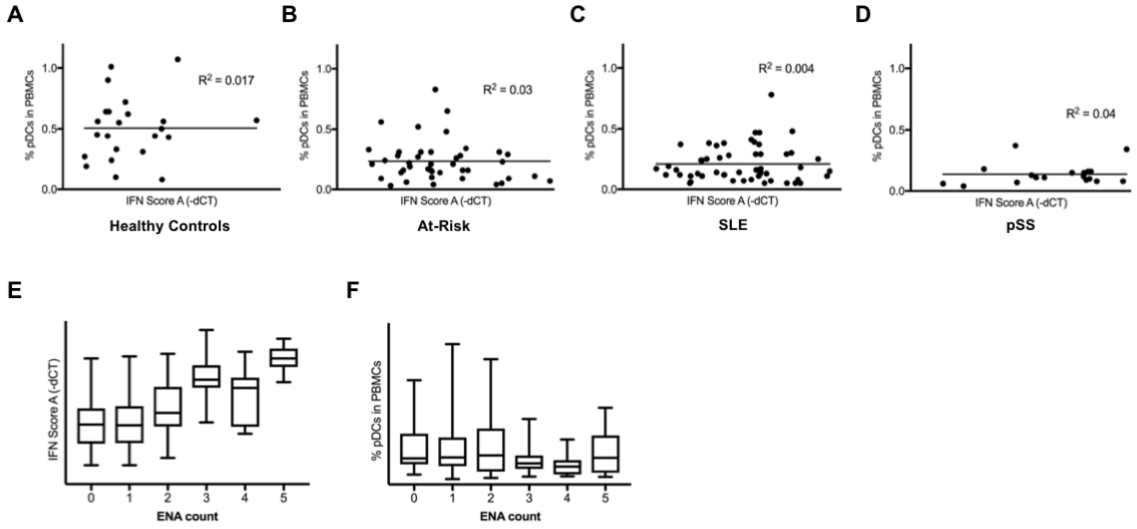


Figure 3.6 pDC numbers are decreased in SLE independently of IFN activity. No association was found between the percentage of pDCs in PBMCs and type I IFN activity measured by IFN Score A in: (A) healthy controls (HC; n = 37), (B) At-Risk individuals (At-Risk; n = 64), (C) patients with systemic lupus erythematosus (SLE; n = 81), and (D) primary Sjögren's Syndrome (pSS; n = 21). (E) Higher expression of IFN Score A was associated with higher number of autoantibodies (ENA count) in patients with SLE and pSS as well as At-Risk individuals. (F) No association was found between ENA count and the percentage of peripheral blood pDCs in patients with SLE and pSS as well as At-Risk individuals.

Particularly in SLE patients, the reduction of circulating pDCs was found to be independent of disease activity (**Figure 3.7A**). In addition, treatment with hydroxychloroquine (**Figure 3.7B**), other immunosuppressants (**Figure 3.7C**) or prednisolone dose (**Figure 3.7D**) did not correlate with the reduction of circulating pDCs either.

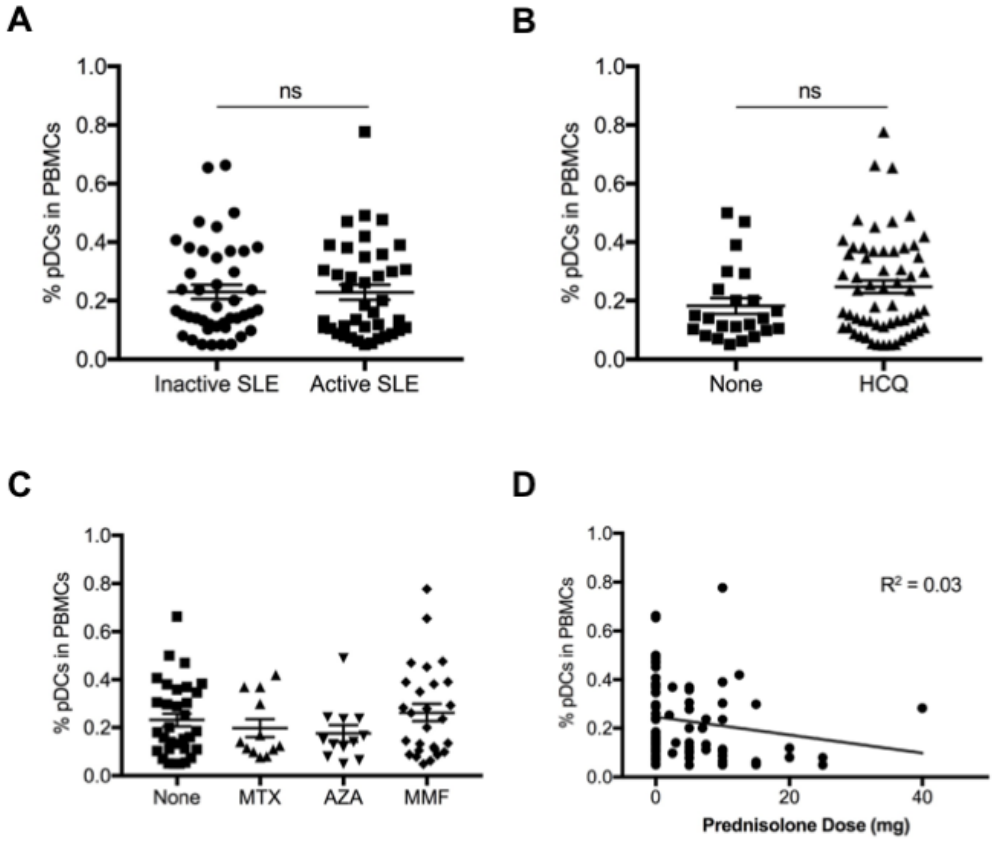


Figure 3.7 pDC numbers are decreased in SLE independently of disease activity and treatment. (A) Percentage of pDCs in PBMCs in SLE patients with inactive and active disease. (B) Percentage of pDCs in PBMCs in SLE patients treated with or without hydroxychloroquine (HCQ). (C) Percentage of pDCs in PBMCs in SLE patients treated with other immunosuppressants (MTX, methotrexate; AZA, azathioprine; MMF, mycophenolate mofetil; None, no immunosuppression). (D) Association of pDCs in PBMCs and prednisolone dose in patients with SLE. ns = not significant.

Finally, the reduction of circulating pDCs in At-Risk individuals, SLE and pSS patients was not associated with the general lymphocyte count and especially, the lymphopenia particularly seen in SLE patients suggesting the decreased pDCs in peripheral blood is an independent feature of autoimmunity (**Figure 3.8**).

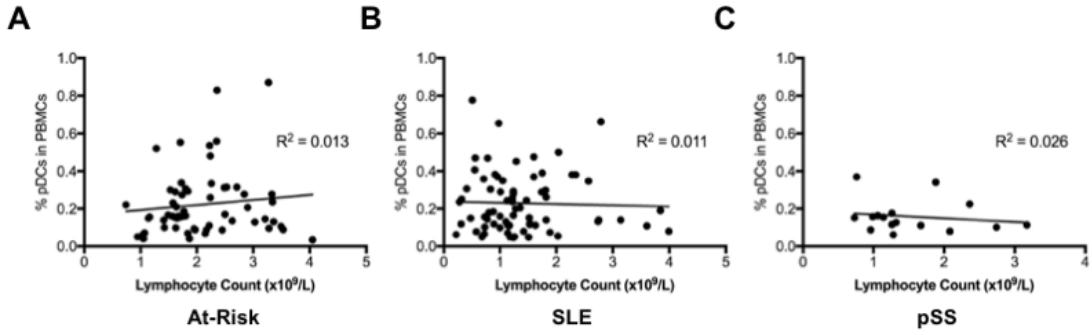


Figure 3.8 pDC numbers are decreased in SLE independently of the lymphocyte count. No association was found between the percentage of pDCs in PBMCs and the lymphocyte count in: (A) At-Risk individuals, (B) patients with SLE, and (C) patients with pSS.

3.2.4 TLR-stimulated pDCs present decreased cytokine production in preclinical autoimmunity and SLE

The production of IFN-α and other pro-inflammatory cytokines, for example TNF-α, in response to TLR-mediated stimulation is the hallmark of pDC function. To evaluate the capacity of cytokine production by pDCs, I stimulated freshly isolated PBMCs from At-Risk individuals (n = 26), patients with established SLE (n = 40) and pSS (n = 7) alongside healthy controls (n = 14) for 6 hours with TLR9 (ODN 2216) or TLR7 (ORN R-2336) agonists. I measured both IFN-α and TNF-α produced by lineage$^-$HLA-DR$^+$CD123$^+$CD303$^+$ pDCs using intracellular staining according to the protocol described in Materials and Methods.

Whereas pDCs from healthy controls produced large amounts of IFN-α in response to TLR9 or TLR7 agonists, pDCs from SLE patients showed little or no cytokine production (**Figure 3.9A**). In more detail, TLR9- and TLR7-mediated IFN-α production was diminished in pDCs from patients with pSS similarly to SLE (**Figure 3.9C-D**). Although TLR9-mediated IFN-α production showed similar results in At-Risk

individuals, their pDCs seemed to partially maintain some TLR7-mediated IFN-α production (**Figure 3.9A-C**).

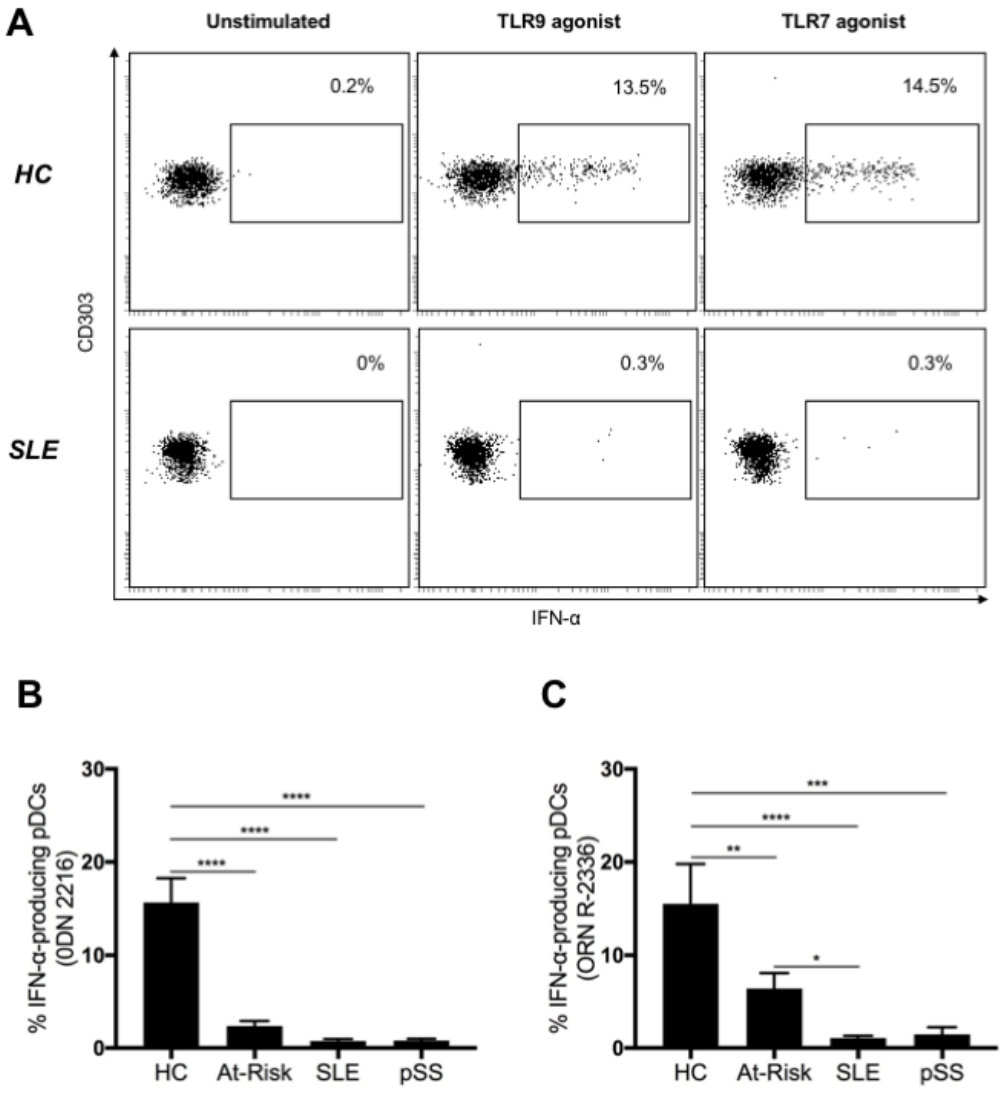


Figure 3.9 pDCs from At-Risk individuals, SLE and pSS patients produce less IFN-α after stimulation with synthetic TLR agonists. (A) Freshly isolated PBMCs were cultured in the absence or presence of TLR9 (ODN 2216) or TLR7 (ORN R-2336) agonists for 6 hours, then IFN-α production by pDCs was measured using intracellular staining. Results shown are representative of a healthy control (HC) and a patient with SLE. Average percentage of IFN-α produced by TLR9-stimulated (B) and TLR7-stimulated (C) pDCs in HC (n = 14), At-Risk (n = 26), SLE (n = 40) and pSS (n = 7) patients. *$P < 0.05$; **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$.

TLR9- and TLR7-mediated TNF-α production was also significantly decreased in pDCs from patients with SLE compared to healthy controls (**Figure 3.10A**). pDCs from pSS patients showed a significant decrease in TNF-α production similar to that of SLE patients, whilst pDCs from At-Risk individuals showed the same trend as IFN-α production, partially maintaining some TLR7-mediated TNF-α production (**Figure 3.10B-C**). No IFN-α and/or TNF-α production by pDCs was detected in any of the samples without external stimulation.

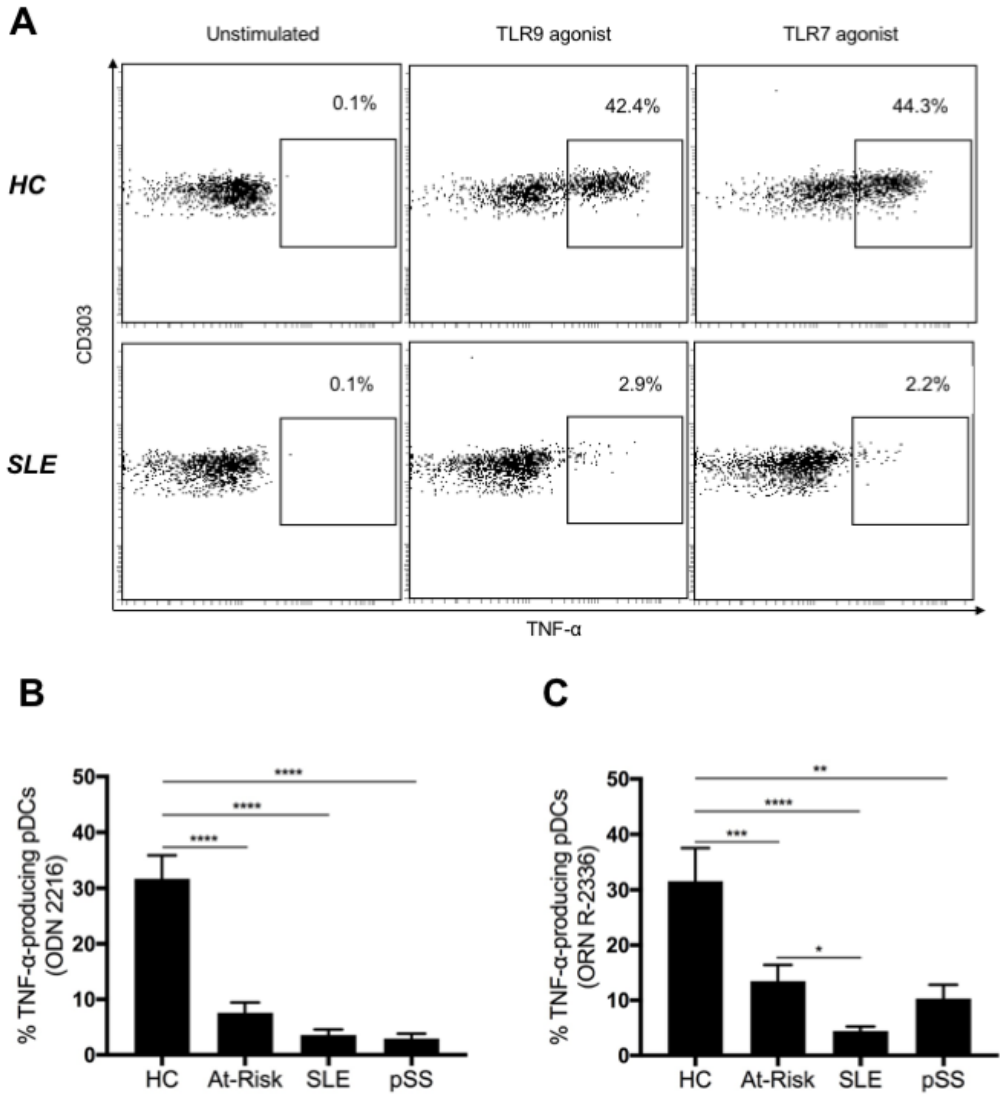


Figure 3.10 pDCs from At-Risk individuals, SLE and pSS patients produce less TNF-α after stimulation with synthetic TLR agonists. (A) Freshly isolated PBMCs were cultured in the absence or presence of TLR9 (ODN 2216) or TLR7 (ORN R-2336) agonists for 6 hours, then TNF-α production by pDCs was measured using intracellular staining. Results shown are representative of a healthy control (HC) and a patient with SLE. Average percentage of TNF-α produced by TLR9-stimulated (B) and TLR7-stimulated (C) pDCs in HC (n = 14), At-Risk (n = 26), SLE (n = 40) and pSS (n = 7) patients. **P* < 0.05; ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001.

I also evaluated whether there was any association between type I IFN activity and IFN-α production by pDCs. No association was found between the levels of TLR-mediated IFN-α production and the level of IFN Score A in patients with SLE as well as At-Risk individuals (**Figure 3.11**).

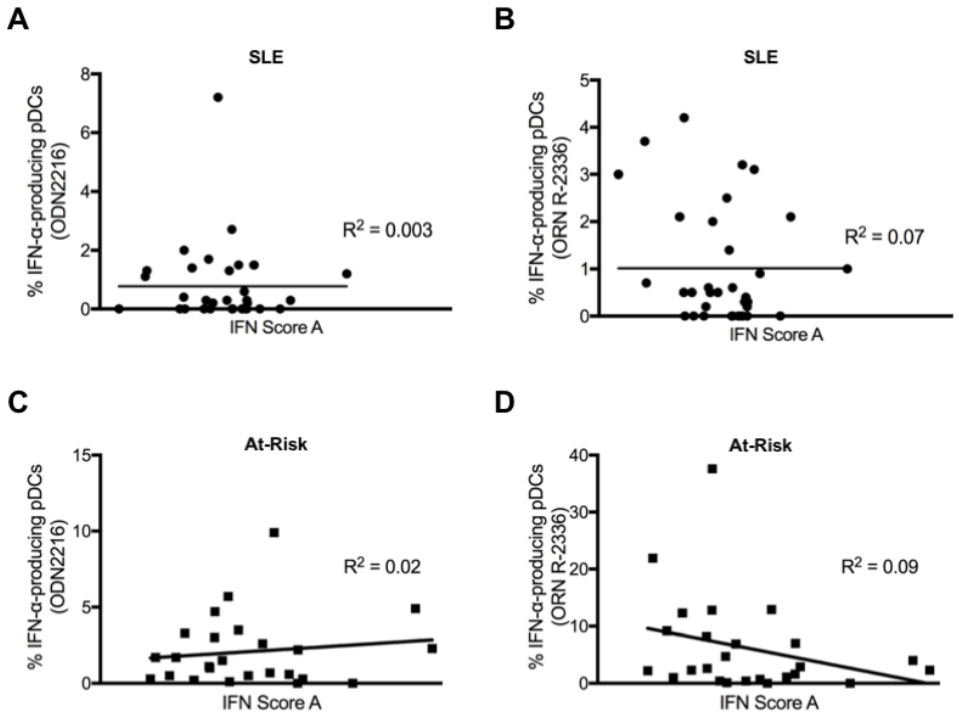


Figure 3.11 No association between TLR9- and TLR7-mediated IFN-α production and IFN Score A in SLE patients and At-Risk individuals.

To further investigate the decreased production of cytokines by TLR-stimulated pDCs in SLE, I measured the intracellular expression levels of both TLR9 and TLR7 using flow cytometry. pDCs (lineage$^-$HLA-DR$^+$CD123$^+$CD303$^+$) from At-Risk individuals and SLE patients showed similar expression levels of both receptors compared to those of healthy controls (**Figure 3.12**).

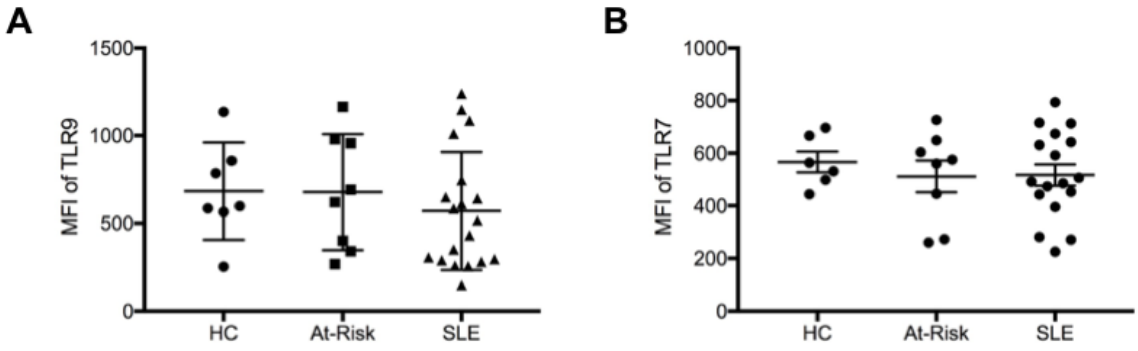


Figure 3.12 No difference in the expression of TLR7 and TLR9 in pDCs of At-Risk and SLE patients compared to healthy controls. Intracellular expression of TLR9 and TLR7 in pDCs was measured using flow cytometry in healthy controls (HC; n = 7), At-Risk (n = 8) and SLE (n = 19) patients.

Interestingly, while culturing PBMCs, a population within monocytes was aroused which was characterised by no expression of HLA-DR but positive expression of CD303 (BDCA-2), which was previously thought to be a pDC-specific marker. These cells showed no response to TLR stimulation, as neither IFN-α nor TNF-α production was detected (**Figure 3.13**).

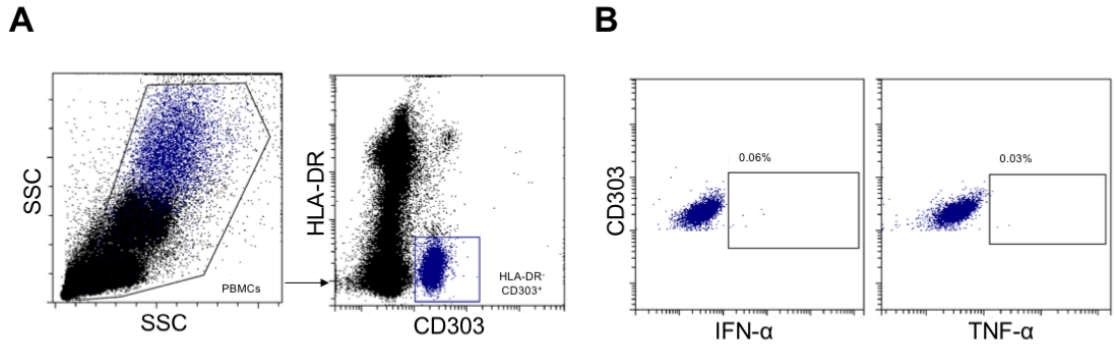


Figure 3.13 CD303$^+$ cells arise from the monocytic population after culture. (A) Gating of HLA-DR$^-$CD303$^+$ cells from cultured PBMCs. (B) No production of IFN-α or TNF-α was detected by HLA-DR$^-$CD303$^+$ cells after TLR-mediated stimulation (ODN 2216).

3.2.5 IL-3 triggers TLR-independent production of IL-6 by pDCs

IL-3 is known to maintain pDC survival *in vitro* and to enhance IFN-α production upon TLR-mediated stimulation (327, 328). I confirmed that pre-treatment for 24 hours with IL-3 amplified IFN-α production by both TLR9- and TLR7-stimulated pDCs from healthy controls (n = 6). However, a statistically significant increase in IFN-α production was not seen in pDCs of At-Risk individuals (n = 4) and SLE patients (n = 7) (**Figure 3.14A-B**). Furthermore, I discovered a new effect of IL-3 on pDCs' function; IL-3 triggered the spontaneous production of IL-6 by pDCs without any exogenous TLR-mediated stimulation. In contrast to the defective IFN-α and TNF-α production in pDCs from SLE patients described above, this TLR-independent IL-6 production by IL-3 stimulation was not impaired in the patient samples (**Figure 3.14C-D**).

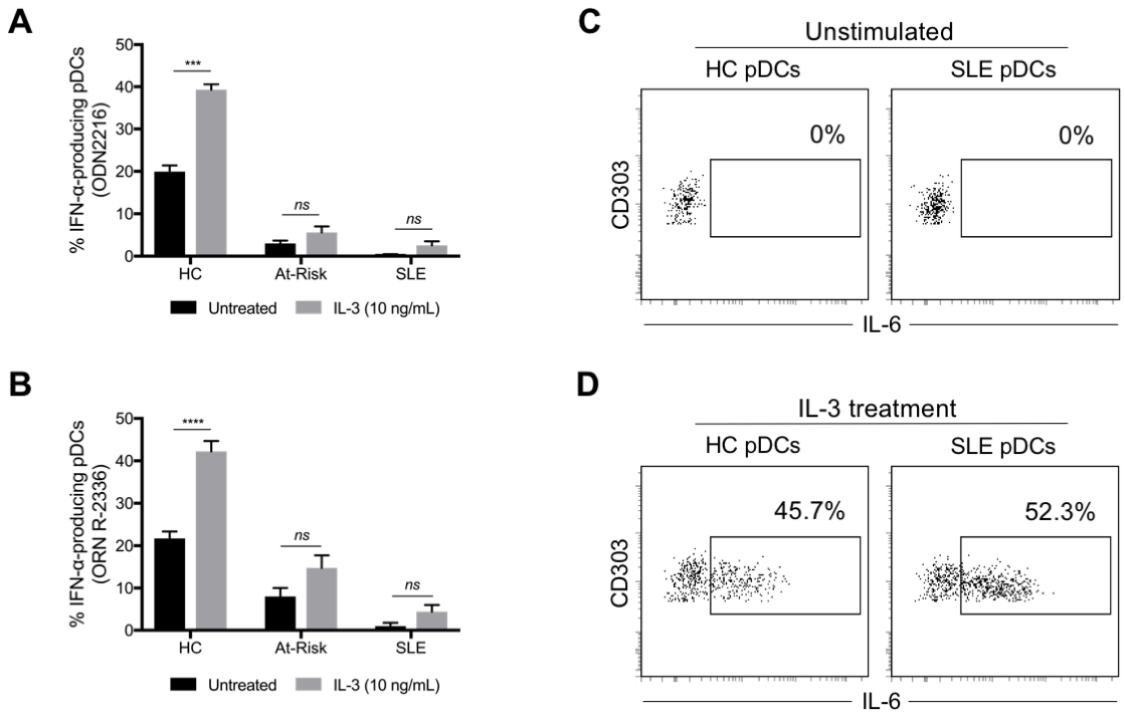


Figure 3.14 IL-3 triggers TLR-independent production of IL-6 by pDCs. PBMCs from healthy controls (HC; n = 6), At-Risk individuals (At-Risk; n = 4) and SLE patients (n = 7) were cultured for 18 hours in the absence or presence of IL-3 (10 ng/mL). The cells were then stimulated by TLR9 (ODN 2216) or TLR7 (ORN R-2336) agonists for 6 additional hours. The production of cytokines was measured by intracellular staining. (A) IL-3 significantly enhanced TLR9-mediated IFN-α production by pDCs of healthy controls ($P < 0.001$); this effect was not seen in pDCs of At-Risk ($P = 0.3$) and SLE ($P = 0.4$) patients. (B) IL-3 significantly enhanced TLR7-mediated IFN-α production by pDCs of healthy controls ($P < 0.0001$); this effect was not that prominent in pDCs of At-Risk ($P = 0.09$) and SLE ($P = 0.6$) patients. (C and D) Treatment with IL-3 (10 ng/mL) induced the production of IL- 6 by pDCs of both healthy controls and SLE patients without exogenous TLR stimulation. The production of IL-6 was detected by intracellular staining. Data are represented as mean ±SEM. ***P < 0.001; ***P < 0.0001; ns = not significant.

3.2.6 pDCs from SLE patients have decreased capacity of inducing T cell proliferation and activation

Although pDCs possess antigen-presentation properties and can trigger T cell responses, little is known about the capacity of pDCs in SLE to induce T cell proliferation and activation. Firstly, I co-cultured freshly isolated pDCs from SLE patients with active disease and healthy controls with allogeneic CellTrace Violet-labelled naïve $CD4^+$ T cells in the presence of anti-CD3/CD28 beads (cell-to-bead ratio 2:1). After 5 days, the proliferation of T cells was measured based on CellTrace Violet dilution (**Figure 3.15A**). Even though pDCs from both groups induced T cell proliferation, pDCs from SLE patients were substantially less efficient, based on the lower percentage of co-cultured T cells that proliferated (**Figure 3.15B**).

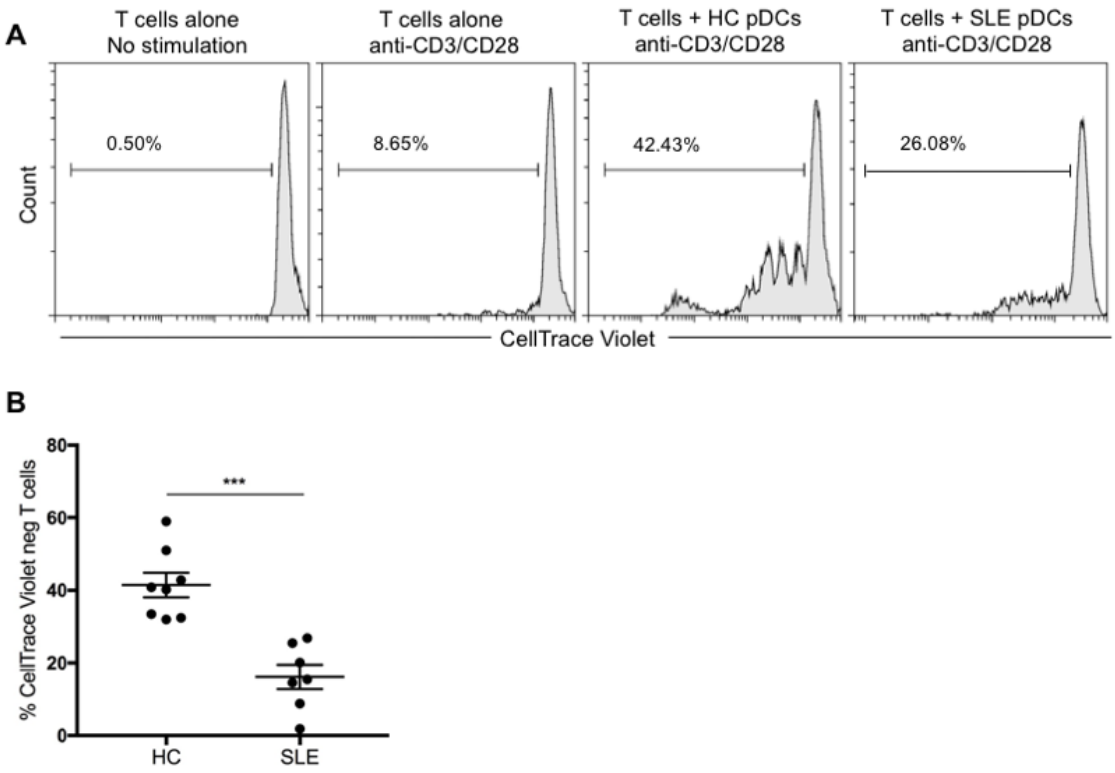


Figure 3.15 pDCs from SLE patients display decreased ability to induce T cell proliferation. (A) Allogeneic naïve $CD4^+$ T cells were labelled with CellTrace Violet and cultured alone or with pDCs purified from healthy controls (HC) or patients with active SLE for 5 days in the presence of anti-CD3/CD38 beads at ratio 2:1 to avoid excessive T cell activation and expansion. T cell proliferation was analysed by flow cytometry based on CellTrace Violet dilution. One representative experiment is shown out of four independent experiments. (B) Average percentage of proliferated $CD4^+$ T cells co-cultured with pDCs from healthy controls (n = 8) and SLE patients (n = 7). ***$P < 0.001$.

Secondly, as pDCs are also known to trigger the induction $FoxP3^+$ T cells, I co-cultured pDCs from SLE patients with active disease and healthy controls with allogeneic naïve $CD4^+$ T cells in the presence of anti-CD3/CD28 beads (cell-to-bead ratio 2:1). In accordance to the findings above, less $CD25^{high}FoxP3^+$ cells were generated from naïve $CD4^+$ T cells after 5 days of co-culturing with pDCs from SLE patients in comparison with pDCs from healthy controls (**Figure 3.16A-B**).

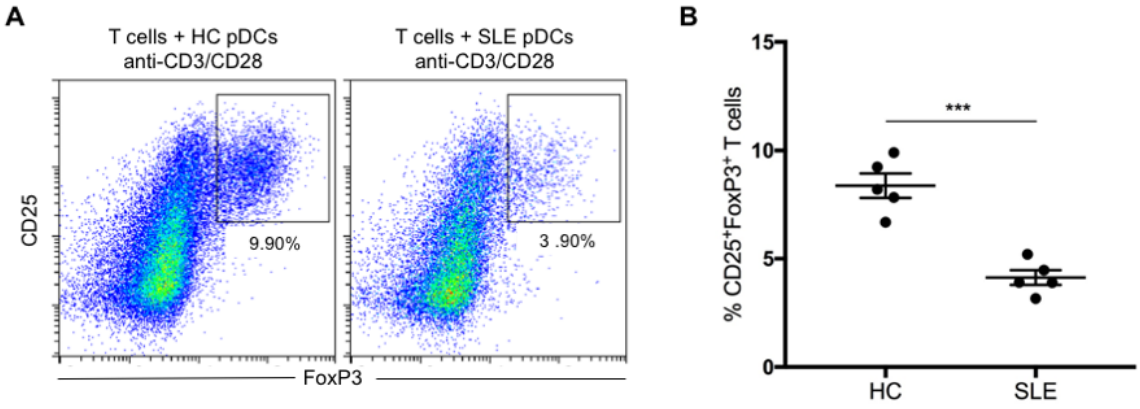


Figure 3.16 pDCs from SLE patients display decreased ability to induce FoxP3$^+$ T cells. (A) Induction of CD4$^+$CD25^{high}FoxP3$^+$ T cells from naïve CD4$^+$ T cells co-cultured for 5 days with pDCs from healthy controls or SLE patients in the presence of anti-CD3/CD38 beads at ratio 2:1. One representative experiment is shown out of three independent experiments. (B) Percentage of CD4$^+$CD25^{high}FoxP3$^+$ T cells derived from the co-culture with pDCs from healthy controls (n = 5) and SLE patients (n = 5). ***P < 0.001.

Lastly, to investigate the ability of pDCs to trigger cytokine production in T cells, I co-cultured pDCs from patients with active SLE and healthy controls with allogeneic naïve CD4$^+$ T cells in the presence of anti-CD3/CD28 beads (cell-to-bead ratio 2:1) for 5 days. On the fifth day, cells were stimulated with PMA/Ionomycin for 5 hours and the production of TNF-α, IFN-γ and IL-10 was measured by intracellular staining following the protocol described in detail in Materials and Methods. In comparison with T cells alone, pDCs from healthy controls enhanced the production of TNF-α (34.07% vs. 48.18%), IFN-γ (5.05% vs. 6.01%) and IL-10 (1.75% vs. 3.42%) from the co-cultured T cells. However, pDCs from SLE patients suppressed the production of all cytokines measured; TNF-α (34.07% vs. 27.95%), IFN-γ (5.05% vs. 3.65%) and IL-10 (1.75% vs. 0.83%). The summary of results can be seen in **Figure 3.17**.

Altogether, pDCs from SLE patients exhibit decreased capacity of triggering T cell proliferation and activation, whilst they might actually contribute to regulation of T cell responses.

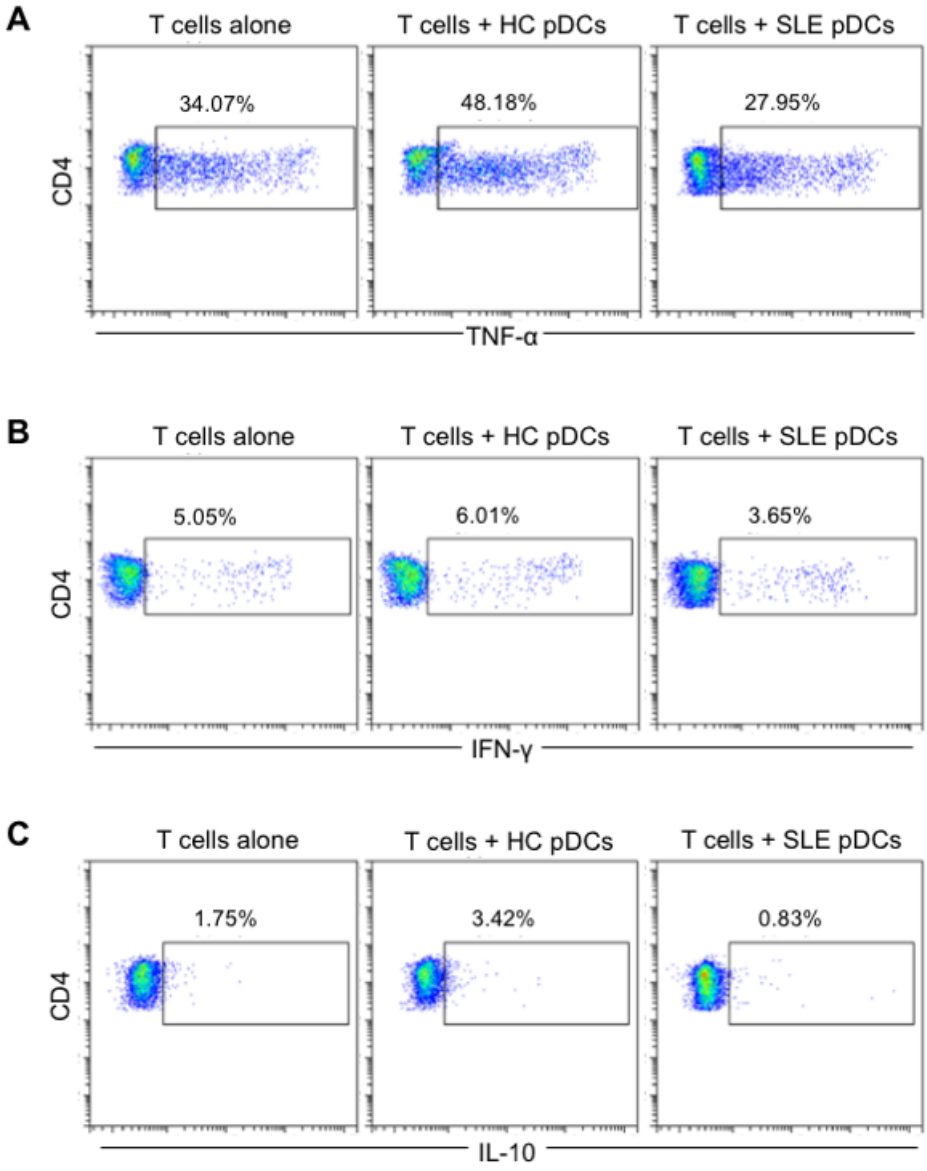


Figure 3.17 pDCs from SLE patients display decreased ability to induce T cell activation. (A-C) Allogeneic naïve $CD4^+$ T cells were cultured alone or with pDCs from healthy controls or SLE patients for 5 days in the presence of anti-CD3/CD38 beads at ratio 2:1. On the fifth day, the cells were stimulated with PMA/Ionomycin and the production of TNF-α, IFN-γ and IL-10 by $CD4^+$ T cells was measured by intracellular staining. One representative experiment is shown out of three independent experiments. ***$P < 0.001$.

3.2.7 pDCs from IFN^{low} and IFN^{high} SLE patients present distinct transcriptional profiles

To investigate disease-associated transcriptional changes in pDCs of autoimmune disorders, I purified pDCs from healthy controls (n = 8), At-Risk individuals (n = 4) and SLE patients (n = 13) by negative selection; I then sorted the cells to achieve purity > 99% based on CD304 (BDCA-4) expression, which is known not to have significant effect on type I IFN production. RNA was purified by sorted pDCs using PicoPure RNA Isolation kit and the extracted RNA was then sequenced using Smart-seq2 for sensitive full-length transcriptomic profiling.

Due to the high variability among the samples, each sample was first scored based on the expression profile of a core set of interferon-stimulated genes (IFN Score) similar to the previously described IFN Score A; then each sample was assigned to IFN^{low} or IFN^{high} subgroups (**Figure 3.18A**). pDCs from SLE patients were characterised by a range of IFN Score, but overall exhibiting a higher IFN Score than pDCs from healthy controls and At-Risk individuals (**Figure 3.18B**). pDCs from most At-Risk individuals (3/4) presented a higher IFN Score compared to pDCs from healthy controls and they were assigned to the IFN^{high} subgroup. Common interferon-stimulated genes (*MX1*, *STAT1*, *XAF1*, *IFI44*, *RSAD2*) were found upregulated in the majority of pDCs in IFN^{high} SLE patients and At-Risk individuals, whilst pDCs in IFN^{low} SLE patients showed similar expression levels to those of healthy controls (**Figure 3.18C**).

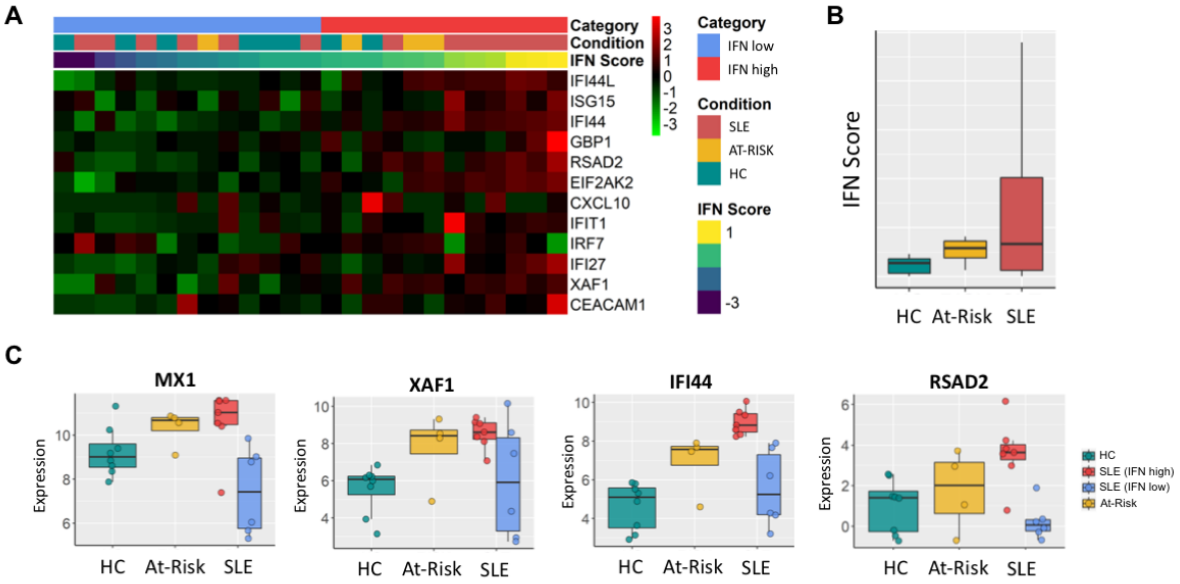


Figure 3.18 pDCs from IFN^{low} and IFN^{high} SLE patients display distinct transcriptomic profiles. (A) Sorted pDCs from HC (n = 7), At-Risk (n = 4) and SLE (n = 13) were classified according to the expression level of the IFN Score described. (B) Average expression level of IFN Score measured in samples described in (A). (C) Expression level of representative ISGs in sorted pDCs from sample groups described in (A).

I next sought to investigate changes in gene expression profiles of pDCs associated with each IFN^{low} or IFN^{high} subgroup compared to those of healthy controls. The analysis of IFN^{low} patients revealed 543 dysregulated transcripts (**Figure 3.19A**), which were particularly enriched for MAPK family signalling cascades, IL-4 and IL-13 signalling, IL-10 signalling, cell migration and pathogen interaction pathways, amongst others (**Figure 3.19B-C**). Amongst the upregulated genes were chemokines, for instance *CXCL3*, *CXCL2* and *CXCL16* (**Figure 3.12A**). A detailed table of the top genes differentially expressed in pDCs of IFN^{low} SLE patients can be found in **Table 3.1**.

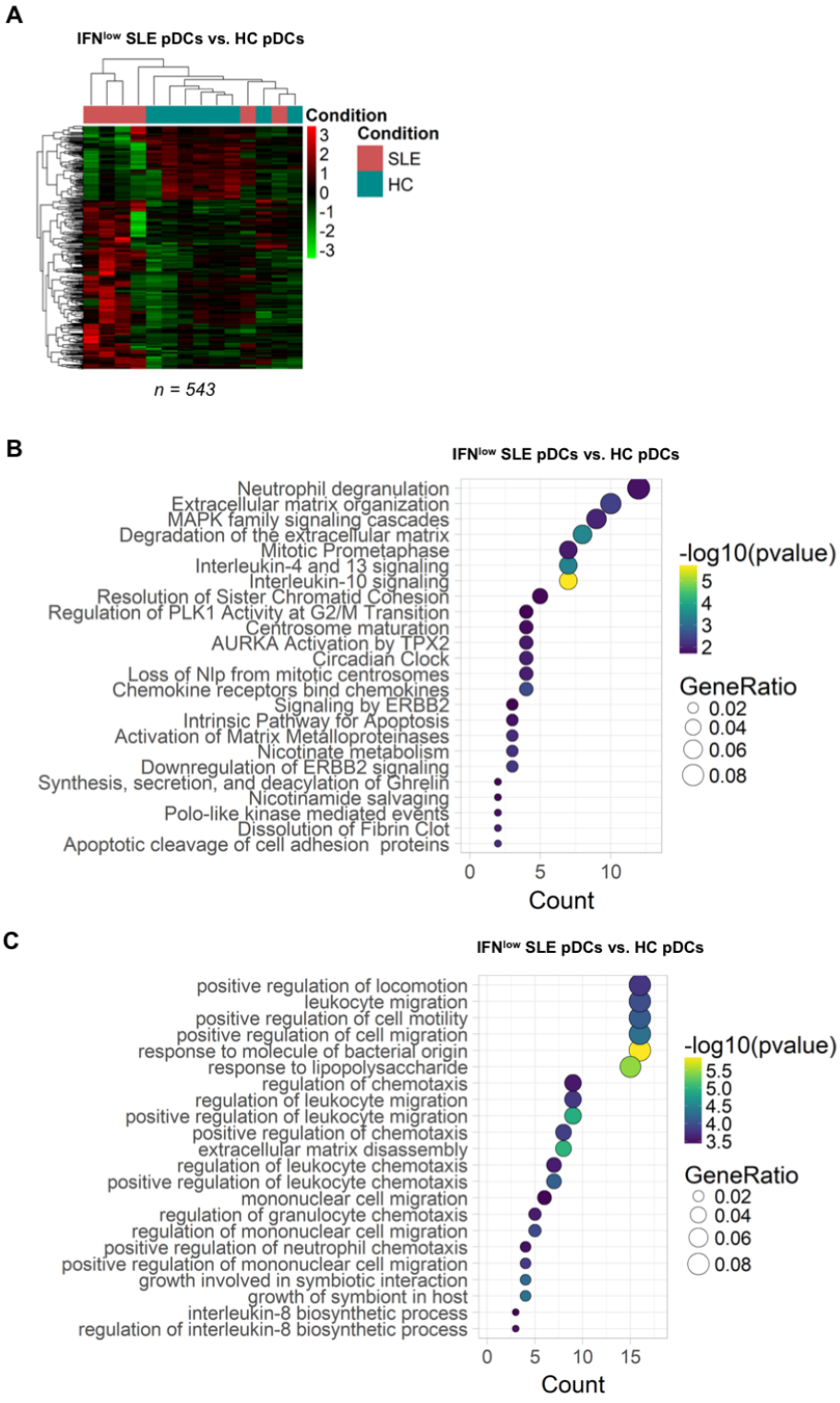


Figure 3.19 Transcriptional profile of pDCs from IFN^{low} SLE patients compared to pDCs of healthy controls (HC). (A) Differentially expressed transcripts (n = 543) in IFN^{low} SLE pDCs compared to HC pDCs. (B) Reactome Pathway Enrichment in differentially expressed genes of IFN^{low} SLE pDCs. (C) Gene Ontology Biological Process Term Enrichment in differentially expressed genes in pDCs of IFN^{low} SLE patients.

In IFN^{high} SLE patients, 674 transcripts were found to be significantly (FDR < 5%) differentially expressed (**Figure 3.20A**). Unsurprisingly, these genes were found to be heavily enriched for interferon response related pathways such as ISG15 antiviral mechanisms, but also pathways related to cellular stress, DNA repair and MAPK pathway signalling (**Figure 3.20B-C**). Several phosphatases known to dephosphorylate MAP kinases (*DUSP1*, *DUSP2*, *DUSP5* and *DUSP8*), transcriptional repressors associated with cell differentiation (*HESX1*, *ETV3*) and NF-κB inhibitors (*NFKBIA*, *NFKBID*) were found to be upregulated in IFN^{high} SLE patients (**Figure 3.21B-D**). A detailed table of the top genes differentially expressed in pDCs of IFN^{low} SLE patients can be found in **Table 3.2**.

Detailed tables of the genes commonly differentially expressed in pDCs of both IFN^{low} and IFN^{high} SLE patients compared to pDCs from healthy controls can be found in **Table 3.3**. Transcripts for any IFN subtype were confirmed not to be expressed in any of the samples.

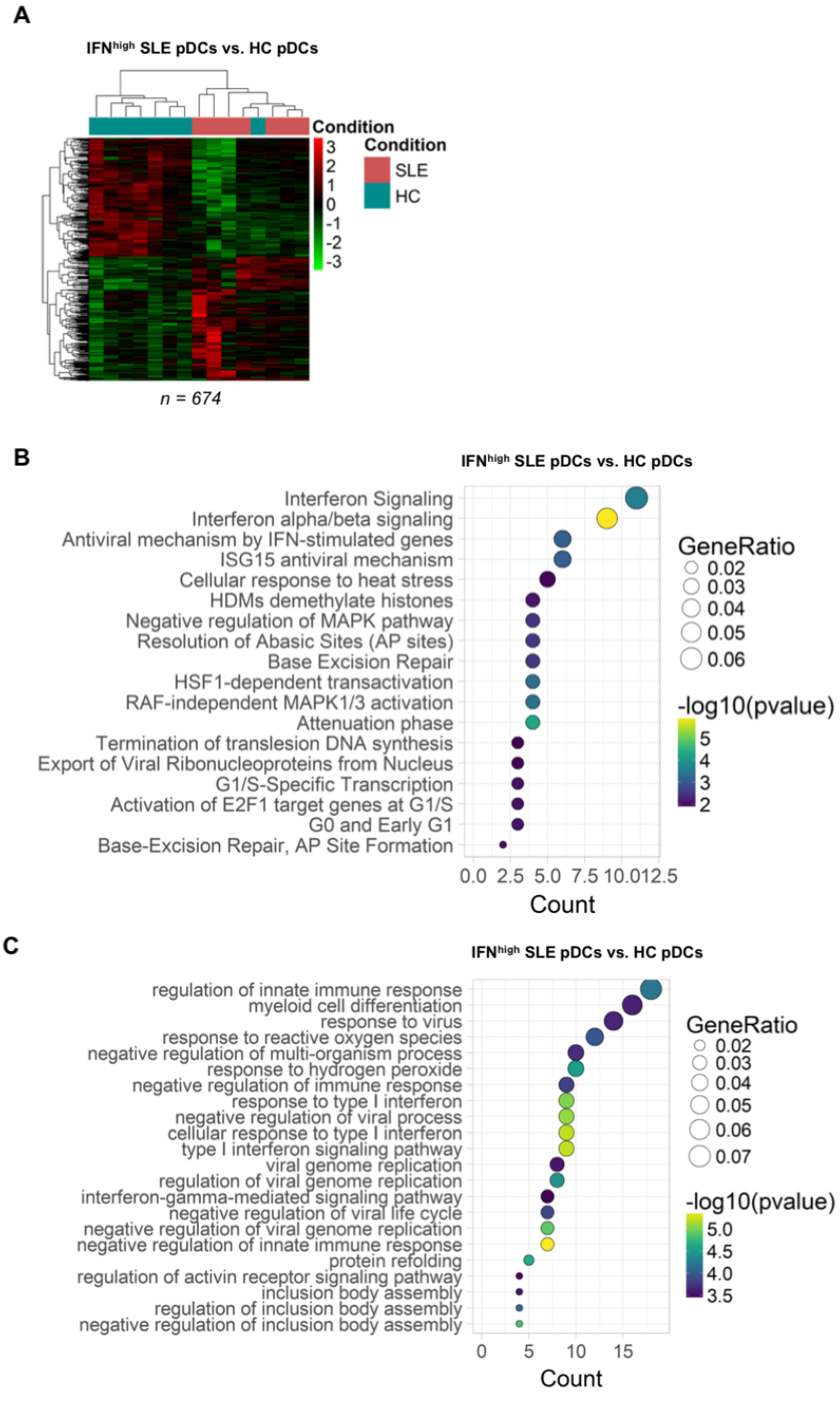


Figure 3.20 Transcriptional profile of pDCs from IFN^{high} SLE patients compared to pDCs of healthy controls (HC). (A) Differentially expressed transcripts (n = 674) in IFN^{high} SLE pDCs compared to HC pDCs. (B) Reactome Pathway Enrichment in differentially expressed genes of IFN^{high} SLE pDCs. (C) Gene Ontology Biological Process Term Enrichment in differentially expressed genes in pDCs of IFN^{high} SLE patients.

	IFN^{low} SLE pDCs vs. HC pDCs		
Gene	**Fold Change (log2)**	***P* value**	**FDR**
ULBP2	19.757	< 0.001	< 0.001
C10orf35	18.302	< 0.001	< 0.001
TPSB2	18.228	< 0.001	< 0.001
TMEM216	-4.981	< 0.001	< 0.001
CXCL2	6.379	< 0.001	< 0.001
PLA2G7	9.712	< 0.001	< 0.001
DNAJB4	1.965	< 0.001	0.001
ZBP1	-7.254	< 0.001	0.001
LUCAT1	6.207	< 0.001	0.006
LOC100861532	2.839	< 0.001	0.007
LGALSL	6.161	< 0.001	0.007
SNORD95	4.390	< 0.001	0.007
LOC100008587	3.428	< 0.001	0.007
BBC3	2.962	< 0.001	0.011
SPR	6.792	< 0.001	0.015
FN1	4.352	< 0.001	0.015
PHF3	1.125	< 0.001	0.015
HSPB1	1.527	< 0.001	0.015
CLEC5A	8.287	< 0.001	0.015
CKAP4	-6.563	< 0.001	0.015
IRAIN	5.493	< 0.001	0.015
FAM157C	3.945	< 0.001	0.015
DNAJB1	2.108	< 0.001	0.015
ETV3	3.037	< 0.001	0.017
TPSAB1	9.650	< 0.001	0.020
SLC6A6	3.758	< 0.001	0.020
NPAS1	5.880	< 0.001	0.020
GADD45B	2.245	< 0.001	0.021
IFITM1	-3.619	< 0.001	0.022
COCH	-7.844	< 0.001	0.022
ANO8	3.516	< 0.001	0.022
LINC00623	3.718	< 0.001	0.022
HSPA4	-2.406	< 0.001	0.023
AOAH	-6.704	< 0.001	0.023
HOXB3	4.793	< 0.001	0.023
SLC8A2	5.243	< 0.001	0.023
NPL	4.663	< 0.001	0.025
ZNF678	4.063	< 0.001	0.025
CNST	2.130	< 0.001	0.025
RNY5	3.224	< 0.001	0.025
PLEKHA8P1	-4.947	< 0.001	0.025
DUSP1	2.903	< 0.001	0.027
LOC100008589	2.695	< 0.001	0.028
NRP2	6.969	< 0.001	0.028
EGR2	7.175	< 0.001	0.029
FCGR2B	-6.697	< 0.001	0.033
NRG2	5.224	< 0.001	0.033
LOC221946	5.139	< 0.001	0.033
PLCXD1	3.230	< 0.001	0.033
LACC1	4.975	< 0.001	0.033

Table 3.1 Top 50 genes that are differentially expressed in pDCs of IFN^{low} SLE patients in comparison with pDCs from healthy controls (HC).

	IFN^{high} SLE pDCs vs. HC pDCs		
Gene	**Fold Change (log2)**	***P* value**	**FDR**
IFI44	5.403	< 0.001	< 0.001
CAMP	7.261	< 0.001	< 0.001
OASL	6.812	< 0.001	< 0.001
SLC8A2	5.644	< 0.001	< 0.001
ATF3	5.870	< 0.001	< 0.001
CMPK2	7.710	< 0.001	< 0.001
BCL2	5.485	< 0.001	< 0.001
LGALSL	6.240	< 0.001	< 0.001
ORM1	8.011	< 0.001	< 0.001
NCMAP	4.931	< 0.001	< 0.001
HCG11	-3.108	< 0.001	< 0.001
PARP14	3.402	< 0.001	< 0.001
MSL2	2.238	< 0.001	0.001
DDX60L	6.470	< 0.001	0.001
PPDPF	1.798	< 0.001	0.001
IER2	2.552	< 0.001	0.001
NR4A1	5.230	< 0.001	0.002
TFB1M	-2.213	< 0.001	0.002
GFOD1	4.279	< 0.001	0.002
TP53INP2	5.236	< 0.001	0.002
TMEM177	-3.452	< 0.001	0.002
LIPT1	-2.499	< 0.001	0.002
JUND	2.486	< 0.001	0.002
THG1L	-2.089	< 0.001	0.002
CCDC121	6.073	< 0.001	0.002
SNORD14C	4.151	< 0.001	0.002
C2orf74	-2.833	< 0.001	0.002
CISD1	-2.392	< 0.001	0.002
HIST1H4F	-2.531	< 0.001	0.002
SEMA7A	3.150	< 0.001	0.002
ETV3L	3.865	< 0.001	0.002
CEACAM1	5.448	< 0.001	0.002
L3MBTL2	-1.887	< 0.001	0.002
ETV3	2.959	< 0.001	0.002
NPAS1	5.469	< 0.001	0.002
QRICH2	4.336	< 0.001	0.002
LTC4S	5.089	< 0.001	0.003
ARID5A	1.781	< 0.001	0.003
EIF2AK2	2.358	< 0.001	0.003
HES4	4.895	< 0.001	0.003
LINC00847	-2.487	< 0.001	0.003
NSUN7	5.334	< 0.001	0.003
DNAJB1	4.229	< 0.001	0.003
KLF8	-4.556	< 0.001	0.003
RSAD2	4.959	< 0.001	0.003
JUN	2.582	< 0.001	0.003
MON1A	-2.248	< 0.001	0.003
IFI44L	3.195	< 0.001	0.004
WDR87	6.155	< 0.001	0.004
IRAIN	4.774	< 0.001	0.004

Table 3.2 Top 50 genes that are differentially expressed in pDCs of IFN^{high} SLE patients in comparison with pDCs from healthy controls (HC).

	IFN^{low} SLE pDCs vs. HC pDCs			IFN^{high} SLE pDCs vs. HC pDCs		
Gene	**Fold Change (log2)**	***P* value**	**FDR**	**Fold Change (log2)**	***P* value**	**FDR**
ETV3	3.037	< 0.001	0.017	2.959	< 0.001	0.002
ATF3	3.914	< 0.001	0.052	5.209	< 0.001	0.000
LIN9	-3.716	< 0.001	0.058	-2.423	< 0.001	0.031
LGALSL	6.161	< 0.001	0.007	6.240	< 0.001	< 0.001
ZNF2	-4.108	< 0.001	0.058	-2.907	< 0.001	0.020
LIPT1	-3.224	< 0.001	0.050	-2.499	< 0.001	0.002
SEC24D	-2.559	< 0.001	0.051	-1.605	0.001	0.038
CXCL2	6.379	< 0.001	< 0.001	4.163	< 0.001	0.019
CBR4	-3.260	< 0.001	0.052	-1.546	< 0.001	0.011
DUSP1	2.903	< 0.001	0.027	2.386	< 0.001	0.021
LTC4S	5.509	< 0.001	0.033	5.089	< 0.001	0.003
SNORD95	4.390	< 0.001	0.007	3.106	< 0.001	0.016
PHF3	1.125	< 0.001	0.015	1.531	< 0.001	0.028
LOC441242	-2.798	0.001	0.068	-2.494	< 0.001	0.015
FUT10	-3.275	0.002	0.100	-2.388	< 0.001	0.016
LOC102724580	3.699	0.001	0.088	4.080	< 0.001	0.004
CCL19	4.468	< 0.001	0.057	3.828	< 0.001	0.020
CTSL	6.460	< 0.001	0.040	4.114	< 0.001	0.020
PUDP	-2.632	< 0.001	0.058	-2.752	< 0.001	0.008
ATP7A	-4.215	< 0.001	0.035	-2.853	< 0.001	0.020
SAT1	1.642	< 0.001	0.036	1.805	< 0.001	0.009
MIR6087	2.376	< 0.001	0.050	2.411	< 0.001	0.028
ZFP91	2.619	< 0.001	0.046	2.147	0.001	0.043
PAAF1	-3.453	0.001	0.066	-1.973	< 0.001	0.033
DUSP8	6.050	0.001	0.085	6.392	< 0.001	0.006
IRAK3	6.179	< 0.001	0.045	4.152	< 0.001	0.007
PLEKHA8P1	-4.947	< 0.001	0.025	-2.810	< 0.001	0.009
ACVR1B	5.196	0.001	0.059	4.169	< 0.001	0.017
RN7SL1	2.867	< 0.001	0.049	2.648	< 0.001	0.034
RN7SL2	3.107	< 0.001	0.033	2.806	< 0.001	0.021
ATG14	2.520	< 0.001	0.058	1.640	< 0.001	0.028
REREP3	3.757	< 0.001	0.046	3.672	< 0.001	0.019
IRAIN	5.493	< 0.001	0.015	4.774	< 0.001	0.004
CDH1	3.795	0.001	0.061	2.895	< 0.001	0.028
SCARNA21	2.549	0.001	0.074	2.980	0.001	0.041
C5AR1	6.724	0.002	0.098	5.210	< 0.001	0.031
SLC8A2	5.243	< 0.001	0.023	5.644	< 0.001	< 0.001
IER2	2.416	< 0.001	0.050	2.552	< 0.001	0.001
DNAJB1	2.108	< 0.001	0.015	4.097	< 0.001	0.005
NPAS1	5.880	< 0.001	0.020	5.469	< 0.001	0.002
LOC100008589	2.695	< 0.001	0.028	2.969	< 0.001	0.006
MIR3687-1	3.321	0.002	0.098	3.448	< 0.001	0.007
MIR3687-2	3.321	0.002	0.098	3.448	< 0.001	0.007
LOC100861532	2.839	< 0.001	0.007	2.930	< 0.001	0.005
WRB	-2.306	0.001	0.068	-2.027	< 0.001	0.008
pDC-specific transcription factors						
E2-2 (TCF4)	0.691	0.118	0.537	0.313	0.578	0.852
SPIB	0.657	0.279	0.719	0.235	0.744	0.922

Table 3.3 Genes that are differentially expressed in pDCs of both IFN^{low} and IFN^{high} SLE patients in comparison with pDCs from healthy controls (HC).

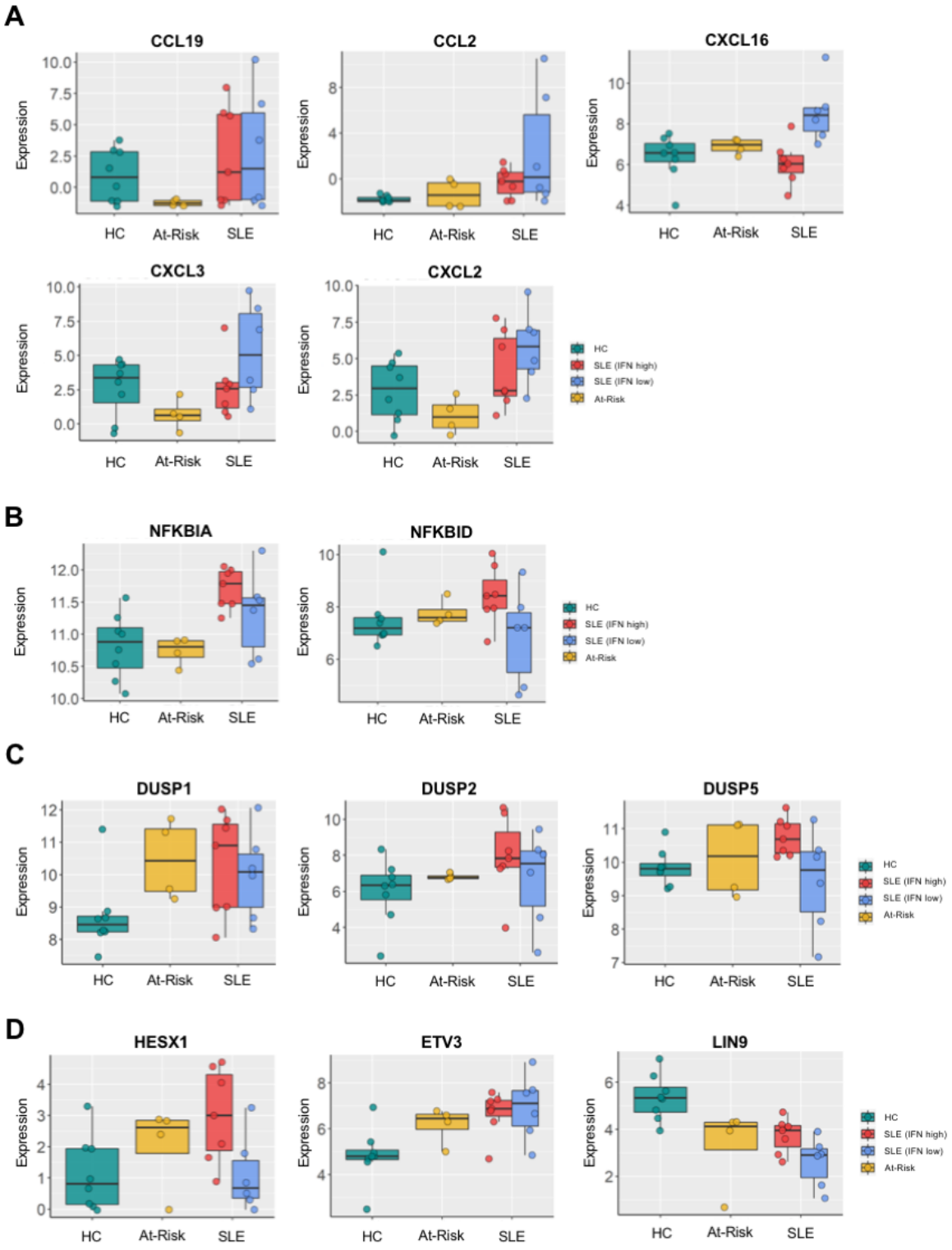


Figure 3.21 Differentially expressed genes in pDCs of healthy controls (HC), At-Risk individuals (At-Risk), IFN^{low} SLE and IFN^{high} SLE patients. (A) Chemokines; (B) NF-κB inhibitors; (C) Phosphatases; (D) Transcriptional repressors.

3.2.8 pDCs from SLE patients present transcriptional and phenotypic features related to immune senescence

In vitro functional assays presented in detail above demonstrated that the decreased secretory function upon TLR stimulation was universally observed in pDCs of SLE patients, independently of the IFN activity measured in the peripheral blood (**Figure 3.9**). To investigate which biological pathways contribute to this defective phenotype, I studied the transcripts differentially expressed in both pDCs of IFN^{low} and IFN^{high} SLE patients compared to those of healthy controls. Surprisingly, little overlap between differentially expressed genes in pDCs of IFN^{low} and IFN^{high} SLE patients was detected (**Figure 3.22A**). Amongst the 80 shared transcripts, there were upregulated genes involved in cellular senescence and stress (*ATG14*, *ATP7A*, *DNAJB1*), protein degradation in lysosomes (*CTSL*), negative regulation of TLR signalling (*IRAK3*), negative regulation MAPK signalling (*DUSP1*, *DUSP8*) and negative regulation non-canonical NF-κB pathway (*ZFP91*), which are known to inhibit the production of type I IFNs and other pro-inflammatory cytokines (**Figure 3.22B**).

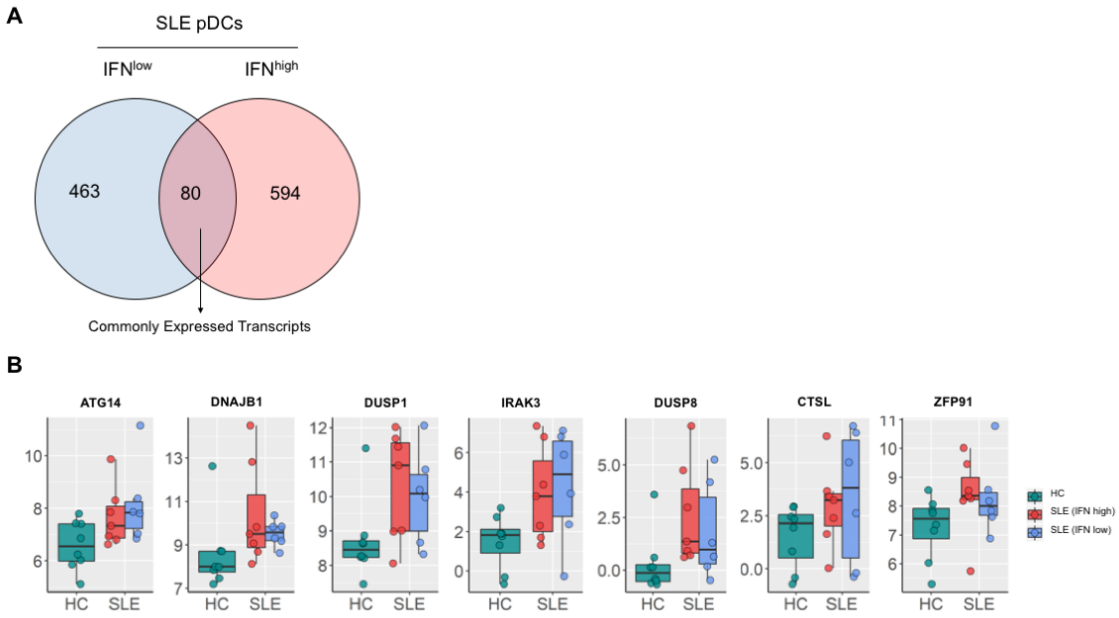


Figure 3.22 Commonly expressed transcripts in pDCs of IFN^{low} and IFN^{high} SLE patients. (A) Venn diagram showing the number of differentially expressed transcripts (n = 80) common to both IFN^{low} and IFN^{high} pDCs from SLE patients compared to pDCs from HC. (B) Expression level of representative genes differentially expressed in both IFN^{low} and IFN^{high} pDCs from SLE patients in comparison with pDCs from HC.

Moreover, the shared transcripts included upregulated genes for *CXCL2* and *CCL19* (**Table 3.3**). Reactome Pathway Enrichment also showed that biological processes related to MAPK family signalling, Toll-like receptor signalling, IL-10 signalling and chemotaxis were significantly enriched (**Figure 3.23**).

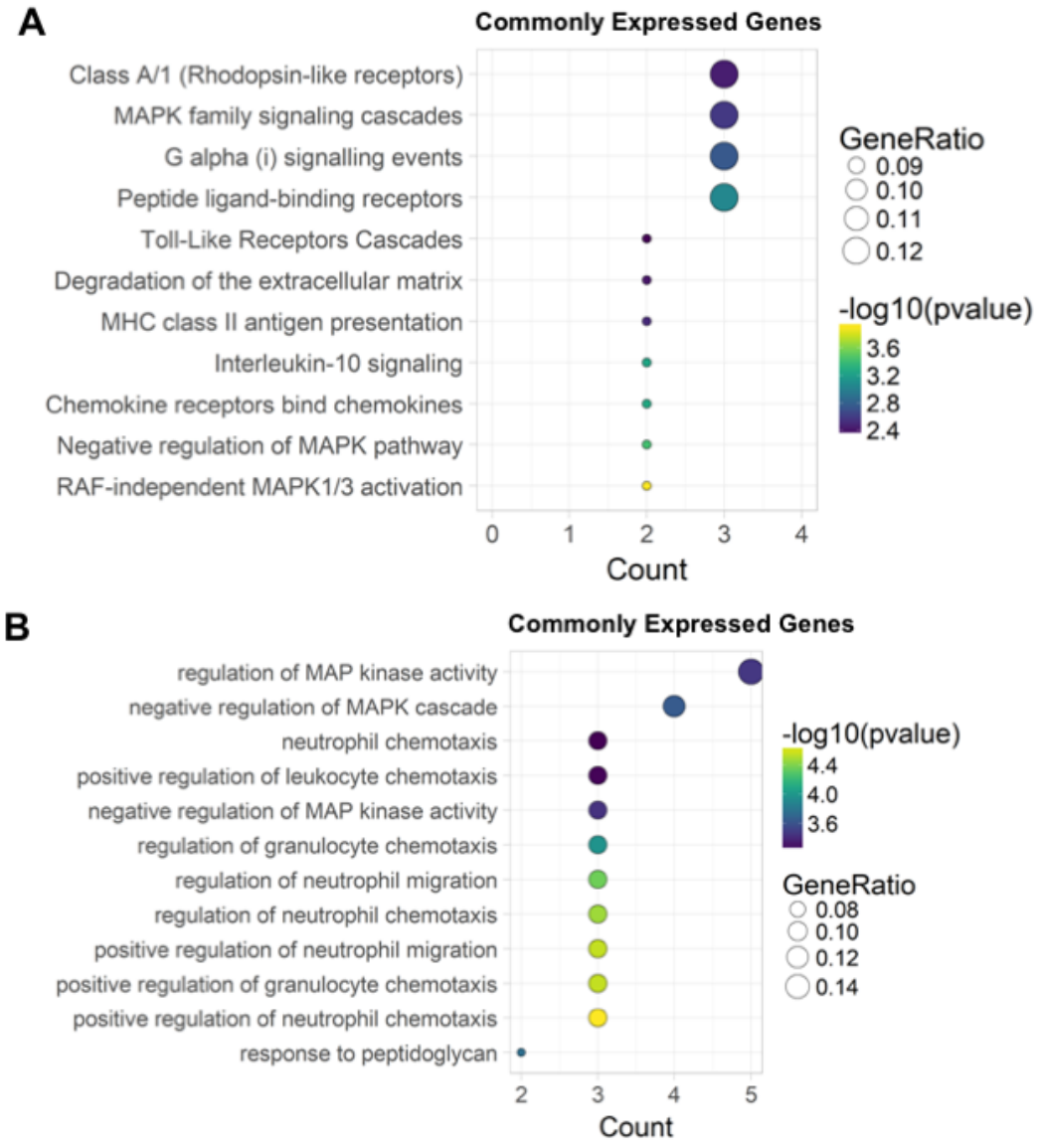


Figure 3.23 Differentially expressed genes in IFN^{low} and IFN^{high} pDCs from SLE patients. (A) Reactome Pathway Enrichment in DEGs in differentially expressed genes in IFN^{low} and IFN^{high} pDCs from SLE patients. (B) Gene Ontology Biological Process Term Enrichment in differentially expressed genes in pDCs of IFN^{low} and IFN^{high} SLE patients.

Increased telomere erosion is known to be related to cellular senescence, a feature which was found in other immune cells of patients with SLE but is still undescribed in pDCs. To address this question, I purified pDCs from healthy controls alongside SLE patients using negative and positive selection to achieve purity > 95%; pDCs were then hybridized with telomere PNA probe before they were analysed by flow cytometry. The determination of the relative telomere length was calculated as the ratio between the telomere signal of pDCs and the tetraploid control cells (1301 cell line) with correction for the DNA index of $G_{0/1}$ cells (**Figure 3.24A**). The analysis confirmed that pDCs from SLE patients had shorter telomere length compared to pDCs from age- and sex-matched healthy controls (**Figure 3.24B**).

RNA-sequencing data analysis also indicated that pathways related to cellular stress was differentially expressed in both IFN^{low} and IFN^{high} pDCs of SLE patients. To further address how this is associated with the secretory function of pDCs, I investigated the effect of oxidative stress on type I IFN production in TLR-stimulated pDCs. Freshly isolated PBMCs from healthy donors were exposed to increasing concentrations of H_2O_2 (0 – 500 μM) for 15 minutes before they were stimulated with ODN 2216 for 6 hours according to the protocol described in Materials and Methods. IFN-α production was measured by intracellular staining. I confirmed that oxidative stress –even at low concentrations of H_2O_2– negatively regulated TLR-mediated responses in viable pDCs leading to a gradual loss of their ability to produce IFN-α (**Figure 3.24C**).

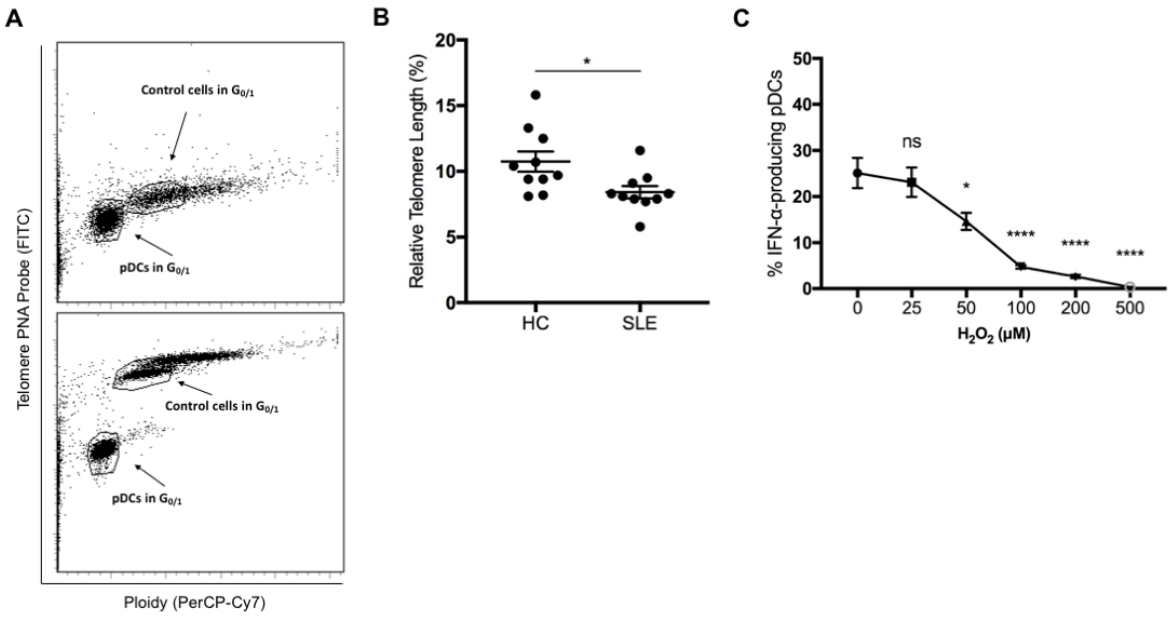


Figure 3.24 pDCs from SLE patients present increased telomere erosion. Purified pDCs from freshly isolated PBMCs were hybridized without (A; upper) or with (A; lower) telomere PNA probe. Gates were set in $G_{0/1}$ phase for both sample cells (pDCs) and tetraploid control cells (1301 cell line). (B) Determination of the relative telomere length as the ratio between the telomere signal of pDCs purified from HC (n = 10) and SLE (n = 10) patients and the control cells (1301 cell line) with correction for the DNA index of $G_{0/1}$ cells. (C) Freshly isolated PBMCs from healthy donors (n = 4) were exposed to H_2O_2 (0 – 500 µM) for 15 minutes. After H_2O_2 exposure, cells were washed thoroughly and resuspended in culture medium before they were stimulated with 2µM ODN 2216 for 6 hours. The production of IFN-α was measured in viable pDCs by intracellular staining. ns = not significant; *$P < 0.05$; ****$P < 0.001$.

3.2.9 High IFN activity in skin characterises preclinical autoimmunity and systemic lupus erythematosus

Since professional IFN-α-producing cells such as pDCs appeared dysfunctional in producing type I IFNs in SLE, the source of the aberrant type I IFN production seen in patients had yet to be identified. To find any potential correlation between type I IFN activity in blood and specific disease manifestations, I compared the level of expression of IFN Score A in blood with the disease activity in the most common affected organs, the skin and the joints. Active disease was defined as BILAG-2004 A or B and inactive disease as BILAG-2004 C-E. I found that IFN Score A was associated with mucocutaneous disease activity (Fold Difference 2.24 (95% CI 1.16 - 4.34); P = 0.017), but not with musculoskeletal disease (Fold Difference 0.97 (95% CI 0.44 - 2.09); P = 0.927) (**Figure 3.25A-B**).

To investigate the above correlation further, I compared the fold increase in IFN Score A in blood and skin biopsies from At-Risk individuals and SLE patients compared to healthy controls. I analysed blood samples from 114 SLE patients, 105 At-Risk individuals, and 49 healthy controls; I also analysed lesional skin biopsies from 10 SLE patients and non-lesional skin biopsies from 10 At-Risk individuals as well as skin biopsies from 6 healthy controls. Total RNA was extracted from the acquired biopsies using RNeasy mini kit (Qiagen) and the RNA quantity was measured and assessed for quality using NanoDrop spectrophotometer, ND-1000. As described in detail in Materials and Methods, TaqMan assays (Applied Biosystems, Invitrogen) were used to perform the quantitative real-time reverse transcriptase-polymerase chain reaction (qPCR) for the same genes used in measuring type I IFN activity in peripheral blood. Data were normalised using Peptidylprolyl isomerase A (PPIA) as a reference

gene to calculate ΔCt. All analyses of IFN Scores were conducted using ΔCt scaling; results were then converted to relative expression ($2^{-\Delta Ct}$) or fold difference ($2^{-\Delta\Delta Ct}$).

In At-Risk individuals compared to healthy controls, mean (95% CI) fold increase in IFN Score A in blood was 2.21 (1.37, 3.53), while in non-lesional skin the fold increase was markedly higher at 28.74 (1.29, 639.48) (**Figure 3.25C)**. The differential increase in ISG expression in blood and skin was even more extreme in SLE patients compared to healthy controls; in some SLE patients ISG expression in skin was more than 5,000 times higher than healthy controls. Mean (95% CI) fold increase was 7.80 (4.75,12.80) in blood compared to 479.33 **(**39.32, 5842.78) in skin (**Figure 3.25D)**. A detailed summary of the expression of each of the gene measured in skin biopsies of healthy controls, SLE patients, and At-Risk individuals can be seen in **Figure 3.26**.

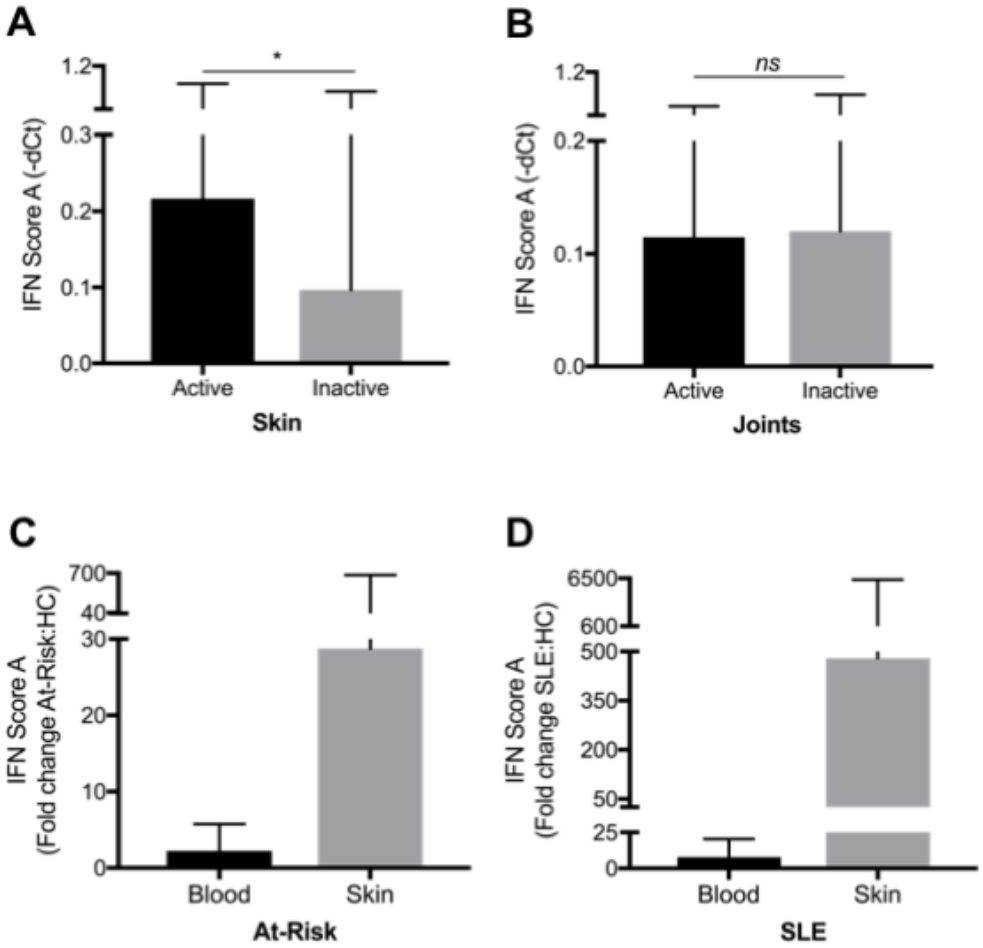


Figure 3.25 Associations of IFN Score A with the two commonest disease manifestations (skin and joints) in patients with SLE. (A) Association of IFN Score A with active and inactive mucocutaneous disease in SLE patients. (B) Association of IFN Score A with active and inactive musculoskeletal disease in SLE patients. (C) Fold increase in IFN Score A of At-Risk individuals in blood (2.21; 1.37, 3.53) and skin (28.74; 1.29, 639.48) compared to healthy controls. (D) Fold increase in IFN Score A of SLE patients (D) in blood (7.80; 4.75,12.80) and skin (479.33; 39.32, 5842.78) compared to healthy controls.

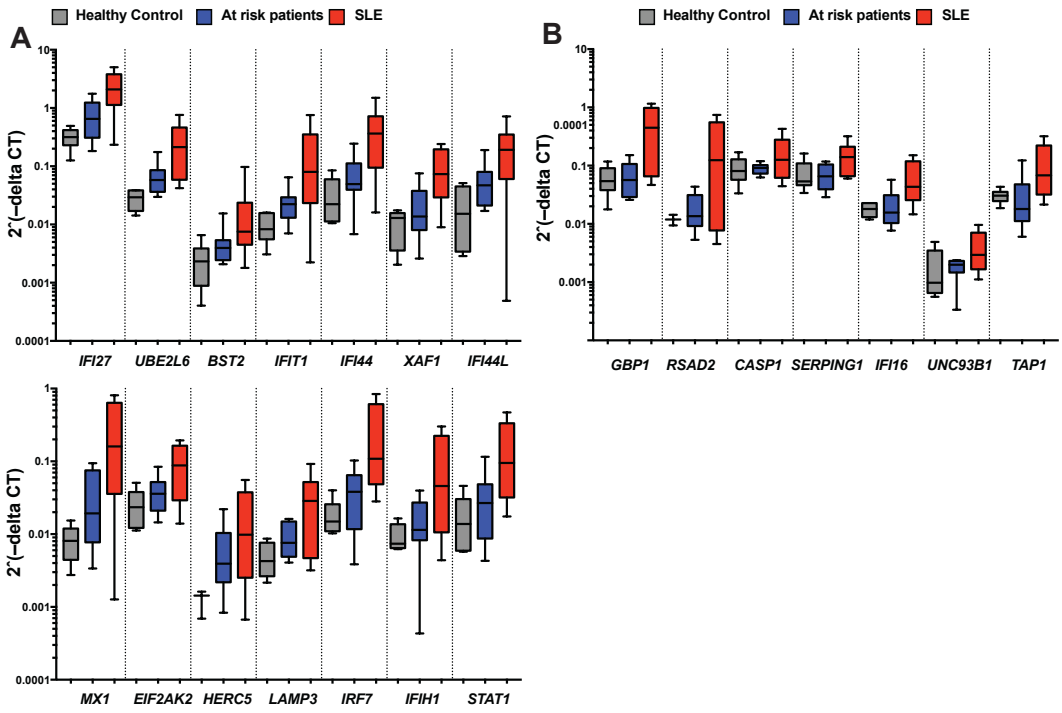


Figure 3.26 Expression of interferon-stimulated genes in skin biopsies. Skin biopsies from healthy controls (HC; n = 7), At-Risk individuals (At-Risk; n = 10), patients with SLE (SLE; n = 10) were obtained and analysed for the expression of 30 ISGs using TaqMan assays. ISGs expressed in skin biopsies were clustered into two distinct groups different to the groups found in peripheral blood: (A) ISGs showed a stepwise increase of expression from healthy controls to At-Risk individuals and then to SLE patients. (B) ISGs showed no significant difference in expression between healthy controls and At-Risk individuals but they were significantly increased in skin biopsies of SLE patients.

3.2.10 Patients with high IFN activity in blood present diffuse expression of type I IFNs in epidermis

Although professional IFN-α-producing cells such as pDCs appear immune senescent in SLE, a prominent source of the aberrant type I IFN production seen in patients is yet to be identified. To study alternative sources of this dysregulation, skin biopsies from healthy controls (n = 4), SLE patients (n = 6) and At-Risk individuals (n = 4) were used as a representative example of tissue inflammation, since I previously showed

that mucocutaneous symptoms in SLE correlate well with IFN activity in peripheral blood (322). Skin biopsies were obtained from areas with an active lesion from SLE patients with a range of IFN Score A in peripheral blood, whilst skin biopsies from At-Risk individuals had no signs of inflammation but they did present high IFN Score A in peripheral blood. Then I performed *in situ* hybridization to visualize the expression of type I IFNs transcripts (*IFNK*, *IFNA2*) in all skin biopsies obtained according to the detailed protocol described in Materials and Methods.

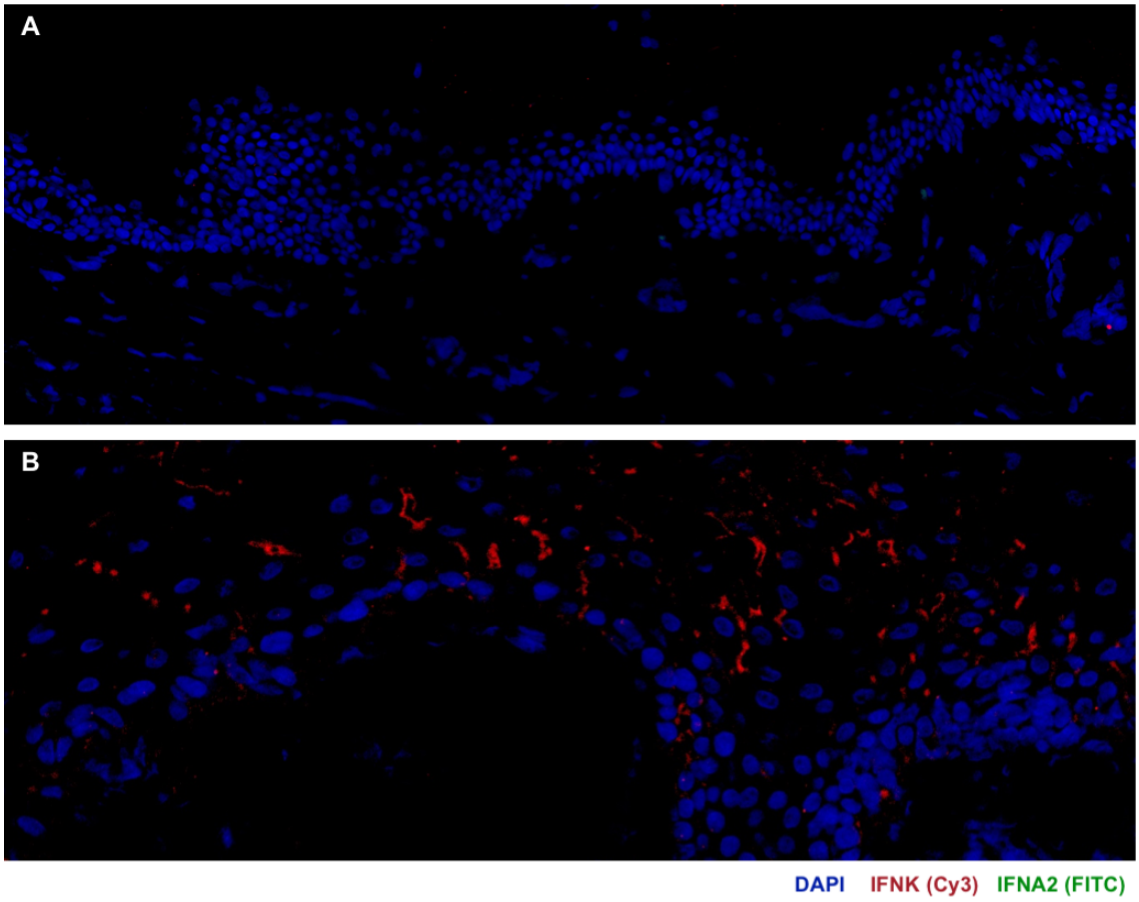


Figure 3.27 SLE patients with high IFN activity in blood present diffuse expression of type I IFNs in epidermis. Skin biopsies were hybridized using RNAScope in situ hybridization technology with custom-designed target probes for IFNA2 and IFNK. Hybridization signals were amplified and detected using TSA Plus fluorescein (FITC) for IFNA2 and TSA Plus Cyanine 3 (Cy3) for IFNK. Nuclei were highlighted using DAPI. Representative in situ hybridization images of: (A) healthy control, (B) IFN^{high} SLE patient with active skin lesion.

As expected, skin biopsies from healthy controls with minimal IFN Score A in peripheral blood showed no expression of either *IFNK* or *IFNA2* (**Figure 3.27A**). In contrast, lesional skin from SLE patients with high IFN Score A presented with diffuse expression of *IFNK* in epidermis among keratinocyte layers (**Figure 3.27B**). However, *IFNK* expression was less prominent in the epidermis of SLE patients with lower IFN Score A. Regarding *IFNA*2, I was able to detect its expression in cells in the dermis, possibly fibroblasts as they were located in areas with dense connective tissue, but not in leucocyte-infiltrating areas (**Figure 3.28**).

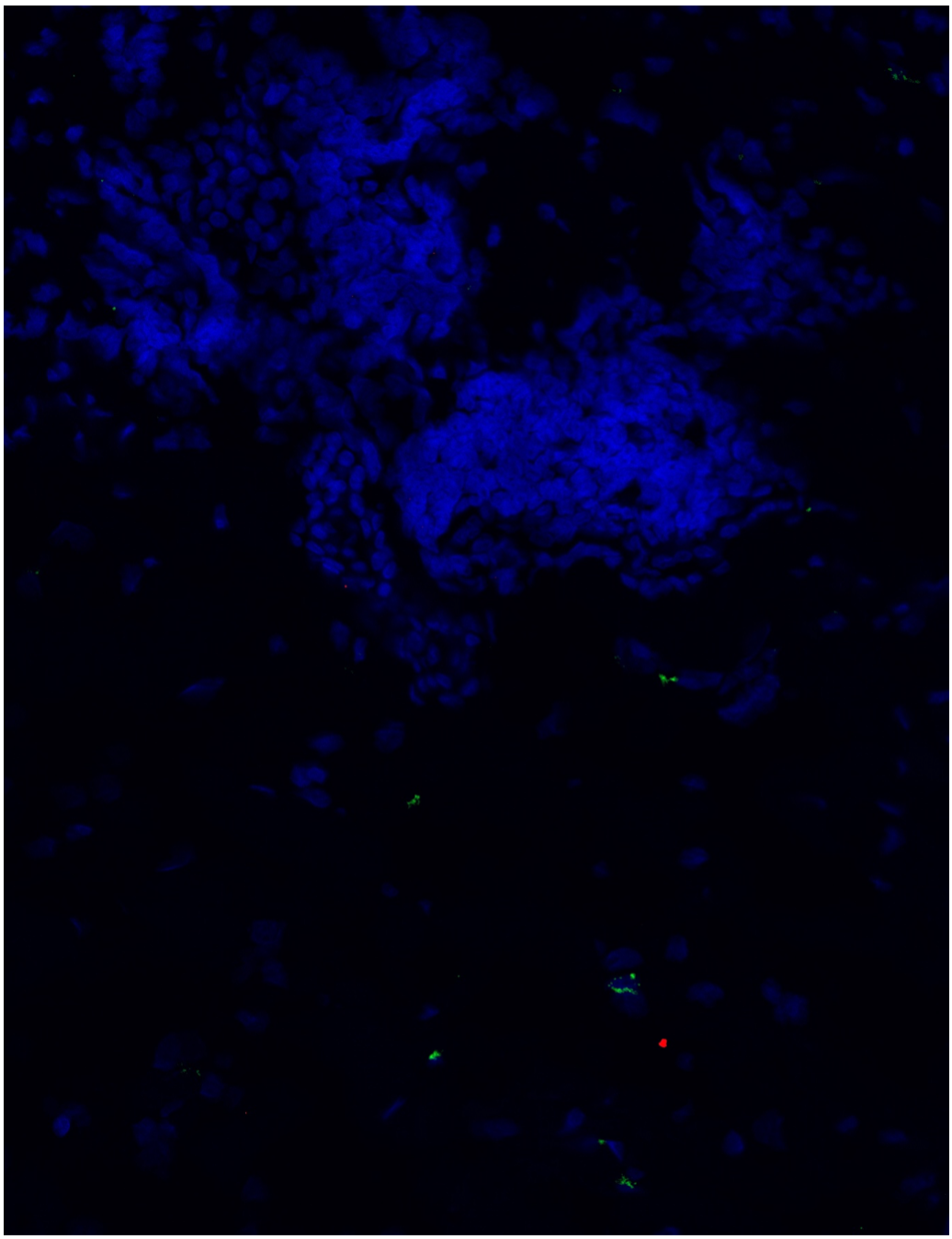


Figure 3.28 Area of lymphocyte infiltration and connective tissue of a patient with SLE with active skin lesion. Skin biopsies were hybridized using RNAscope in situ hybridization technology with custom-designed target probes for IFNA2 and IFNK. Hybridization signals were amplified and detected using TSA Plus fluorescein (FITC) for IFNA2 and TSA Plus Cyanine 3 (Cy3) for IFNK. Nuclei were highlighted using DAPI. IFNA2 expression was detected in cells within the connective tissue but not in infiltrating leucocytes.

Interestingly, the epidermis of At-Risk individuals with high IFN Score A in peripheral blood was also characterised by diffuse expression of *IFNK* among keratinocyte layers, but unlike SLE patients there was no sign of cutaneous inflammation either clinically or histopathologically (**Figure 3.29**).

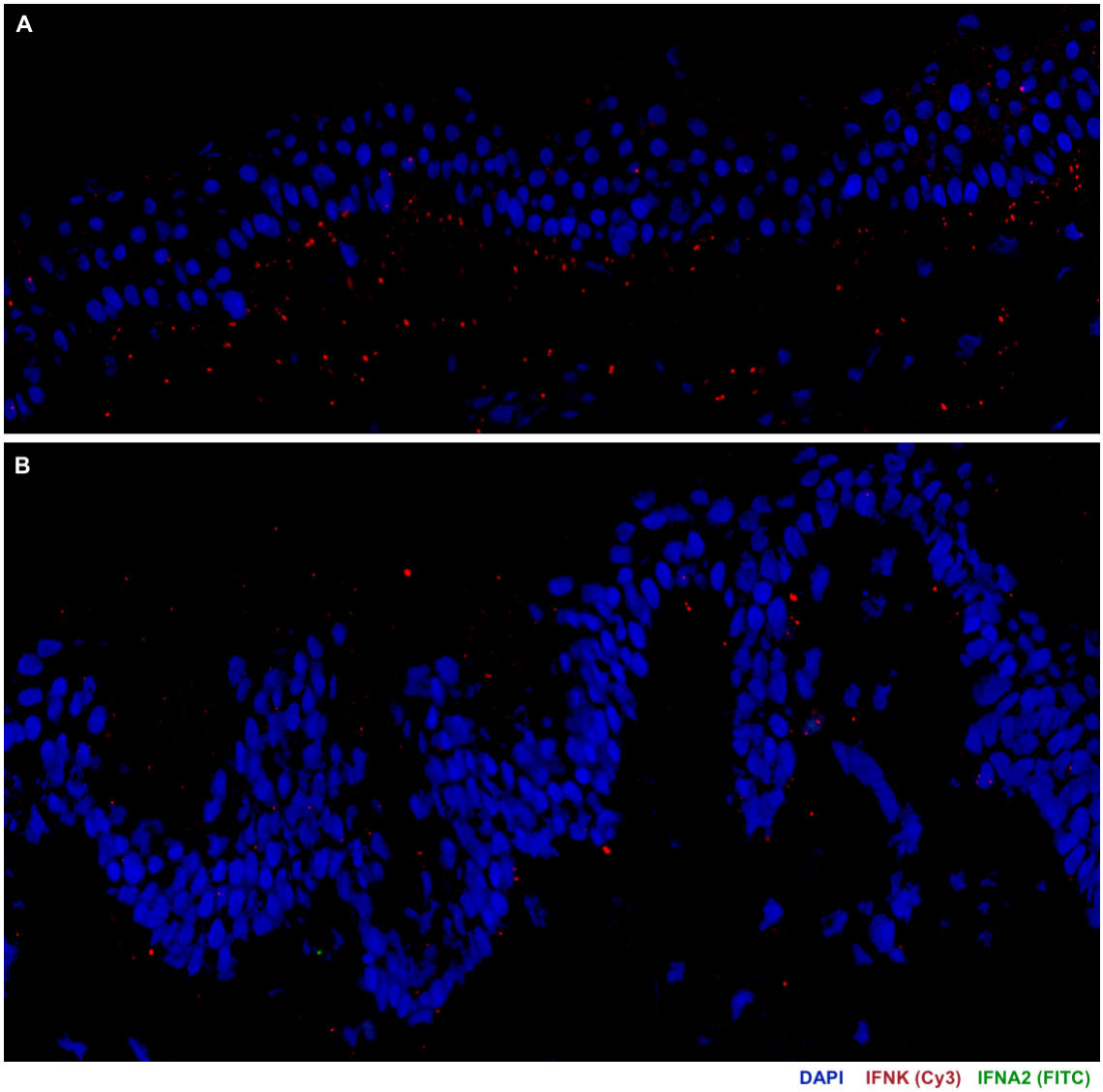


Figure 3.29 At-Risk individuals with high IFN activity in blood present diffuse expression of type I IFNs in epidermis. Skin biopsies were hybridized using RNAscope in situ hybridization technology with custom-designed target probes for IFNA2 and IFNK. (A, B) Representative in situ hybridization images of epidermis from IFN^{high} At-Risk individuals with no clinical or histopathological signs of inflammation.

3.2.11 UV provocation in vivo enhances IFNK expression in keratinocytes

UV provocation was performed using a solar simulator, which replicated the protocol of UV-A and UV-B provocation in a single exposure. After the minimal erythema dose was determined, a 10 cm^2 non-sun exposed area of skin was exposed to the dose x 1.5 on three consecutive days. A biopsy of the pre-exposed and exposed area of skin was obtained when a reaction was seen clinically. The tissue biopsies were cryosectioned and stored at -80^oC until RNAscope protocol for *in situ* hybridization was applied to measure the expression of *IFNK* transcripts in single cell level on the tissue section. A significant enhancement of *IFNK* expression in exposed area of skin of SLE patients was observed compared to the pre-exposed area that showed less *IFNK* expression (**Figure 3.30**). These results not only confirm an important role for UV light known to trigger lupus pathology but also show a new mechanism linking environmental stimuli and excessive type I IFN production by non-haematopoietic tissue cells such as keratinocytes.

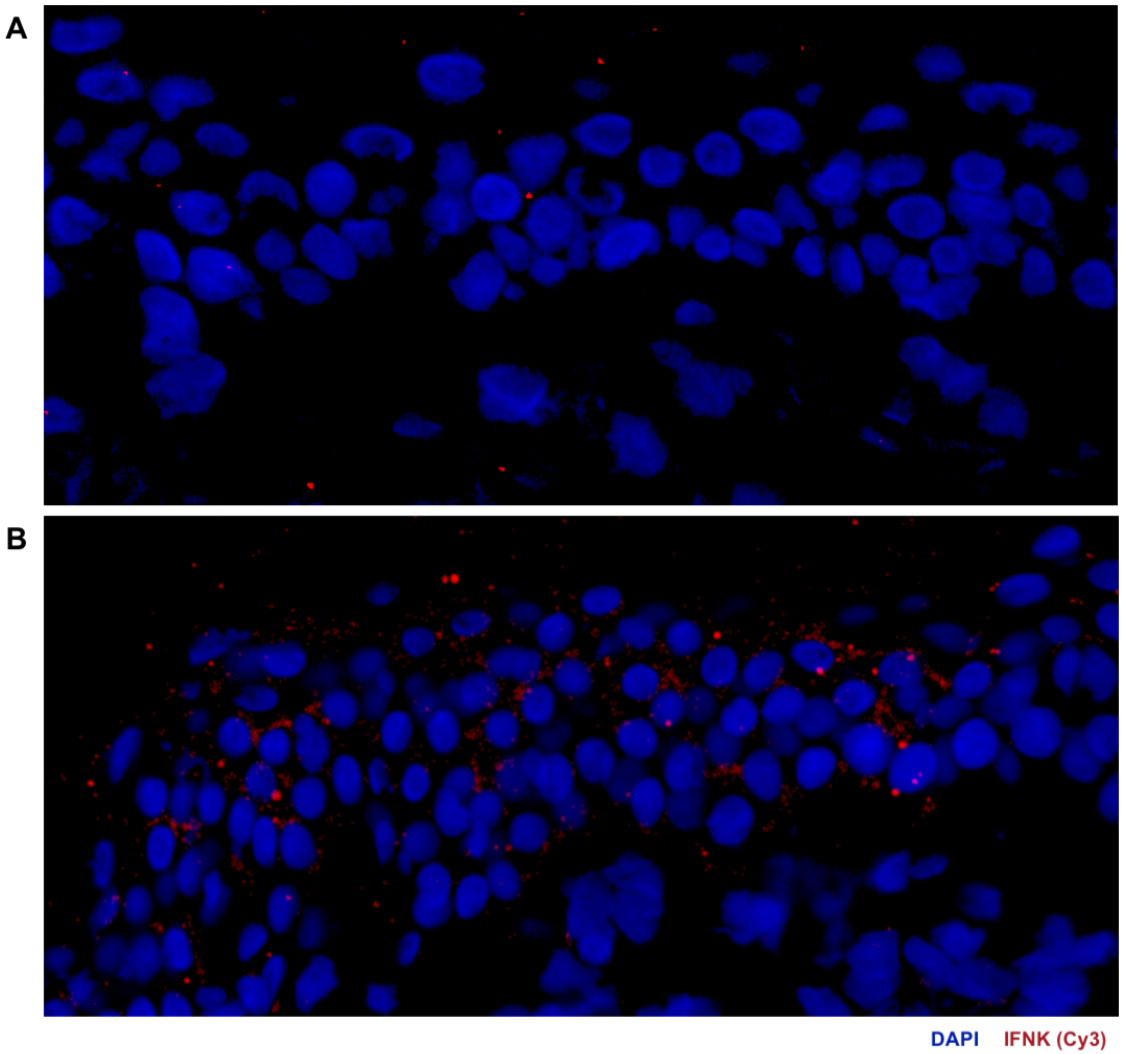


Figure 3.30 UV provocation enhances type I IFN expression in epidermis of SLE patients. Skin biopsies were then hybridized using RNAscope *in situ* hybridization technology with custom-designed target probes for IFNA2 and IFNK. (A) IFNK expression in epidermis of patient with inactive SLE before UV provocation. (B) IFNK expression in epidermis of the same SLE patient after UV provocation.

3.2.12 Keratinocytes from At-Risk and SLE patients present increased expression of IFNs in response to nucleic acids

To validate the results from *in situ* hybridization, human keratinocytes and dermal fibroblasts were isolated from healthy controls (n = 3), At-Risk individuals (n = 5) and SLE patients (n = 5). Cells from patients with cutaneous discoid lupus erythematosus

(CDLE), who were ANA negative and had minimal IFN Score A expression in blood, were also used as an inflammatory control (n = 3). Cells were cultured in the presence or absence of TLR3 stimulation, Poly I:C (1 μg/mL), or RIG-I stimulation, Poly dA:dT (100 ng/ml), for 6 and 24 hours before the expression of IFNK was measured by qPCR. At baseline, without exogenous stimulation, keratinocytes from both At-Risk and SLE patients presented higher expression of IFNK. Interestingly, after both TLR3 and RIG-I stimulation, the expression of IFNK was significantly increased in the keratinocytes of At-Risk and SLE patients. However, keratinocytes from CDLE did not show the same response (**Figure 3.31A**). For *IFNB1*, there was no expression at baseline in any sample. However, after stimulation with Poly(I:C) there was a trend to increased expression for At-Risk keratinocytes and a significant increase for keratinocytes from SLE and CDLE patients. *IFNB1* expression was also increased in keratinocytes of SLE patients after Poly(dA:dT) stimulation but not in other conditions (**Figure 3.31B**).In contrast, *IFNL1* expression was only observed in CDLE keratinocytes following Poly(I:C) stimulation but not in the other conditions or following Poly(dA:dT) stimulation (**Figure 3.31C**). Finally, *IFNA2* expression by keratinocytes was not found in any sample or condition (data not shown).

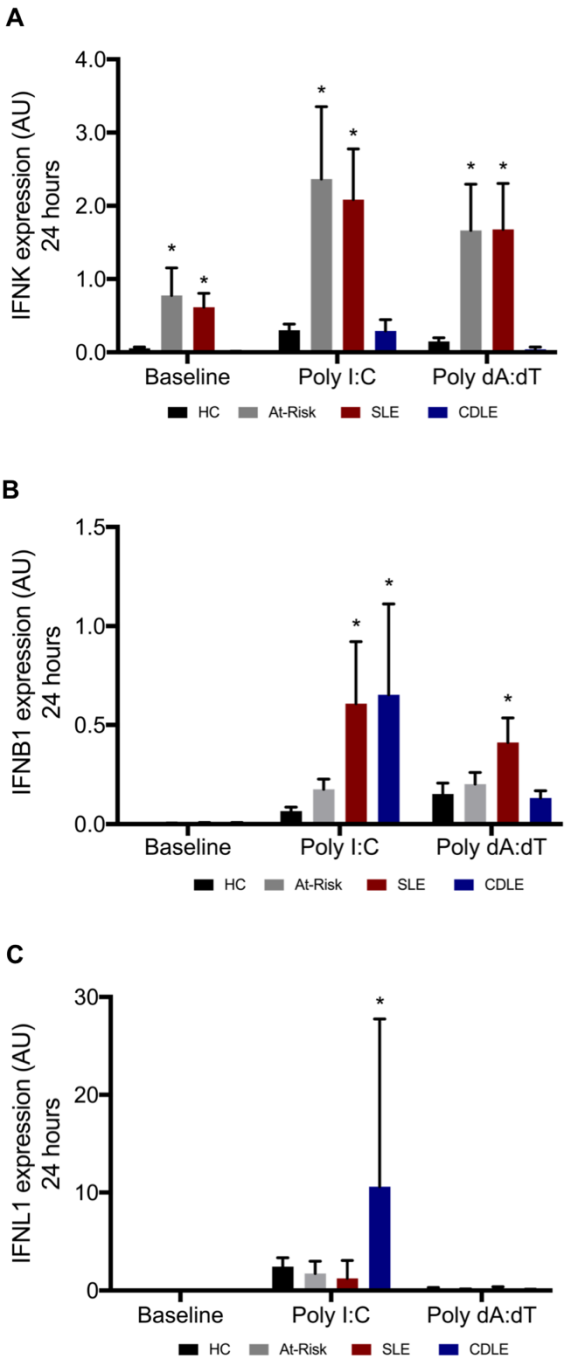


Figure 3.31 In vitro culture and stimulation of human keratinocytes. Human keratinocytes were isolated from fresh skin biopsies and were then cultured in the absence or presence of Poly I:C (1 µg/mL) or Poly dA:dT (100 ng/mL). Expression level of IFNK (A), IFNB1 (B), IFNL1 (C) in keratinocytes from healthy controls (HC), At-Risk individuals (At-Risk), SLE patients (SLE), and patients with cutaneous discoid lupus erythematosus (CDLE) after *in vitro* culture for 24 hours. Data are represented as mean ±SEM. *$P < 0.05$.

3.3 Discussion

Type I IFNs are crucial mediators linking innate and adaptive immune responses and are recognized as key players in the pathogenesis of systemic autoimmune diseases. Although the main production of type I IFNs derives from pDCs during antiviral responses, the mechanism of IFN regulation in SLE seems more complicated than previously thought. Previous studies reported contradictory data about the numbers of pDCs in peripheral blood of SLE patients (329-331). The results presented in the current study confirmed that circulating pDCs were indeed decreased not only in SLE but also in pSS, while this feature was expanded to a stage of benign preclinical autoimmunity. Interestingly, the reduction in pDC numbers showed no association with the status of IFN activity, clinical disease activity, serology, treatment and it was independent of lymphopenia observed in SLE patients. The migration of pDCs to sites of inflammation, for instance skin lesions, was a valid hypothesis (332). Nevertheless, this hardly explains the fact that SLE patients in clinical remission or At-Risk individuals with no evidence of inflammation count low pDC numbers. These observations strongly suggest the lack of association between pDCs and type I IFN activity seen in autoimmunity.

Regarding the secretory function of pDCs in SLE, I reported the lack of IFN-α and TNF-α production upon TLR stimulation. Again, this feature was expanded to pDCs of patients with pSS as well as At-Risk individuals, although the latter could partially maintain a TLR7-mediated cytokine production. Previous data reported an impaired IFN-α-producing capacity of PBMCs in SLE patients in response to TLR9 stimulation, while a novel assay to measure human IFN-α demonstrated a striking presence of IFN-α in pDCs of STING patients, but it failed to confirm this finding in SLE (325, 331).

Trex1-deficient mice failed to regulate STING-mediated antiviral response leading to aberrant type I IFN production that initiated from non-haematopoietic cells (326). Experimental work on lupus-prone mice reported a gradual loss of pDC capacity to produce IFN-α at late stage of disease course (333, 334). Particularly, in the MRL/Mp-*Fas* ^{lpr} (*lpr*) mouse model was found that pDCs expressed an increased level of MHC-II suggesting a functional drift to antigen presentation. However, our pDC-T cell co-culture revealed that pDCs from SLE were significantly defective in triggering T cell proliferation and activation, while they could potentially have a more regulatory role inducing T cell anergy.

Recent findings on Systemic Sclerosis reported the abnormal expression of TLR8 in pDCs that leads to IFN-α production suggesting a key pathological role of RNA-sensing TLR involvement in the establishment of fibrosis (335). However, in our RNA-sequencing data in pDCs sorted from At-Risk individuals or SLE, we could not confirm positive expression of TLR8 in any of the samples.

It is noticeable that impaired pDC-derived IFN-α production is not uncommon in cancer and chronic viral infections. In melanoma and ovarian cancer, tumour-infiltrating pDCs do not produce IFN-α but actually their presence is associated with tumour growth (336-338). Additionally, hepatitis B virus can interfere with TLR9 pathway by blocking MyD88-IRAK4 signalling and Sendai virus by targeting IRF7, while HIV impairs IFN-α production in pDCs via SYK phosphorylation (339, 340).

Within pDC population, there are distinct subsets that can mediate different immune functions (341). Single-cell RNA-sequencing data revealed the diversification of human pDCs in response to influenza virus into three phenotypes (P1-, P2-, P3-pDCs) with distinct transcriptional profiles and functions (342). pDCs from SLE patients

were mostly similar to the P1-phenotype, which represented the conventional secretory function and morphology known about pDCs. This is consistent with the findings presented above that SLE pDCs demonstrated decreased ability to induce $CD4^{+}CD25^{high}FoxP3^{+}$ T cells, the numbers and function of which are known to be impaired in patients with active SLE (343-345). Apart from that, the RNA-sequencing data reported that type I IFN activity was overall increased in pDCs of At-Risk and SLE patients compared to those of healthy controls, even though pDCs from IFN^{low} or IFN^{high} SLE patients had distinct transcriptomic profiles related to different biological pathways. However, pDCs from both subgroups differentially expressed genes that are well known to be involved in cellular senescence and stress, negative regulation of TLR and MAPK pathways as well as IL-10 signalling downstream, which can inhibit cytokine production and survival of pDCs (346-348). Intriguingly, aging was shown to affect IFN-α-producing capacity of human pDCs by impairing IRF7 and PI3K pathways, while this defect was often associated with age-induced cellular stress (349-351). I confirmed that pDCs from SLE had increased telomere erosion, while oxidative stress had a deleterious effect on IFN-α production. Altogether, these findings suggest that pDCs in SLE could present increased biological aging as a regulatory mechanism to control systemic immune activation.

IFN-κ is predominantly expressed by human keratinocytes with pleiotropic effects similar to IFN-α/β (352). Keratinocytes have been implicated in the pathogenesis of skin injury in SLE undergoing apoptosis or necrosis and eventually realising autoantigens (65). Previous studies demonstrated that keratinocytes from patients with cutaneous lupus erythematosus presented increased production of IL-6 compared to healthy controls, with type I IFNs enhancing this process (353). In

addition, *IFNK* expression was reported to be significantly increased in lesional skin of patients with cutaneous lupus erythematosus related to photosensitivity (354).

By using a novel assay for *in situ* hybridization, I was able to visualize the direct expression of type I IFN transcripts in human skin biopsies and not using a surrogate marker for IFN response (e.g. MxA). I demonstrated that keratinocytes of SLE patients exhibited enhanced IFN-κ-producing capacity at baseline or after stimulation with nucleic acids, a feature that was more prominent in cells of At-Risk individuals, possibly due to lack of any medication. Notably, diffuse *IFNK* expression was prominent in individuals with high IFN activity in blood but no skin inflammation, suggesting that keratinocytes can be predisposed to excessive type I IFN production in response to environmental triggers such as UV radiation. Increased recognition of danger signals from keratinocytes of predisposed individuals could potentially trigger enhanced IFNK production. Indeed, I demonstrated that UV provocation, a well-known trigger for lupus pathology, induced higher expression of *IFNK* by keratinocytes *in vivo*. Interestingly, the failure to detect IFNK expression in keratinocytes from ANA negative CDLE patients, who exhibited minimal IFN activity in blood, may suggest an alternative pathophysiology with other inflammatory mediators demonstrating a more prominent role compared to type I IFNs. Further, the types of IFN produced in vitro after culture and stimulated with TLR3 and RIG-I agonists varied between systemic and discoid lupus as well as preclinical autoimmunity. These results therefore indicated production of IFN by non-haematopoietic cells in the absence of production by pDCs or tissue leucocytes early in the initiation of autoimmunity and in a disease-specific manner.

In conclusion, while the importance of type I IFN in SLE is undeniable, the reasons for the failure of normal regulation of its production have never been clear. The current work provides an explanation for this dysregulation by demonstrating an abnormal source of IFN in non-haematopoietic tissue cells; whilst the professional IFN-α-producing pDCs have lost their major immunogenic properties. The presence of this phenotype in the epidermis of At-Risk individuals indicates that these cells, rather than being a passive target of inflammatory processes, play an active role at a very early stage of disease development. In established autoimmunity, these insights indicate potential therapeutic targets outside the conventional immune system. Moreover, in the At-Risk stage, this is the first report on where IFN dysregulation occurs and how it might be targeted for disease prevention.

Key messages:

- Type I IFN activity is increased in patients with SLE and pSS as well as At-Risk individuals compared to healthy controls.
- The number of pDCs in peripheral blood is significantly decreased in patients with SLE and pSS as well as At-Risk individuals independently of disease activity, type I IFN activity, and treatment.
- The capacity of pDCs to produce cytokines in response to TLR stimulation is significantly impaired in patients with SLE and pSS as well as At-Risk individuals compared to healthy controls.
- The capacity of pDCs to induce T cell activation and proliferation is significantly impaired in patients with active SLE compared to healthy controls.
- pDCs from SLE patients with low and high IFN activity present distinct transcriptional signatures.
- pDCs from SLE patients present transcriptional and phenotypic features of immune senescence.
- Type I IFN activity is significantly increased in lesional and non-lesional skin biopsies of SLE patients and At-Risk individuals.
- The epidermis of lesional and non-lesional skin biopsies of SLE patients and At-Risk individuals, but not leucocyte-infiltrating areas, is characterised by diffuse expression of type I IFNs.
- Keratinocytes from SLE patients and At-Risk individuals present increased expression of type I IFNs in response to UV light and nucleic acids.

CHAPTER 4.

TNF-α REGULATES THE PHENOTYPE AND FUNCTION OF HUMAN PLASMACYTOID DENDRITIC CELLS

4.1 Introduction

Human plasmacytoid dendritic cells (pDCs) consist of a distinct dendritic cell population that play a vital role in modulating immune responses. A common DC progenitor in the bone marrow can generate both pDCs and conventional DCs (cDCs), but pDCs are unique in their ability to produce type I interferons (IFNs) in response to viral infection (119). Upon ligation of TLR7 and TLR9 with exogenous or endogenous nucleic acids, pDCs secrete massive amounts of type I IFNs, predominantly IFN-α, and other pro-inflammatory cytokines leading to activation of both innate and adaptive immune compartments such as enhancement of NK cell cytotoxicity, effector $CD4^+$ and $CD8^+$ T cell responses, B cell differentiation into plasma cells and antibody production (115, 155, 355-358).

Although not as efficient as cDCs, pDCs express MHC class II molecules and they are able to capture, process and present antigens to $CD4^+$ T cells inducing their activation (149, 150). TLR-activated pDCs have enhanced antigen-presenting function and can promote Th1 and Th17 differentiation (151-153). Despite their weaker antigen-presenting properties, pDCs can also cross-present exogenous antigens to $CD8^+$ T cells and therefore induce antiviral and antitumor responses (155-157). However, unstimulated or HIV-stimulated pDCs are predominantly known to be involved in the induction of tolerogenic immune responses by expressing indoleamine-2,3-

dioxygenase (IDO) and promoting $CD4^+$ T cell anergy and T_{reg} differentiation (158-162). In addition, pDCs can secrete granzyme B, which impairs their capacity to induce T cell proliferation (359).

As the main drivers of type I IFN responses, pDCs have been implicated in many diseases, especially chronic viral infections, cancer and autoimmunity (321, 360, 361). Multiple regulatory surface receptors (e.g., BDCA-2, ILT7, BST2, NKp44) control the aberrant production of type I IFNs by TLR-activated pDCs (362-364). Cross-regulation of TNF-α and IFN-α appears to be important in many immune-mediated diseases (365, 366). TNF-α downregulates the influenza-induced IFNα production and strongly inhibits the *in vitro* generation of pDCs by $CD34^+$ hematopoietic progenitors (367).

In this study, I investigated the regulatory role of TNF-α on the phenotype and function of blood-purified human pDCs. I found that TNF-α is a major cytokine produced alongside IFN-α by TLR9- or TLR7-stimulated pDCs and that exogenous TNF-α strongly inhibited both IFN-α and TNF-α production, an effect which is predominantly TLR9- and less TLR7-mediated. Additionally, TNF-α induced a distinct transcriptomic profile to pDCs by promoting pathways related to antigen processing and presentation as well as T cell activation and differentiation. Even though TNF-treated pDCs failed to produce type I IFNs, they indeed induced higher T cell proliferation, activation and differentiation towards Th1 and Th17. Our findings demonstrate that TNF-α is a major regulator of human pDCs and can enhance their function by switching their main role as IFN-α-producing cells to a more conventional DC phenotype.

4.2 Results

4.2.1 Human pDCs produce both IFN-α and TNF-α in response to TLR9 and TLR7 agonists

Although pDCs are mostly recognised for their IFN-α-producing capacity, they are also capable of producing other pro-inflammatory cytokines. To evaluate the level of this, peripheral blood pDCs were analysed by flow cytometry for the production of both IFN-α and TNF-α upon stimulation with TLR9 (ODN 2216) or TLR7 (ORN R-2336) agonists. Freshly isolated PBMCs were cultured with 2µM ODN 2216 or 2µM ORN R-2336 agonists for 6 hours and then pDCs were gated as lineage^{-}HLA-DR^{+}CD123^{hi}CD303^{+} cells (**Figure 4.1A**).

Cytokine production was measured using intracellular staining. As previously described, circulating pDCs produced no IFN-α and/or TNF-α without external stimulation (**Figure 4.1B**). After external stimulation with TLR9 agonist, three major populations of pDCs could be observed: (1) non-producers, (2) TNF-α-producers, (3) IFN-α- and TNF-α-producers (**Figure 4.1C**). Similar results could be seen when the cells were stimulated with TLR7 agonist (**Figure 4.1D**). Thus, TNF-α is a major cytokine produced by TLR9 or TLR7 stimulated pDCs, while the co-expression with IFN-α may suggest a cross-regulation between the two.

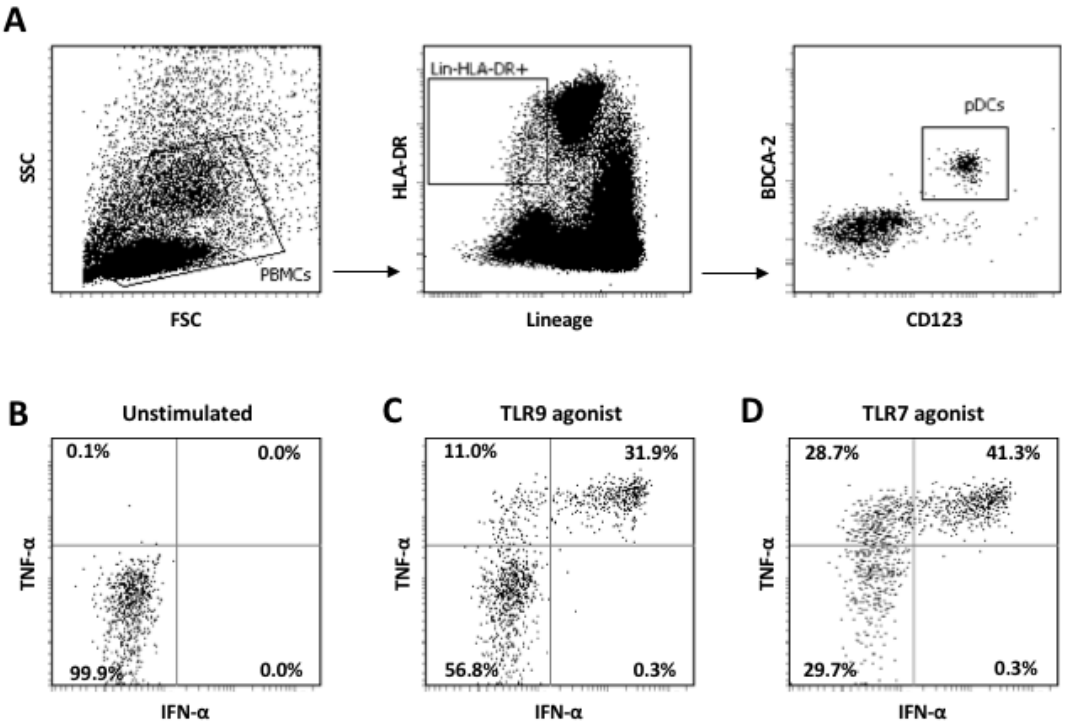


Figure 4.1 Human pDCs produce both IFN-α and TNF-α in response to TLR9 and TLR7 agonists. (A) Gating strategy for human pDCs; pDCs are characterised by the lack of lineage markers (CD3, CD19, CD14, CD56, CD11c), intermediate to high expression of MHC class II (HLA-DR), high expression of CD123 and CD303 (BDCA-2). Freshly isolated PBMCs were cultured and stimulated with TLR9 (ODN 2216) or TLR7 (ORN R-2336) agonists for 6 hours, then IFN-α and TNF-α production was detected using intracellular staining. (B) Unstimulated pDCs produced no IFN-α and/or TNF-α. (C and D) Upon stimulation with TLR9 or TLR7 agonists, there were 3 major pDC populations: (1) non-producers, (2) TNF-α-producers, (3) IFN-α- and TNF-α-producers. Results shown are representative of three independent experiments.

4.2.2 TNF-α regulates IFN-α and TNF-α production in TLR-stimulated pDCs

To unravel the role of TNF-α on pDC function, I first investigated the effect of TNF-α on cytokine production in the presence or absence of TLR stimulation. Freshly isolated PBMCs were cultured in the absence or presence of different concentrations of recombinant human TNF-α (1 – 50 ng/mL) for 24 hours. No induction of IFN-α

and/or TNF-α production was observed in TNF-treated pDCs without exogenous stimulation. However, treatment with TNF-α significantly altered the function of pDCs to produce pro-inflammatory cytokines in response to stimulation with TLR9 or TLR7 agonists, as detected by intracellular staining (**Figures 4.2 & 4.3)**.

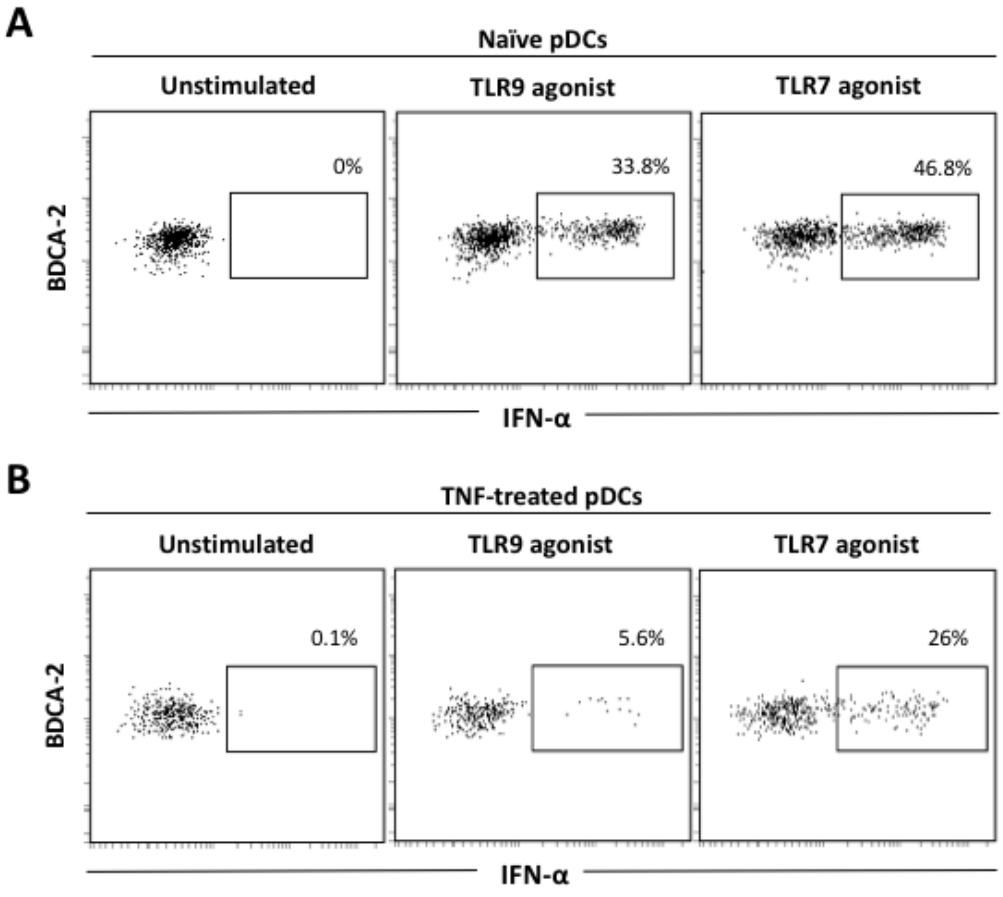


Figure 4.2 TNF-α regulates IFN-α production in TLR-stimulated pDCs. (A-B) Freshly isolated PBMCs were cultured in the absence or presence of recombinant human TNF-α. After 24 hours, PMBCs were washed twice and stimulated with TLR9 (ODN 2216) or TLR7 (ORN R-2336) agonists for 6 hours, then IFN-α production by pDCs was measured using intracellular staining. Results shown are representative of three independent experiments.

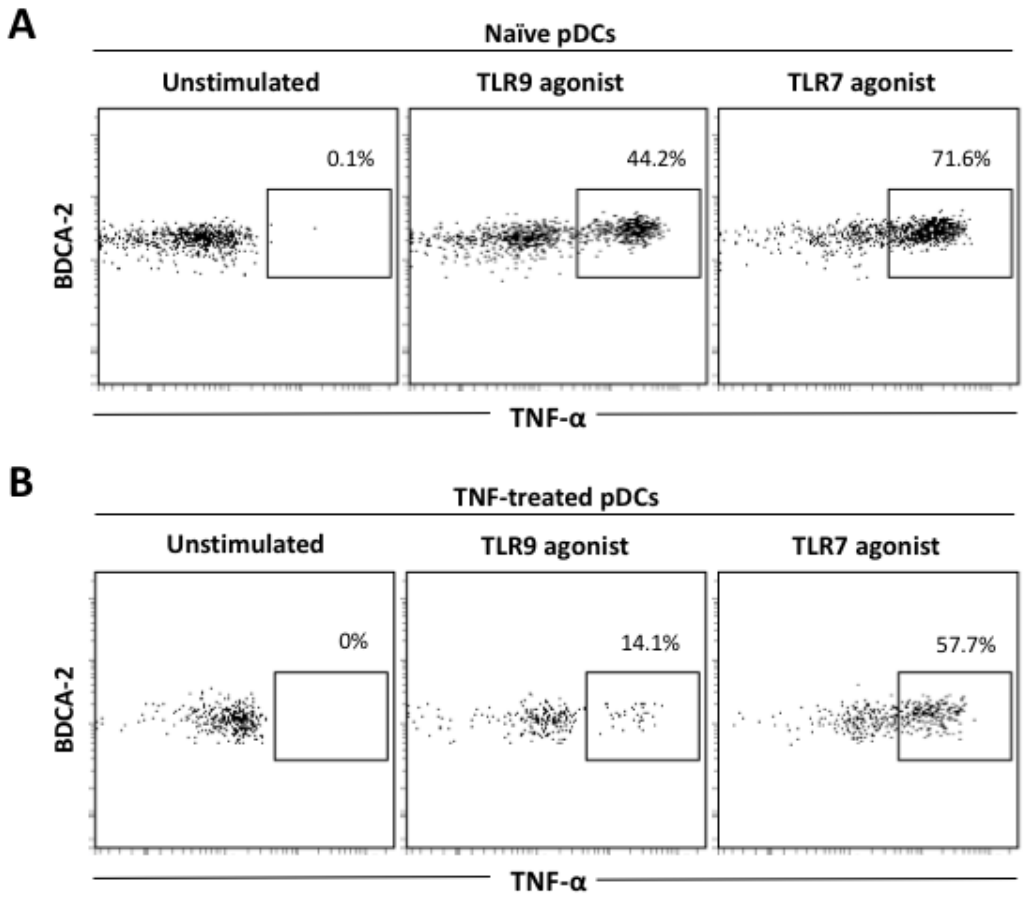


Figure 4.3 TNF-α regulates TNF-α production in TLR-stimulated pDCs. (A-B) Freshly isolated PBMCs were cultured in the absence or presence of recombinant human TNF-α. After 24 hours, PMBCs were washed twice and stimulated with TLR9 (ODN 2216) or TLR7 (ORN R-2336) agonists for 6 hours, then TNF-α production by pDCs was measured using intracellular staining. Results shown are representative of three independent experiments.

In particular for TLR9 activation, exogenous TNF-α (1 ng/mL) strongly inhibited both IFN-α (**Figure 4.4A**) and TNF-α (**Figure 4.4B**) production by pDCs, while no significant further reduction was observed at higher concentrations (10 ng/mL – 50 ng/mL). For TLR7 activation, exogenous TNF-α (1 ng/mL) had a similar effect on inhibiting IFN-α production with a further reduction at higher concentrations (20 – 50 ng/mL); a significant reduction in TNF-α production by TLR7-stimulated pDCs was only seen at higher concentrations (20 – 50 ng/mL) of exogenous TNF-α.

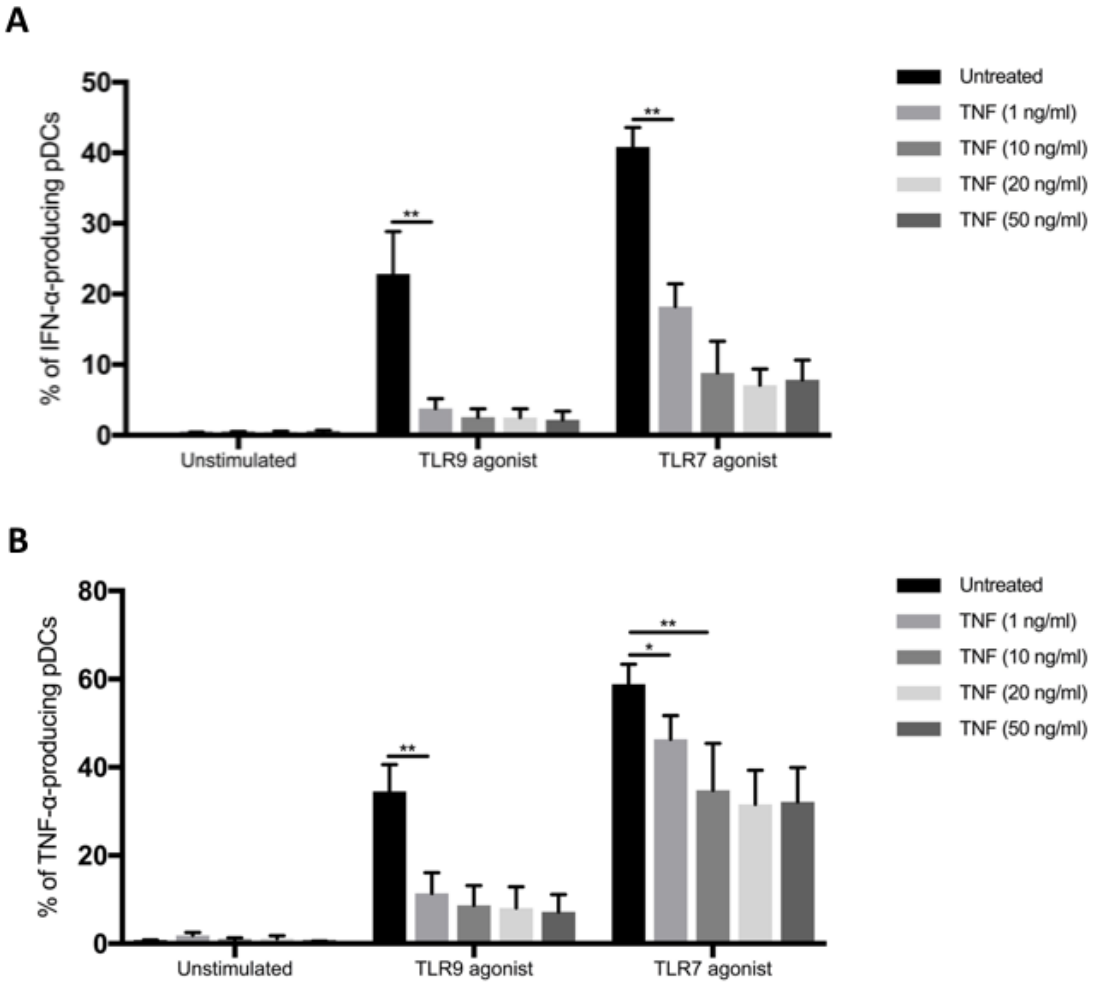


Figure 4.4 TNF-α regulates IFN-α and TNF-α production in TLR-stimulated pDCs. (A-B) Freshly isolated PBMCs were cultured in the absence or presence of recombinant human TNF-α (1 ng/ml, 10 ng/ml, 20 ng/ml, 50 ng/ml). After 24 hours, PMBCs were washed twice and stimulated with TLR9 (ODN 2216) or TLR7 (ORN R-2336) agonists for 6 hours, then IFN-α and TNF-α production by pDCs was measured using intracellular staining). Bars represent median value with 95% CI. *P < 0.05, **P < 0.01

Considering the above findings of the effect exogenous TNF-α on pDCs, I examined whether neutralization of endogenous TNF-α had an impact on IFN-α production. I isolated a pDC-enriched population from PBMCs by negative selection using magnetic beads (purity >92%) and the cells were stimulated with ODN 2216 or ORN R-2336 in the absence or presence of anti-TNF antibody or isotype control. After 24

hours, the supernatants were collected and IFN-α production was measured by ELISA (**Figure 4.5A**). The cells were then washed twice, re-stimulated with ODN 2216 or ORN R-2336 and the supernatants were collected after additional 24 hours. IFN-α production was measured by ELISA (**Figure 4.5B**). In the first culture (0 – 24 h), neither anti-TNF neutralizing antibody nor isotype control altered the levels of IFN-α secreted. However, in the secondary culture (24 – 48 h), anti-TNF-treated pDCs re-stimulated with ODN 2216 (TLR9 agonist) could partially maintain IFN-α secretion in comparison with the control-treated pDCs. This effect could not be seen in pDCs re-stimulated with ORN R-2336 (TLR7 agonist), as the levels of IFN-α secreted were similar in both anti-TNF- and control-treated pDCs. Taken all together, TNF-α regulates IFN-α production in a manner that is predominantly TLR9-mediated.

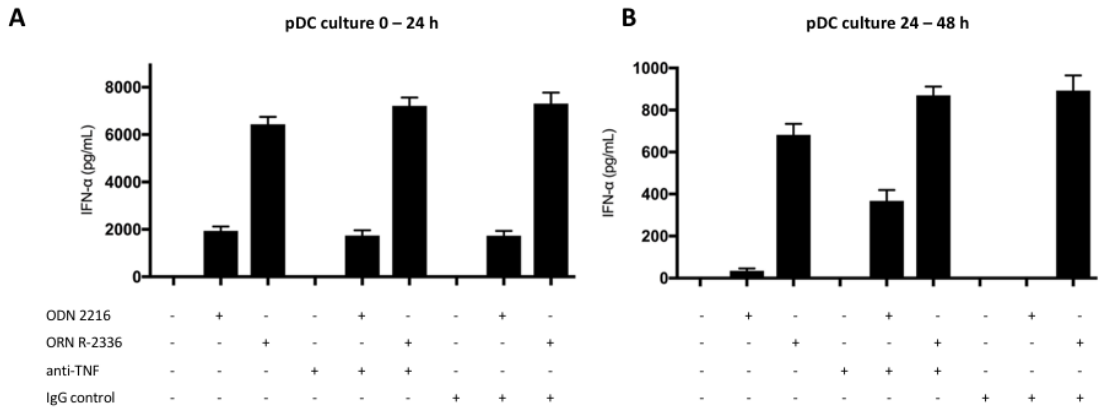


Figure 4.5 Purified pDCs were stimulated with TLR9 or TLR7 agonists in the absence or presence of anti-TNF antibody or isotype control. (A) After 24 hours, the supernatants were collected and IFN-α production was measured by ELISA (0 – 24 h). (B) pDCs were washed twice, re-stimulated with TLR9 and TLR7 agonists and the supernatants were collected after additional 24 hours. IFN-α production was measured by ELISA (24 -48 h). Bars represent median value with 95% CI.

4.2.3 RNA-sequencing: data generation

The next step was to investigate how TNF-α regulates TLR-mediated cytokine production and induces further transcriptional changes in human pDCs. pDCs from healthy subjects (n = 3) were purified by negative selection using magnetic beads (Miltenyi Biotec). The pre-enriched pDCs (purity > 85%) from each donor were counted using an automated cell counter (Beckmann Coulter) and then divided into two aliquots before they were cultured in RPMI medium 1640 with GlutaMAX supplement (ThermoFisher Scientific) containing 10% (vol/vol) FBS and 100 U/ml penicillin/streptomycin in the presence or absence of human recombinant TNF-α (10 µL/mL) for 18 hours. After incubation, untreated and TNF-treated pre-enriched pDCs from all three donors (n = 6) were washed thoroughly before they were finally sorted using BD Influx 6 Way Cell Sorter (BD Biosciences) based on CD304 (BDCA-4) expression to achieve purity > 99%. Unlike CD303 (BDCA-2) that is known to inhibit IFN-α production, CD304 (BDCA-4) does not interfere with the type I IFN pathway. After sorting, RNA was purified by sorted pDCs using PicoPure RNA Isolation kit (ThermoFisher Scientific) and quantified using Qubit RNA HS Assay Kit (Thermo Fisher Scientific). RNA libraries were made by using SMART-Seq V4 ultra low Input RNA Kit (Takara Bio USA) and Nextera XT DNA Library Preparation Kit (Illumina) for Next Generation Sequencing. Indexed sequencing libraries were pooled and sequenced on a single lane on HiSeq 3000 instrument as 151bp paired-end reads. Pooled sequence data was demultiplexed using Illumina bcl2fastq software allowing no mismatches in the read index sequences.

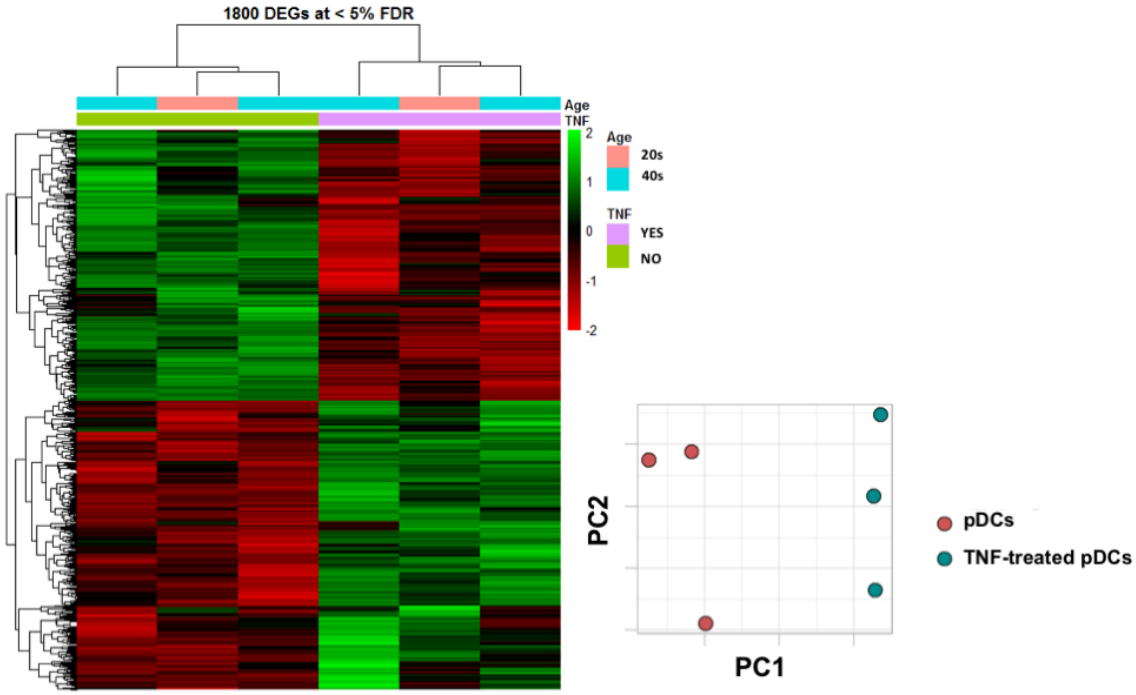


Figure 4.6 All 1,800 differentially expressed genes (DEGs) at < 5% false discovery rate (FDR) between untreated and TNF-treated pDCs. Principal component analysis (PCA) shows that the main source of variation in the data derives from the treatment with TNF-α.

Principal component analysis (PCA) demonstrated that the main source of variation in each sample derived from the treatment with TNF-α (**Figure 4.6**). In total, the analysis indicated 1,800 differentially expressed genes (DEGs) at < 5% false discovery rate (FDR) between untreated and TNF-treated pDCs. The top 100 upregulated and downregulated genes by TNF-α in pDCs can be found in **Table 4.1** and **Table 4.2** respectively.

	pDCs vs. TNF-treated pDCs		
Gene	**Fold Change (log2)**	***P* value**	**FDR**
CRIP2	6.846	< 0.001	< 0.001
FXYD2	3.599	< 0.001	< 0.001
FXYD6-FXYD2	3.575	< 0.001	< 0.001
ANO9	4.520	< 0.001	< 0.001
CRIP1	2.803	< 0.001	< 0.001
TYW3	2.740	< 0.001	< 0.001
TNFRSF4	3.115	< 0.001	< 0.001
HDGFRP3	4.350	< 0.001	< 0.001
HLA-DQA1	2.169	< 0.001	< 0.001
CCDC28B	2.696	< 0.001	< 0.001
BCL2A1	2.589	< 0.001	< 0.001
TMEM138	2.036	< 0.001	< 0.001
TFEC	2.382	< 0.001	< 0.001
UNQ6494	5.108	< 0.001	< 0.001
CTSH	2.375	< 0.001	< 0.001
KDM2B	2.617	< 0.001	< 0.001
PDGFA	4.535	< 0.001	< 0.001
CDKN1A	2.015	< 0.001	< 0.001
HLA-DQB1	1.814	< 0.001	< 0.001
NEK6	2.364	< 0.001	< 0.001
CD83	1.929	< 0.001	< 0.001
EDNRB	4.598	< 0.001	< 0.001
FAS	3.784	< 0.001	< 0.001
LAGE3	2.247	< 0.001	< 0.001
RELB	2.367	< 0.001	< 0.001
CRYZ	1.981	< 0.001	< 0.001
IL6ST	2.175	< 0.001	< 0.001
GPX4	1.646	< 0.001	< 0.001
LSR	2.904	< 0.001	< 0.001
GGT1	3.310	< 0.001	< 0.001
KREMEN2	3.689	< 0.001	< 0.001
BID	1.578	< 0.001	< 0.001
MAD2L2	1.817	< 0.001	< 0.001
UQCC2	2.382	< 0.001	< 0.001
DPCD	3.829	< 0.001	< 0.001
SEMA4A	1.986	< 0.001	< 0.001
DNASE1L3	1.411	< 0.001	< 0.001
HSD17B10	1.858	< 0.001	< 0.001
BTN2A2	2.168	< 0.001	< 0.001
TFRC	1.855	< 0.001	< 0.001
SSH1	2.579	< 0.001	< 0.001
CX3CR1	2.330	< 0.001	< 0.001
WNT10A	2.311	< 0.001	< 0.001
ANK3	3.667	< 0.001	< 0.001

	pDCs vs. TNF-treated pDCs		
Gene	**Fold Change (log2)**	***P* value**	**FDR**
MARCKS	2.336	< 0.001	< 0.001
CD59	2.135	< 0.001	< 0.001
TIMM10	1.838	< 0.001	< 0.001
TNFAIP2	2.723	< 0.001	< 0.001
HLA-DQB2	1.817	< 0.001	< 0.001
HLA-DQA2	1.857	< 0.001	< 0.001
HIVEP3	3.310	< 0.001	< 0.001
SWAP70	2.380	< 0.001	< 0.001
HLA-DRB1	1.180	< 0.001	< 0.001
MGLL	1.619	< 0.001	< 0.001
SYNGR2	1.270	< 0.001	< 0.001
FSCN1	2.109	< 0.001	< 0.001
NME1	2.258	< 0.001	< 0.001
TVP23A	2.441	< 0.001	< 0.001
BLVRB	2.908	< 0.001	< 0.001
SEC61B	1.157	< 0.001	< 0.001
TMEM120B	1.773	< 0.001	< 0.001
GPATCH4	2.068	< 0.001	< 0.001
NCCRP1	3.587	< 0.001	< 0.001
RHOF	1.689	< 0.001	< 0.001
DGAT2	3.027	< 0.001	< 0.001
TXN	1.243	< 0.001	< 0.001
DDB2	3.466	< 0.001	< 0.001
NFKBIA	1.352	< 0.001	< 0.001
DCANP1	2.159	< 0.001	< 0.001
DNPH1	1.714	< 0.001	< 0.001
TIGIT	3.193	< 0.001	< 0.001
ICAM1	1.579	< 0.001	< 0.001
GRHPR	1.572	< 0.001	< 0.001
HLA-F	1.592	< 0.001	< 0.001
TLCD1	2.062	< 0.001	< 0.001
FDPS	1.324	< 0.001	< 0.001
CYB5A	1.984	< 0.001	< 0.001
CFLAR	1.430	< 0.001	< 0.001
PPIL1	3.010	< 0.001	< 0.001
TTC39A	2.011	< 0.001	< 0.001
PPA1	2.043	< 0.001	< 0.001
RPSA	1.146	< 0.001	< 0.001
LRRC75A-AS1	1.299	< 0.001	< 0.001
BIRC3	1.575	< 0.001	< 0.001
TCEB3	1.299	< 0.001	< 0.001
HLA-DPB1	1.245	< 0.001	< 0.001
RSL1D1	1.270	< 0.001	< 0.001
ADAT2	2.994	< 0.001	< 0.001

	pDCs vs. TNF-treated pDCs		
Gene	**Fold Change (log2)**	***P* value**	**FDR**
SMS	2.337	< 0.001	< 0.001
FEZ1	3.242	< 0.001	< 0.001
ATOX1	1.333	< 0.001	< 0.001
LINC01268	3.205	< 0.001	< 0.001
MRPL14	1.615	< 0.001	< 0.001
HLA-DRA	1.116	< 0.001	< 0.001
IL10RA	1.289	< 0.001	< 0.001
HLA-DRB5	1.042	< 0.001	< 0.001
CLIC2	1.878	< 0.001	< 0.001
SPRED2	2.875	< 0.001	< 0.001
GRPEL1	1.667	< 0.001	< 0.001
NKG7	2.605	< 0.001	< 0.001

Table 4.1 Top 100 genes upregulated by TNF-α in pDCs.

	pDCs vs. TNF-treated pDCs		
Gene	**Fold Change (log2)**	***P* value**	**FDR**
CST3	-2.557	< 0.001	< 0.001
HS3ST1	-4.792	< 0.001	< 0.001
S100A4	-2.993	< 0.001	< 0.001
RASD1	-3.091	< 0.001	< 0.001
S100A6	-2.513	< 0.001	< 0.001
LOC100507600	-3.040	< 0.001	< 0.001
BTLA	-4.467	< 0.001	< 0.001
SCN9A	-2.466	< 0.001	< 0.001
PLXNA4	-3.152	< 0.001	< 0.001
IFITM2	-2.324	< 0.001	< 0.001
ACY3	-2.246	< 0.001	< 0.001
PLD4	-1.910	< 0.001	< 0.001
SPNS3	-2.950	< 0.001	< 0.001
PLP2	-1.707	< 0.001	< 0.001
LIME1	-2.156	< 0.001	< 0.001
MS4A6A	-1.850	< 0.001	< 0.001
TXNIP	-1.819	< 0.001	< 0.001
METTL7A	-2.991	< 0.001	< 0.001
RNA5S1	-1.692	< 0.001	< 0.001
RNA5S10	-1.692	< 0.001	< 0.001
RNA5S11	-1.692	< 0.001	< 0.001
RNA5S12	-1.692	< 0.001	< 0.001
RNA5S13	-1.692	< 0.001	< 0.001
RNA5S14	-1.692	< 0.001	< 0.001
RNA5S15	-1.692	< 0.001	< 0.001
RNA5S16	-1.692	< 0.001	< 0.001
RNA5S17	-1.692	< 0.001	< 0.001
RNA5S2	-1.692	< 0.001	< 0.001
RNA5S3	-1.692	< 0.001	< 0.001
RNA5S4	-1.692	< 0.001	< 0.001
RNA5S5	-1.692	< 0.001	< 0.001
RNA5S6	-1.692	< 0.001	< 0.001
RNA5S7	-1.692	< 0.001	< 0.001
RNA5S8	-1.692	< 0.001	< 0.001
CD300A	-2.557	< 0.001	< 0.001
TGFBR3	-3.922	< 0.001	< 0.001
CD99	-1.737	< 0.001	< 0.001
OAS1	-3.590	< 0.001	< 0.001
MIR7641-2	-1.636	< 0.001	< 0.001
PLAC8	-1.626	< 0.001	< 0.001
KIAA0125	-2.059	< 0.001	< 0.001
MMP23A	-3.094	< 0.001	< 0.001
GLIPR1	-1.619	< 0.001	< 0.001
THBS1	-2.809	< 0.001	< 0.001

FCER1A	-2.407	< 0.001	< 0.001
CMTM3	-2.069	< 0.001	< 0.001
ATP13A2	-2.403	< 0.001	< 0.001
DERL3	-1.505	< 0.001	< 0.001
PECAM1	-2.112	< 0.001	< 0.001
C12orf75	-1.411	< 0.001	< 0.001
IGFBP3	-2.742	< 0.001	< 0.001
NCF1B	-1.548	< 0.001	< 0.001
RUNX2	-1.786	< 0.001	< 0.001
SYNGR1	-2.075	< 0.001	< 0.001
RN7SL1	-1.195	< 0.001	< 0.001
GAPT	-1.333	< 0.001	< 0.001
CXCL16	-2.246	< 0.001	< 0.001
LOC101928034	-2.745	< 0.001	< 0.001
LILRB2	-3.286	< 0.001	< 0.001
RNF166	-2.115	< 0.001	< 0.001
RAB11FIP4	-3.369	< 0.001	< 0.001
GAS6	-1.888	< 0.001	< 0.001
CMTM7	-2.490	< 0.001	< 0.001
MAPKAPK2	-2.060	< 0.001	< 0.001
LILRA4	-1.351	< 0.001	< 0.001
EPS8L2	-2.669	< 0.001	< 0.001
FCER1G	-1.302	< 0.001	< 0.001
C1orf162	-1.706	< 0.001	< 0.001
TNFRSF17	-2.301	< 0.001	< 0.001
HHEX	-1.698	< 0.001	< 0.001
LINGO3	-3.276	< 0.001	< 0.001
HPCAL1	-2.144	< 0.001	< 0.001
CD164	-1.315	< 0.001	< 0.001
RN7SL2	-1.166	< 0.001	< 0.001
UCP2	-1.378	< 0.001	< 0.001
RGS14	-2.451	< 0.001	< 0.001
NCF1C	-1.264	< 0.001	< 0.001
LRRC25	-3.003	< 0.001	< 0.001
LOC102724297	-2.073	< 0.001	< 0.001
SPON2	-3.220	< 0.001	< 0.001
CD300LB	-3.146	< 0.001	< 0.001
CCR2	-2.201	< 0.001	< 0.001
ABHD15	-1.832	< 0.001	< 0.001
ZFP36L2	-1.464	< 0.001	< 0.001
PRICKLE3	-1.474	< 0.001	< 0.001
IFI44L	-3.043	< 0.001	< 0.001
CCR5	-3.003	< 0.001	< 0.001
ID3	-1.874	< 0.001	< 0.001
TXNDC5	-1.269	< 0.001	< 0.001
RAC2	-1.244	< 0.001	< 0.001

CYTH4	-1.356	< 0.001	< 0.001
TRAF3IP3	-1.442	< 0.001	< 0.001
PLCG2	-1.413	< 0.001	< 0.001
PROC	-1.653	< 0.001	< 0.001
MMP23B	-2.807	< 0.001	< 0.001
BLOC1S5-TXNDC5	-1.240	< 0.001	< 0.001
DUSP1	-1.689	< 0.001	< 0.001
CD52	-1.388	< 0.001	< 0.001
APBB1IP	-1.265	< 0.001	< 0.001
ANXA1	-1.885	< 0.001	< 0.001

Table 4.2 Top 100 genes downregulated by TNF-α in pDCs.

4.2.4 TNF-α promotes transcriptional changes associated with antigen processing and presentation

TNF-α induced the upregulation of genes in pDCs, which were particularly enriched for pathways associated with MHC class II antigen processing and presentation, Th17 differentiation, Th1 and Th2 differentiation, MHC class I antigen processing and cross-presentation, induction of TCR signalling and co-stimulation of CD28, phosphorylation of CD3 and TCR zeta chains, translocation of ZAP70 to immunological synapse amongst other pathways. A detailed presentation of enriched KEGG pathways and Reactome pathways in differentially expressed genes can be seen in **Figure 4.7** and **Figure 4.8** respectively. Among the most enriched biological processes induced by TNF-α were found to be lymphocyte aggregation, T cell activation, immune response-activating cell surface receptor signalling, antigen processing and presentation of exogenous antigen, T cell co-stimulation. Regarding the enriched cellular components in differentially expressed genes, these included units and functions mainly related to antigen processing and presentation (**Figure 4.9**); for instance, endocytic vesicle membrane, MHC class II protein complex, clathrin-coated vesicle membrane and other.

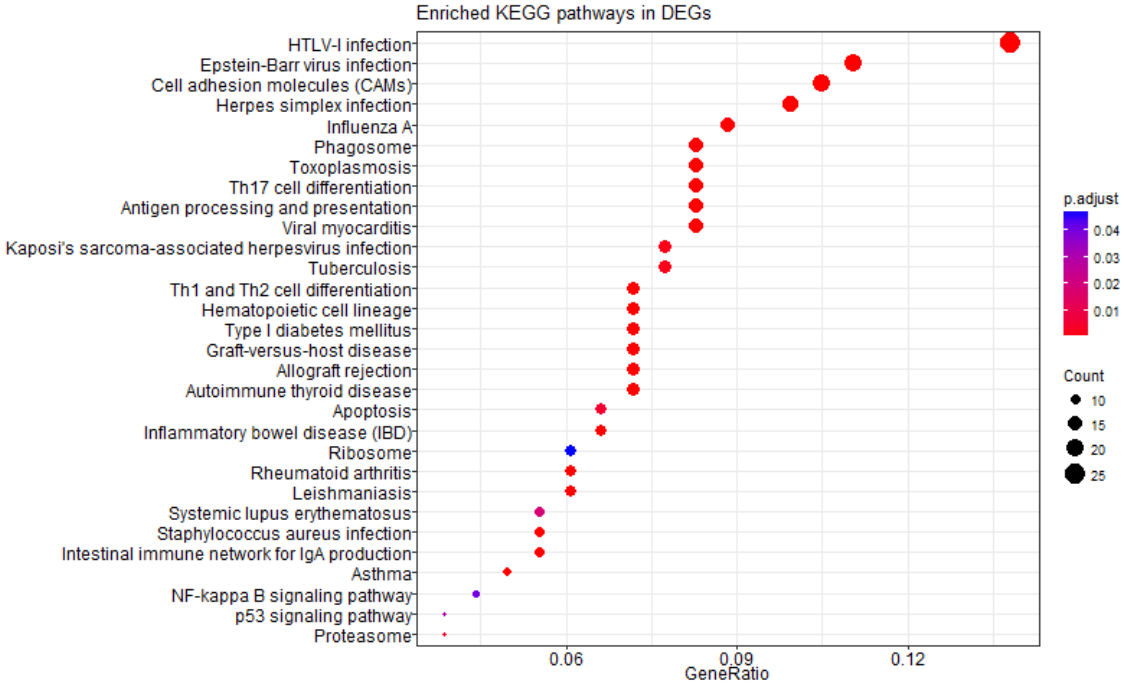


Figure 4.7 Enriched KEGG pathways in differentially expressed genes (DEGs) upregulated by TNF-α in pDCs.

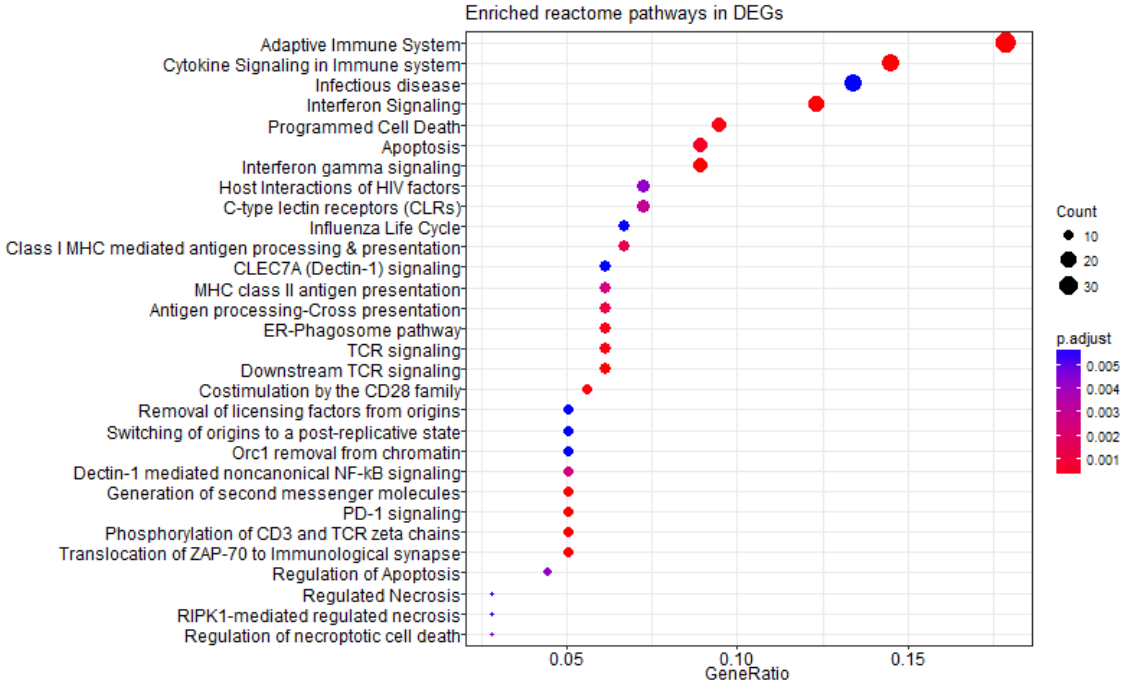


Figure 4.8 Enriched Reactome pathways in differentially expressed genes (DEGs) upregulated by TNF-α in pDCs.

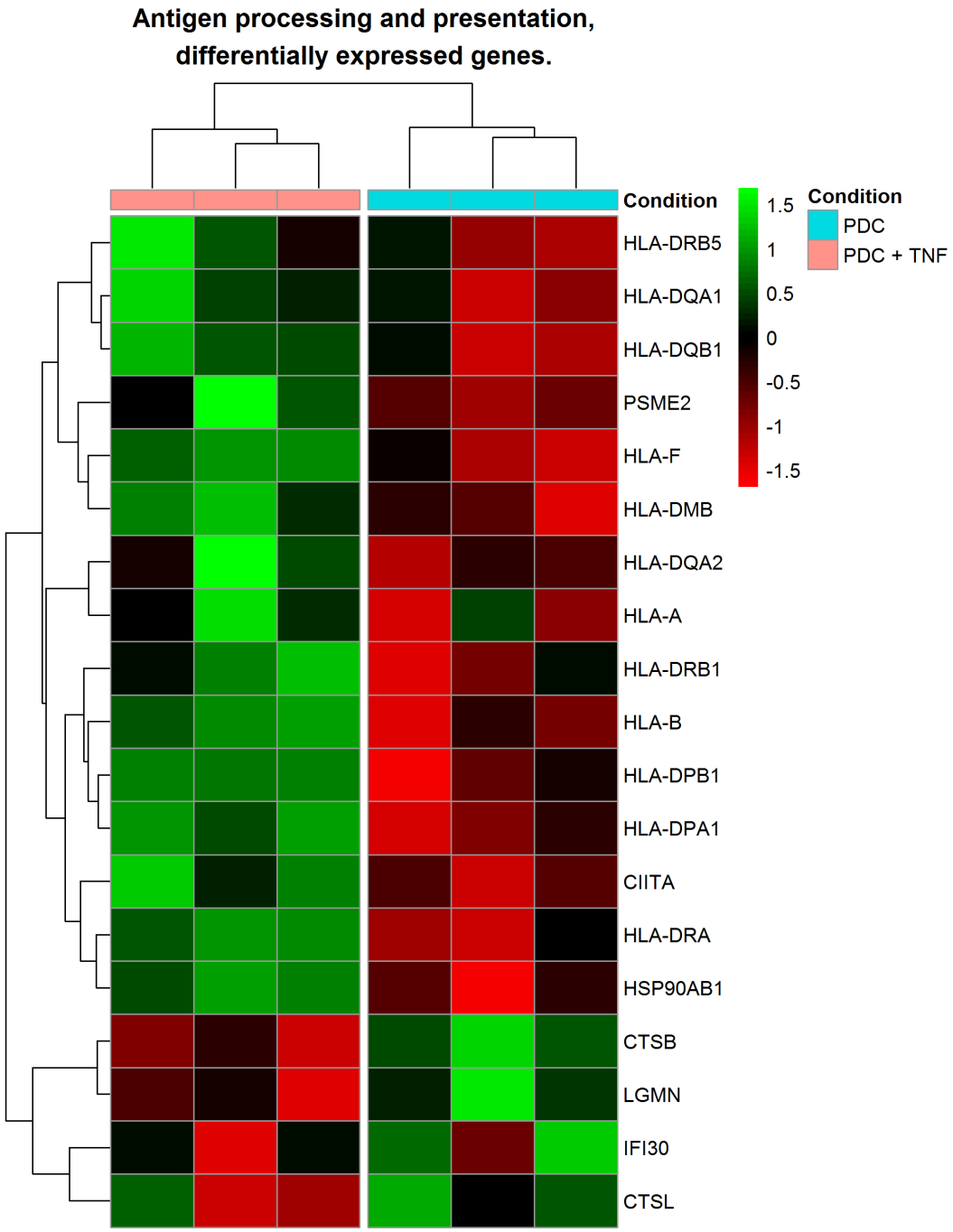


Figure 4.9 Heatmap showing that TNF-α promotes differentially expressed genes associated with antigen processing and presentation pathways in pDCs.

Among the upregulated genes in TNF-treated pDCs compared to the untreated ones were HLAs corresponding to MHC class I (*HLA-A*, *HLA-B*, *HLA-F*) as well as MHC class II (*HLA-DPA1*, *HLA-DPB1*, *HLAD-DRA*, *HLA-DRB1*, *HLA-DRB5*, *HLA-DQA1*, *HLA-DQA2*, *HLA-DQB1*, *HLA-DMB*).

4.2.5 TNF-α promotes transcriptional changes associated with T activation and differentiation

Analysing further the RNA-sequencing data for biological effects of TNF-α on the function of pDCs, differentially expressed genes associated with positive regulation of T cell proliferation and activation were particularly enriched (**Figure 4.10**). Apart from the upregulated MHC class II molecules, upregulated genes included co-stimulatory molecules (CD80, CD86, CD83), molecules promoting endocytosis (CD59) and cell adhesion (ADAM8), ICOS ligand (ICOSLG) as well as IL-27 subunit beta (EBI3). In contrast, downregulated genes included *CCR2*, *PTPRC*, *SYK*, *IL1B*, *LILRB2*, *SOCS1* amongst others.

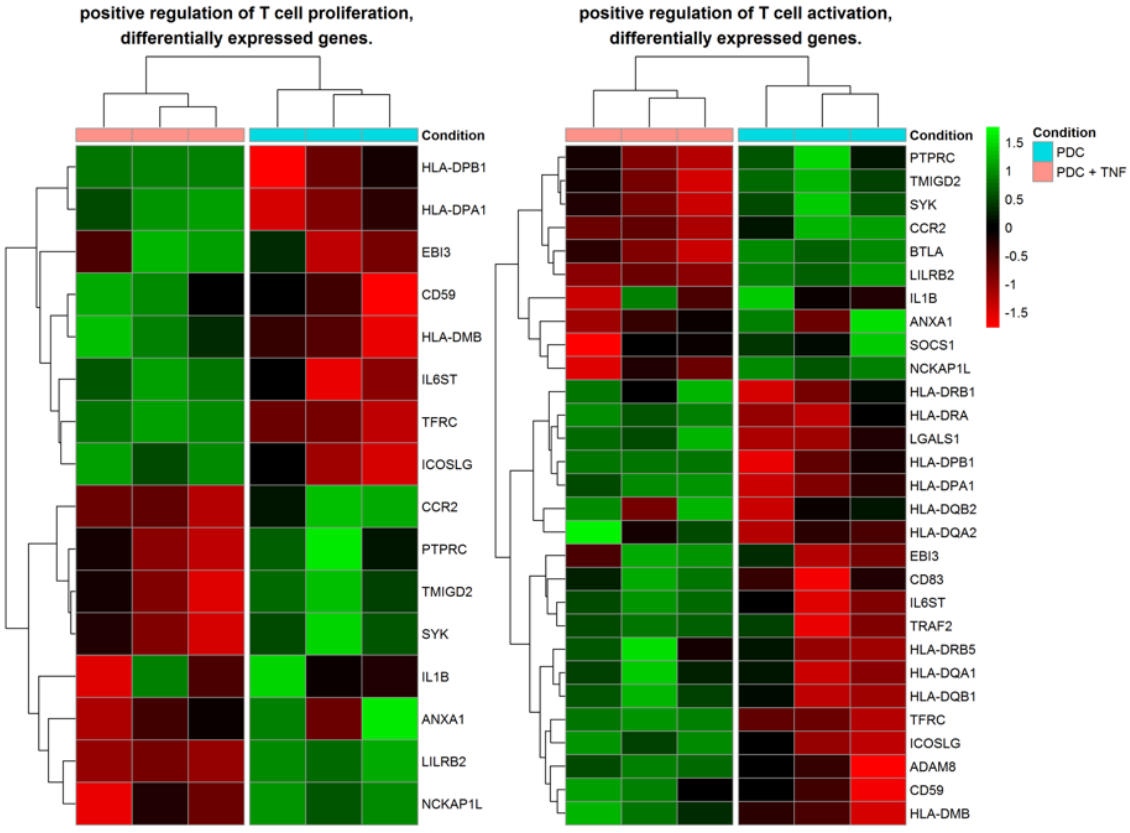


Figure 4.10 Heatmap of differentially expressed genes in TNF-treated vs. untreated pDCs showed significant enrichment in positive regulation of T cell proliferation and activation.

TNF-α also upregulated genes associated with T cell differentiation, for instance *RPL22*, *ADAM8*, *IRF4*, *CD83*, *RELB*, *FAS*, *SEMA4A,* whilst it downregulated others such as *SOX4, BATF, RUNX2, FCER1G* (**Figure 4.11**). In detail, RNA-sequencing data analysis suggested induction of T cell differentiation towards Th17, Th1 and Th2 (**Figure 4.12**).

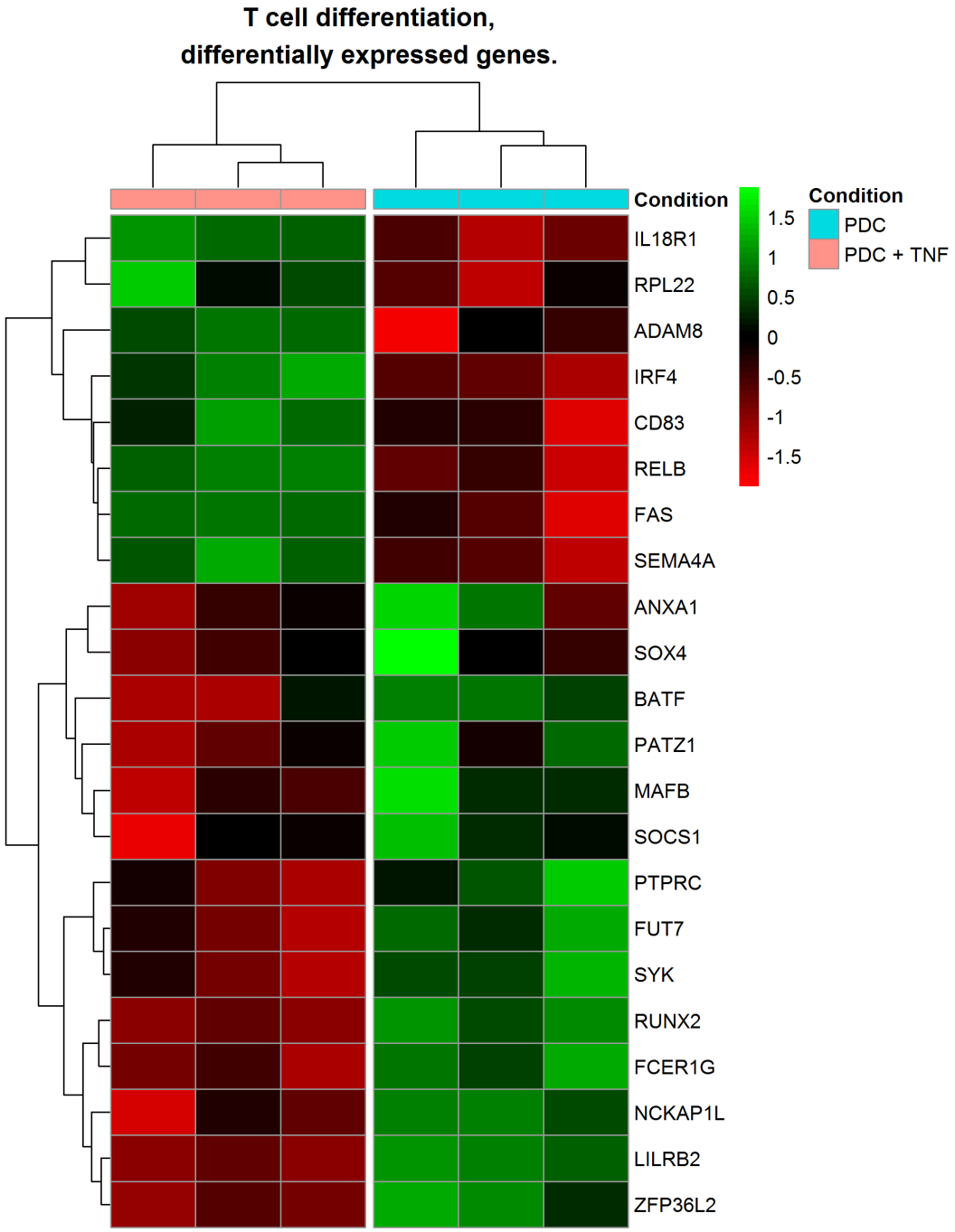


Figure 4.11 Heatmap of differentially expressed genes in TNF-treated vs. untreated pDCs showed significant enrichment in positive regulation of T cell differentiation.

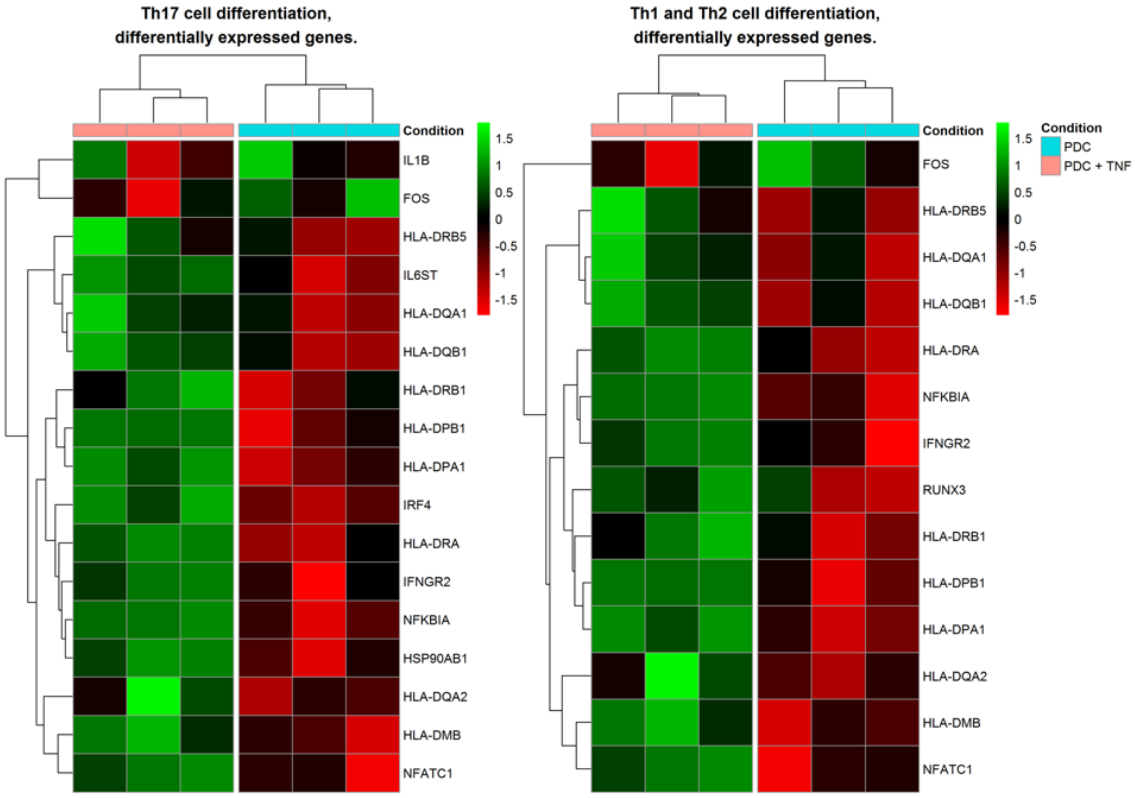


Figure 4.12 Heatmap of differentially expressed genes in TNF-treated vs. untreated pDCs showed significant enrichment in induction of Th17, Th1 and Th2 cell differentiation.

4.2.6 TNF-α inhibits TLR cascade signalling pathways

Despite the transcriptional changes towards antigen processing and presentation as well as T cell activation and differentiation, TNF-α seems to negative regulate other functions of pDCs. Among the downregulated genes in TNF-treated pDCs compared to untreated pDCs there were enriched pathways associated with negative regulation of G protein–coupled receptor (GPCR) cascade signalling, negative regulation of TLR cascade signalling and IFN-α/β secretion (MyD88 and MAPK signalling pathways), phagosomal maturation (early endosomal stage) as well as negative regulation of trafficking and processing of endosomal TLRs (**Figure 4.13**).

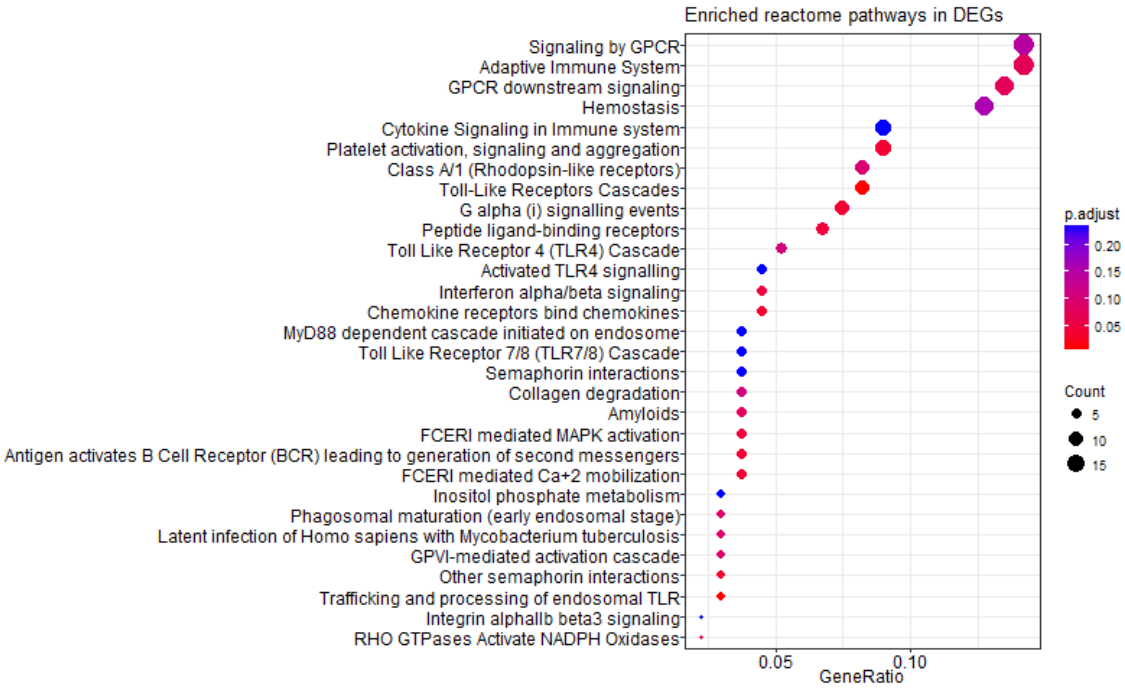


Figure 4.13 Enriched Reactome pathways in differentially expressed genes (DEGs) downregulated by TNF-α in pDCs.

As type I IFN and pro-inflammatory cytokine production by pDCs is primarily mediated by TLR7 and TLR9 ligation with nucleic acids in early endosomes, the effect of TNF-α in TLR-cascade signalling was investigated in the RNA-sequencing data analysis. Not surprisingly, as the in vitro experiments confirmed that TNF-α inhibits the secretory function of pDCs, there was a significant downregulation of genes encoding intracellular proteins and kinases mediating the phosphorylation of IRF7, NF-κB and AP-1 with eventual outcome the production of type I IFNs and pro-inflammatory cytokines (**Figure 4.14**). In more detail, there was a statistically significant reduction in expression levels of TLR7 and TLR9, MyD88-IRAK1/2/4 complex as well as IRF7. On the other hand, there was upregulation of NF-κB inhibitor (*NFKBIA*) known to block the translocation of NF-κB to the nucleus (**Figure 4.15**).

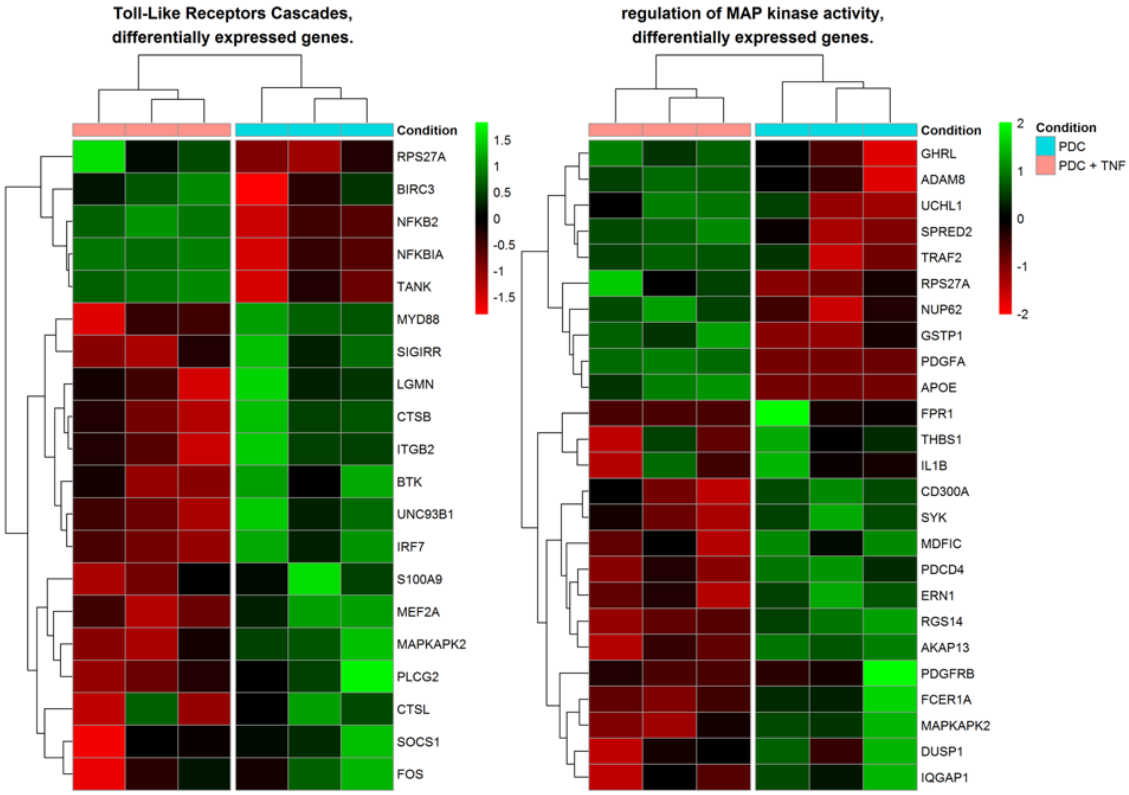


Figure 4.14 Heatmap of differentially expressed genes in TNF-treated vs. untreated pDCs showed negative regulation of TLR cascade signalling and MAPK signalling pathway.

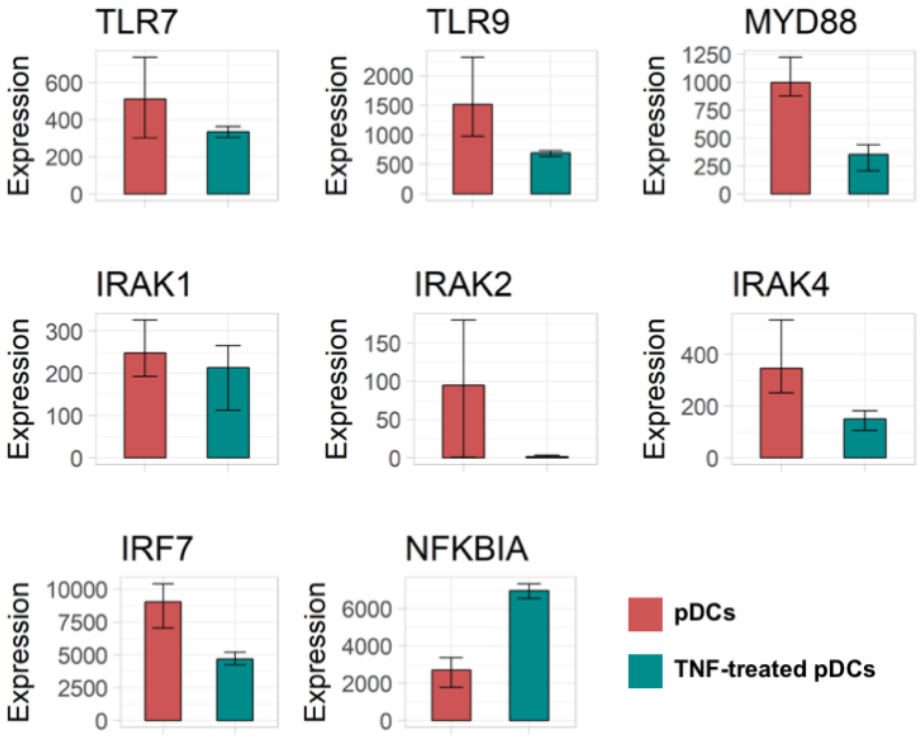


Figure 4.15 Differentially expressed genes in TNF-treated vs. untreated pDCs associated with negative regulation of TLR-mediated type I IFN production.

In addition, TNF-treated pDCs presented a significant downregulation of common interferon-stimulated genes (ISGs) including *SOCS*, *IFI30*, *IRF7*, *IFITM2*, *IFITM3*, *OAS1*, whilst there was upregulation of other ISGs such as *BST2*, *IRF4*, *NUP62* and *IFNGR2* (**Figure 4.16**).

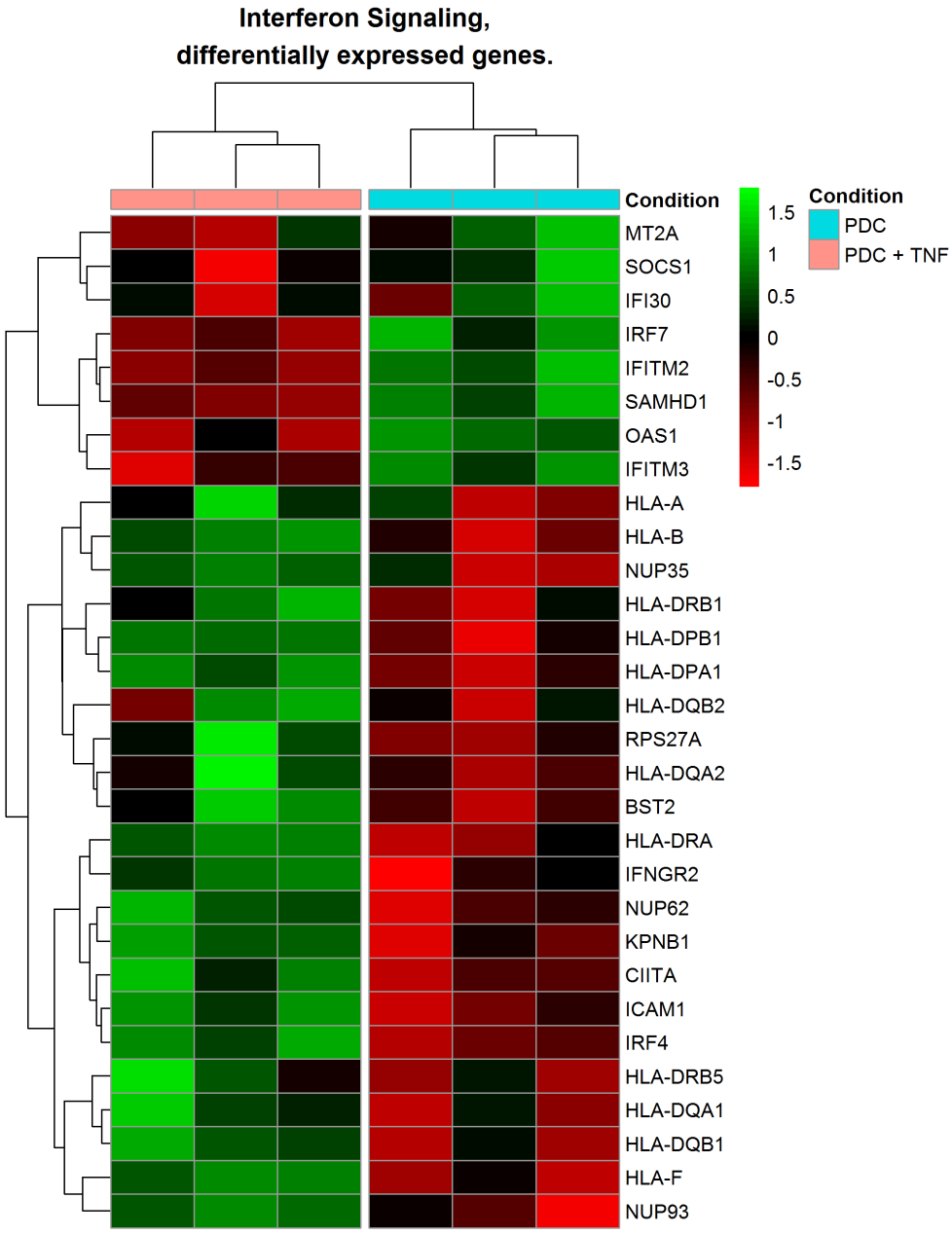


Figure 4.16 Heatmap of differentially expressed genes in TNF-treated vs. untreated pDCs showed negative regulation of TLR cascade signalling and MAPK signalling pathway.

4.2.7 TNF-α promotes the upregulation of co-stimulatory and maturation markers on pDCs

RNA-sequencing data analysis indicated that TNF-α upregulated transcripts of maturation markers and costimulatory molecules in pDCs. To validate the data at protein level, pDCs were enriched from PBMCs by negative selection using magnetic beads (purity >92%) and were cultured in the presence or absence of exogenous TNF-α for 24 hours. The expression of surface molecules was then measured by flow cytometry (**Figure 4.17**). The flow cytometric analysis confirmed that TNF-α strongly upregulated maturation markers such as HLA-DR (MHC-II) and CCR7 (CD197) as well as the costimulatory molecules CD80 and CD86 on pDCs. In addition, TNF-α induced the upregulation of molecules related to IFN-α negative regulation such as ILT7 (CD85j) and CD317 (BST2, tetherin). On the other hand, TNF-α induced the downregulation of the pDC-specific marker BDCA-2 (CD303), while the expression of BDCA-4 (CD304) remained unchanged (data not shown).

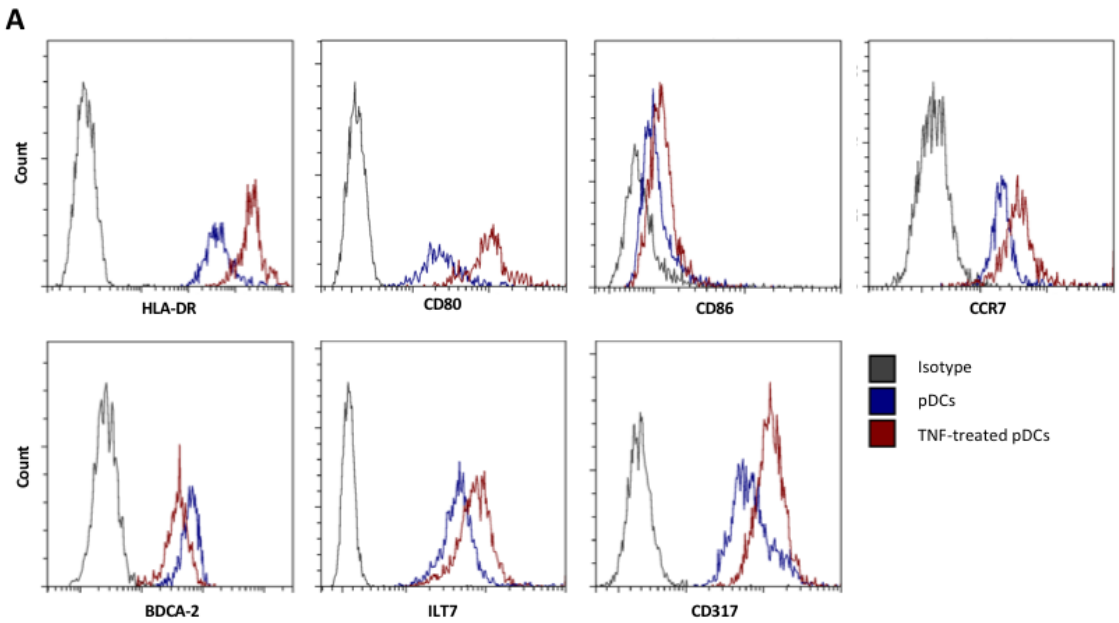


Figure 4.17 TNF-α promotes the maturation of pDCs. pDCs were purified from freshly isolated PBMCs and cultured in the absence or presence of recombinant human TNF-α. After 24 hours, pDCs were analysed by flow cytometry. Fluorescence intensity is shown on the x axis. Results shown are representative of three independent experiments. (A) TNF-α upregulates HLA-DR (MHC-II), costimulatory molecules such as CD80 and CD86, CCR7, but downregulates pDC-specific markers such as BDCA-2 (CD303). TNF-α also upregulates receptors related to type I IFN regulation such as ILT7 (CD85g) and CD317 (BST2, tetherin).

4.2.8 TNF-α-treated pDCs enhance T cell proliferation and activation

Human pDCs express HLA-DR and are able to induce $CD4^+$ T cell activation. Following the RNA-sequencing data analysis, I performed a series of *in vitro* allogeneic pDC – naïve $CD4^+$ T cell co-cultures to evaluate whether TNF-α could enhance the T cell activation properties of pDCs. First, pDCs were cultured in the presence or absence of exogenous TNF-α (10 ng/mL) for 24 hours. After washing thoroughly, pDCs were co-cultured with allogeneic naïve $CD4^+$ T cells for 5 days. T cell proliferation was assessed based on CellTrace Violet dilution upon cell division using flow cytometry. As expected, naïve $CD4^+$ T cells alone did not proliferate in the absence of pDCs. On

the other hand, both pDC groups could induce T cell proliferation without exogenous stimulation (**Figure 4.18A**). However, TNF-treated pDCs were more efficient, as they induced a significantly higher percentage of proliferating T cells (**Figure 4.18B**).

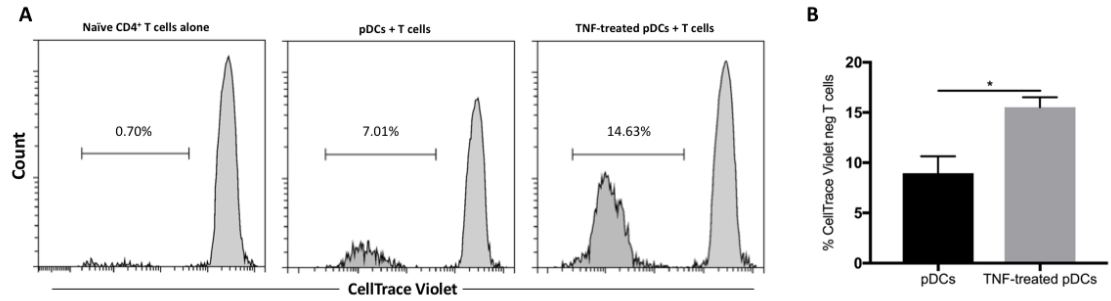


Figure 4.18 TNF-α-treated pDCs enhance T cell proliferation. (A) Allogeneic naïve $CD4^+$ T cells were labeled with CellTrace Violet and cultured alone or with pDCs or TNF-α-treated pDCs for 5 days. T cell proliferation was analysed by flow cytometry based on CellTrace Violet dilution. One representative experiment is shown out of three independent experiments. (B) Average percentage of proliferated $CD4^+$ T cells co-cultured with pDCs or TNF-α-treated pDCs (n = 3). $*P < 0.05$.

Similarly, although pDCs promoted T cell activation, TNF-treated pDCs showed better capacity of promoting T cell activation as measured by surface expression of CD69 (**Figure 4.19**).

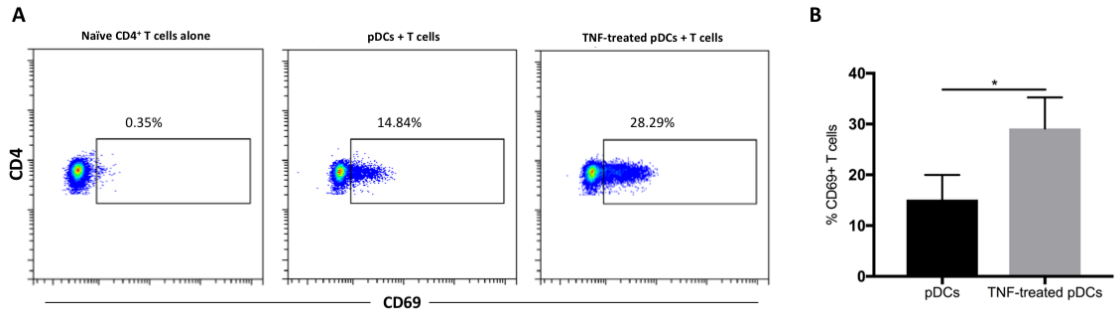


Figure 4.19 TNF-α-treated pDCs enhance T cell activation. (A) Expression of CD69 on $CD4^+$ T cells from the cultures shown in A. One representative experiment is shown out of three independent experiments. (B) Average expression of CD69 on $CD4^+$ T cells co-cultured with pDCs or TNF-α-treated pDCs (n = 3). $*P < 0.05$.

Furthermore, I investigated whether TNF-treated pDCs favoured or enhanced specific T cell differentiation. I co-cultured allogeneic naïve $CD4^+$ T cells with pDCs or TNF-treated pDCs for 5 days and I then measured multiple cytokine production in T cells by intracellular staining. Although both pDC groups induced T cell activation, TNF-treated pDCs induced notably higher production of TNF-α (**Figure 4.20A**, 2.39% vs. 4.74%) and IFN-γ (**Figure 4.20B**, 9.54% vs. 13.05%) as well as IL-17A (**Figure 4.20C**, 1.05% vs. 2.08%). Collectively, these results confirmed that TNF-α enhanced the properties of pDCs to induce T cell proliferation and activation and to favour T cell differentiation towards Th1 and Th17 phenotype.

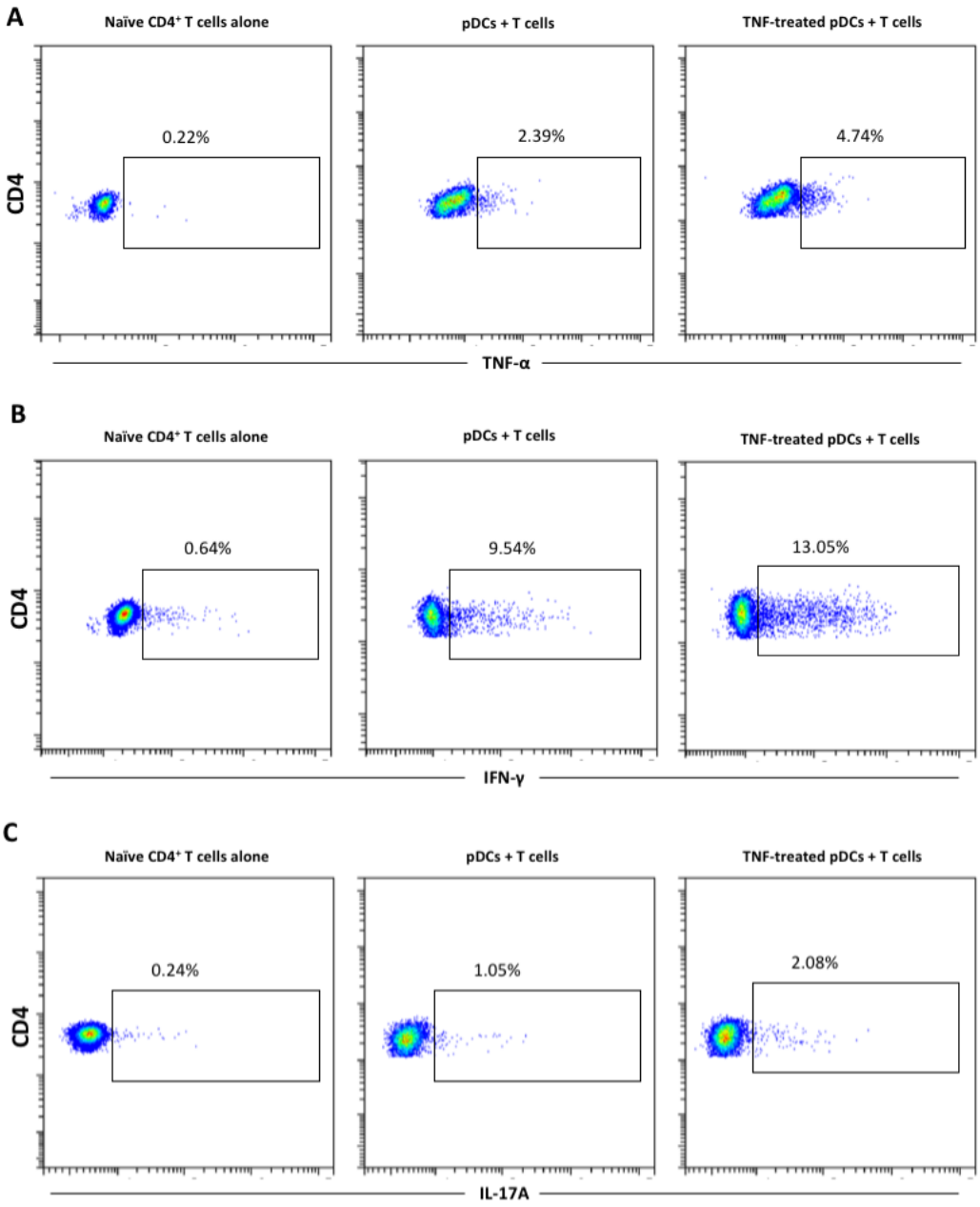


Figure 4.20 TNF-α-treated pDCs enhance the production of Th1 and Th17 cytokines. Allogeneic naïve $CD4^+$ T cells were cultured alone or with pDCs or TNF-α-treated pDCs for 5 days. Percentage of TNF-α, IFN-γ and IL-17A production by $CD4^+$ T cells was measured by intracellular staining. One representative experiment is shown out of three independent experiments.

4.3 Discussion

Apart from the major role of pDCs as type I IFN-producing cells, pDCs are able to capture, process and present antigens to $CD4^+$ T cells. However, the regulation of each of these functions still remains elusive. Previous studies reported the cross-regulation of TNF-α and IFN-α during *in vitro* stimulation of pDCs with influenza virus, however the precise mechanism for this effect was entirely addressed (367). Here I demonstrated that TNF-α not only inhibited IFN-α production even at minimal concentrations, predominantly in a TLR9-mediated manner, but also had a similar regulatory effect on autologous TNF-α production.

Novel single cell RNA-sequencing data revealed new subsets of DCs with distinct transcriptomic profiles and immune functions (368). Apart from the traditional pDC population which is characterised by expression of genes associated with pathogen sensing and induction of type I IFNs as well as the master regulator transcription factor *TCF4*, a novel DC subset was described with a unique gene signature (*AXL*, *SIGLEC1*, *SIGLEC6*, *CD22/SIGLEC2*) sharing features of both cDCs and pDCs. Moreover, a novel $CD2^{hi}CD5^+CD81^+$ pDC subset was shown to induce strong T and B cell activation but not to be able to secrete type I IFNs (341).

The RNA-sequencing data of TNF-treated pDCs revealed a downregulation of this pDC-specific gene signature associated with pathogen sensing (*IRF7*, *TLR7*, *SLC15A4*, *PACSIN1*) and secretion (*DERL3*, *LAMP5*, *SCAMP5*) as well as *TCF4* alongside its binding targets (*SLA2*, *PTCRA*, *PTPRCAP*). Hence, it can be concluded that TNF-α strongly influences the transcriptional profile of pDCs by downregulating their classical pathways and upregulating genes (e.g., *LY86*) mostly related to the conventional DC phenotype.

Other cytokines such as IL-21 had a regulatory impact on the function of pDCs promoting their tolerogenic properties (359). In contrast, TNF-α enhanced the immunogenic properties of pDCs towards antigen presentation and T cell activation. Regarding TNF receptor superfamily, TNF-α downregulated *TNFRSF1A* (TNF receptor 1A; CD120a) but not *TNFRSF1B* (TNF receptor 1B; CD120b), *TNFRSF6B* (Decoy receptor 3; TR6; M68), whilst it upregulated *TNFRSF4* (OX40; CD134), *FAS* (Fas receptor; Apo-1; CD95), and *CD40* (Bp50; CD40). Interestingly, TNF-α promoted the downregulation of *IFNAR1* and *IFNAR2* but the upregulation of *IFNGR1* and *IFNGR2* indicating a potential synergistic effect with other major pro-inflammatory cytokines such as IFN-γ.

Linking these data to human disease, anti-TNF treatment has been associated with lupus-like symptoms as well as an induction of IFN signature in peripheral blood (366, 369, 370). Synovium of rheumatoid arthritis patients was reported to contain pDCs able to activate T cells more efficiently (371). Notably, pDCs were found to localise in RA synovium expressing Il-18 in close proximity to clusters of $CD3^{+}CD8^{+}$ T cells. TNF-α as one of the main pathogenic cytokines driving synovial inflammation can alter pDC function enhancing antigen presentation and promoting Th1 and Th17 cell differentiation.

Although pDCs possess weak antigen-presenting properties, TNF-α can enhance their maturation by switching their main role as IFN-α-producing cells to a more conventional DC phenotype. The functional status of pDCs might be strongly influenced by the overall inflammatory environment, whilst TNF-α might regulate IFN-α-mediated aspects of a range of autoimmune and inflammatory diseases.

Key messages:

- TNF-α is a major cytokine produced by human pDCs upon TLR stimulation.
- TNF-α inhibits IFN-α and TNF-α production by TLR-stimulated pDCs by negatively regulating IRF7 and NF-κB pathways.
- TNF-α upregulates pathways in pDCs related to antigen processing and presentation as well as T cell activation and differentiation.
- TNF-α upregulates costimulatory molecules and maturation markers on human pDCs.
- TNF-α-treated pDCs induce higher $CD4^+$ T cell proliferation and activation enhancing the production of Th1 and Th17 cytokines.

CHAPTER 5.

GENERAL DISCUSSION

The data presented in the current thesis highlight novel findings in the regulation of type I IFNs in patients with SLE. As described in full detail in Chapter 1, type I IFNs are a large group of molecules with pleiotropic functions on the immune system linking innate and adaptive immune responses. As the predominant antiviral and antitumor cytokines, they also appear to be key players in systemic autoimmune diseases triggering autoreactive phenomena and possibly leading to certain pathologies and organ involvement in SLE. In addition, they might have an important role in initiating autoimmunity, since individuals with ANA positivity but no clinically overt disease present with a high IFN signature in peripheral blood.

It has long been speculated that pDCs, the professional type I IFN-producing cells of the immune system, are primed to produce large amounts of these molecules and hence responsible for the increased IFN activity observed in patients with SLE. However, previous studies suggesting the above statement had major limitations; they isolated pDCs from healthy donors and they mimicked SLE conditions *in vitro*.

In addition, the numeration of circulating pDCs, even in blood samples of patients with SLE, was often problematic; an appropriate flow cytometry gating to identify the pure pDC population within the PBMCs was not followed, neglecting potential contamination with cells belonging to another lineage, such as cDCs or monocytes. For the purpose of this thesis, to resolve this problem an extensive immunophenotyping was used carefully to exclude lineage negative cells (B cells, T cells, NK cells, monocytes conventional DCs) and choose double positive cells for

both CD123 and CD303 within the HLA-DR positive population. As a result, a clear reduction in the circulating pDCs was observed analysing a large number of fresh samples obtained from patients with established autoimmune rheumatic diseases (SLE and pSS) as well as individuals with preclinical autoimmunity (At-Risk). Importantly, the data from the current PhD thesis clearly demonstrated that pDCs were significantly defective in promoting TLR-mediated cytokine production, both IFN-α and TNF-α, while their phenotype did not associate with any clinical or immunological features of SLE patients, such as disease activity, treatment with immunosuppressive drugs, autoantibody profile, and IFN activity in peripheral blood. This is the first known study to provide a comprehensive analysis of pDCs in human autoimmune conditions, not only studying TLR-mediated responses, but also providing valuable associations with the clinical background as well as other immunological and treatment-related factors that could affect the results.

Interestingly, I showed for the first time a new function of human pDCs; IL-3 is not only an important cytokine enhancing pDC survival and IFN-a production as previously thought, but also a robust stimulus for the induction of IL-6 production. This novel feature was independent of TLR stimulation of pDCs and it did not appear to be defective in pDCs from SLE patient and At-Risk individuals.

Furthermore, pDCs from SLE patients presented other immunological deficiencies including their inability to induce sufficient T cell activation and proliferation in comparison with pDCs from healthy individuals. On the other hand, a novel regulatory role for a conventional pro-inflammatory cytokine such as TNF-α was also described. TNF-α can change the transcriptional profile of human pDCs from their IFN-producing role towards a functional drift antigen presentation inducing stronger

T cell activation. However, one of the major limitations of this PhD thesis was the design of pDC-T cell co-cultures. Human pDCs are hard to be cultured *in vitro* because they cannot survive for more 48 hours after isolation. Apart from that, pDCs are dramatically reduced in the circulation of SLE patients resulting in unexpected difficulties in isolating adequate numbers of the cells to perform the co-culture. As a consequence, T cells were practically exposed to pDCs only for the first 48 hours regarding the co-culture in Chapter 3, whilst that period of time was practically reduced to 24 hours for the experiment in Chapter 4 (pDCs were first treated with TNF for 24 hours before they were co-cultured with T cells). This is the main reason for the discrepancies observed in the co-cultures. To further optimise the results in Chapter 3, I decided to add anti-CD3/CD28 soluble beads at low concentration, so I can boost the survival of T cells but not affecting the primary effect of pDCs on T cells at the same time. Indeed, with the optimisation described above, the T cells proliferated stronger and produced more cytokines upon secondary stimulation with PMA/Ionomycin.

Another novelty of the current PhD thesis was the transcriptomic analysis of pDCs from patients with SLE as well as individuals at preclinical stage. However, data analysis of the RNA-sequencing data was challenging. Human samples from all donors were highly heterogeneous and the further clustering into groups simply by a disease diagnosis was ineffective. As a consequence, a new approach based on the expression of an IFN Score (describing the IFN activity in the pDCs of each donor) was adopted to resolve the classification issue and acquire more information about transcriptional differences between autoimmune and healthy pDCs. The highly sensitive full-length RNA-sequencing demonstrated transcriptional changes

associated with immune senescence and immune tolerance. These insights were in accordance with the *in vitro* work that clearly demonstrated that pDCs presented dysfunctional TLR responses in patients with SLE and At-Risk individuals. All data together pointed that pDCs in human preclinical and established autoimmunity exhibit an exhausted phenotype. A better investigation would require a single-cell based RNA-sequencing analysis that would enable a diversification of possibly distinct pDC populations in patients with SLE. That would be a challenging approach requiring an in-depth analysis of single cells from many patients, as SLE is a very heterogenous condition. Although IFN activity seems to be able to classify the patients into to major groups (low and high), other immunological or clinical parameters could be investigated in relation to pDC phenotype.

One of the first findings observed was the strong correlation between type I IFN activity in peripheral blood -measured as IFN Score A- and mucocutaneous disease activity. This correlation could possibly indicate a link between skin tissue and type I IFN dysregulation SLE. Acquiring skin biopsies from non-lesional skin of both SLE and At-Risk patients, I observed a significant upregulation of ISGs in samples of both groups compared to those of healthy donors. The overall expression level of IFN Score A in skin biopsies of At-Risk individuals was notoriously higher [29.5 fold increase (1.3 - 635.0)] in comparison with the IFN signature in peripheral blood [2.2 fold increase (2.0 – 2.3). Nonetheless, measuring ISGs and creating an IFN score does not provide an answer to the fundamental question what the primary source of type I IFN overproduction is.

To address this complicated question, I utilised a novel *in situ* hybridization assay (RNAscope) enabling to detect transcripts of the actual type I IFN in the skin biopsies.

Surprisingly, when I hybridized the lesional skin biopsies obtained from SLE patients, I found out a diffuse expression of IFNK across the epidermis but not in leucocyte-infiltrating areas. These data were in accordance of the *in vitro* work carried out from pDCs isolated from peripheral blood showing lack of TLR stimulation and a phenotype associated with immune senescence. However, the most interesting data were generated by the hybridization of non-lesional skin biopsies of the At-Risk individuals. Gene expression analysis had previously demonstrated a massive upregulation of ISGs in those skin biopsies. In addition, the RNAscope assay provided vital information regarding the localization of the type I IFN transcripts -not the outcome of their effect (ISGs)- indicating that the source of type I IFN production is not the infiltrating leucocytes but the non-haematopoietic tissue cells. These results are strongly supported by the fact that keratinocytes were primed to express high levels of *IFNK* even in skin biopsies of At-Risk individuals who had no signs of clinical and/or histopathological abnormalities.

Alongside the *in situ* hybridization data, the *in vitro* culture of isolated human keratinocytes from four different conditions (healthy, SLE, CDLE, At-Risk) confirmed and complemented the imaging results. Keratinocytes from non-lesional skin biopsies of both SLE patients and At-Risk individuals showed a significantly higher expression of IFNK in response to nucleic acids, whilst SLE patients demonstrated an upregulation of IFNB as well. In contract, keratinocytes from CDLE showed an immune response towards type III IFNs. From these data, it can be concluded that keratinocytes in preclinical autoimmunity and SLE can respond to environmental triggers (e.g. UV light) or self-nucleic acids through endoplasmic TLRs or cytoplasmic receptors and produce excessive amounts of type I IFNs, particularly IFN-κ.

Considering all the data discussed above, the role of non-haematopoietic cells in initiation of autoimmunity seems to be prominent and hence this requires further investigation. Keratinocytes do not consist of homogenous population of cells but they present different transcriptomic profiles and functional features. To decipher that, single-cell RNA-sequencing of keratinocytes from both patients with SLE and more importantly treatment-naïve At-Risk individuals would be an excellent tool elucidate many pathways and pathogenic phenotypes contributing to the excessive type I IFN production. In addition, single-cell RNA-sequencing can provide novel information about unique cellular population among the conventional keratinocytes and also elucidate novel functions that play a key role in the pathogenesis of human autoimmunity and cutaneous inflammation. On the other hand, it would be crucial to investigate how these non-haematopoietic tissue resident cells shape immune responses and affect the microenvironment of skin inflammation. T and B lymphocyte clonal expansion can be seen early in SLE, while their role in escalating inflammation and tissue damage is undeniable. Therefore, the cellular interactions between keratinocytes and cells of the adaptive immune system can be studied *in vitro* concluding important results about the escalation of immune response and factors determining persistent inflammation.

Last but not least, the role of epithelial tissues other than the skin should be investigated further to shed more light on specific organ involvement and the role of its tissue in driving the inflammatory process. SLE is such a heterogeneous disease and even though mucocutaneous manifestations are among the commonest, other pathologies including lupus nephritis appear to be more severe and often life threatening. As a consequence, similarly to the novel role of keratinocytes in the

regulation of type I IFNs in systemic autoimmunity, other cell types such as glomerular epithelial cells can potentially contribute to type I IFN dysregulation.

The data analysed in this PhD thesis fundamentally change our understanding of IFN-mediated autoimmunity in humans. In the established disease, these insights might indicate potential therapeutic targets outside the conventional immune system. Moreover, at the preclinical stage, this is the first report on where IFN dysregulation occurs and how it might be targeted for disease prevention. These results have greater translational implications as I used exclusively human samples -not an animal lupus model- from different autoimmune rheumatic diseases, such as SLE and primary Sjögren's Syndrome, but most importantly samples from ANA positive individuals at preclinical stages, the majority of whom will remain at this benign autoimmune phase. This could indicate that even though type I IFN dysregulation may be an essential feature for development of autoimmunity at early stages, it may not be sufficient for progression to a clinical syndrome.

CHAPTER 6.

REFERENCES

1. Danchenko N, Satia JA, Anthony MS. Epidemiology of systemic lupus erythematosus: a comparison of worldwide disease burden. Lupus. 2006;15(5):308-18.
2. Feldman CH, Hiraki LT, Liu J, Fischer MA, Solomon DH, Alarcon GS, et al. Epidemiology and sociodemographics of systemic lupus erythematosus and lupus nephritis among US adults with Medicaid coverage, 2000-2004. Arthritis Rheum. 2013;65(3):753-63.
3. Uramoto KM, Michet CJ, Jr., Thumboo J, Sunku J, O'Fallon WM, Gabriel SE. Trends in the incidence and mortality of systemic lupus erythematosus, 1950-1992. Arthritis Rheum. 1999;42(1):46-50.
4. Pons-Estel GJ, Alarcon GS, Scofield L, Reinlib L, Cooper GS. Understanding the epidemiology and progression of systemic lupus erythematosus. Seminars in arthritis and rheumatism. 2010;39(4):257-68.
5. McCarty DJ, Manzi S, Medsger TA, Jr., Ramsey-Goldman R, LaPorte RE, Kwoh CK. Incidence of systemic lupus erythematosus. Race and gender differences. Arthritis Rheum. 1995;38(9):1260-70.
6. Rees F, Doherty M, Grainge M, Davenport G, Lanyon P, Zhang W. The incidence and prevalence of systemic lupus erythematosus in the UK, 1999-2012. Ann Rheum Dis. 2016;75(1):136-41.
7. Cohen A, Reynolds, WE and Franklin, EC. Preliminary criteria for the classification of SLE. Bulletin of the Rheumatic Diseases. 1971;21:643-5.
8. Tan EM, Cohen AS, Fries JF, Masi AT, McShane DJ, Rothfield NF, et al. The 1982 revised criteria for the classification of systemic lupus erythematosus. Arthritis Rheum. 1982;25(11):1271-7.
9. Hochberg MC. Updating the American College of Rheumatology revised criteria for the classification of systemic lupus erythematosus. Arthritis Rheum. 1997;40(9):1725.
10. Petri M, Orbai AM, Alarcon GS, Gordon C, Merrill JT, Fortin PR, et al. Derivation and validation of the Systemic Lupus International Collaborating Clinics classification criteria for systemic lupus erythematosus. Arthritis Rheum. 2012;64(8):2677-86.
11. Gabriela S, F. HB, Martin A, R. JS, I. DD, Thomas D. Multi-center Delphi Exercise Reveals Important Key Items for Classifying Systemic Lupus Erythematosus. Arthritis Care & Research.0(ja).
12. Dubois EL, Tuffanelli DL. Clinical Manifestations of Systemic Lupus Erythematosus. Computer Analysis of 520 Cases. JAMA. 1964;190:104-11.
13. Cervera R, Khamashta MA, Font J, Sebastiani GD, Gil A, Lavilla P, et al. Morbidity and mortality in systemic lupus erythematosus during a 10-year period: a comparison of early and late manifestations in a cohort of 1,000 patients. Medicine (Baltimore). 2003;82(5):299-308.

14. Uva L, Miguel D, Pinheiro C, Freitas JP, Marques Gomes M, Filipe P. Cutaneous manifestations of systemic lupus erythematosus. Autoimmune Dis. 2012;2012:834291.
15. Man BL, Mok CC. Serositis related to systemic lupus erythematosus: prevalence and outcome. Lupus. 2005;14(10):822-6.
16. Santiago MB. Miscellaneous non-inflammatory musculoskeletal conditions. Jaccoud's arthropathy. Best practice & research Clinical rheumatology. 2011;25(5):715-25.
17. Pipili C, Sfritzeri A, Cholongitas E. Deforming arthropathy in systemic lupus erythematosus. Eur J Intern Med. 2008;19(7):482-7.
18. Ostendorf B, Scherer A, Specker C, Modder U, Schneider M. Jaccoud's arthropathy in systemic lupus erythematosus: differentiation of deforming and erosive patterns by magnetic resonance imaging. Arthritis Rheum. 2003;48(1):157-65.
19. Fayyaz A, Igoe A, Kurien BT, Danda D, James JA, Stafford HA, et al. Haematological manifestations of lupus. Lupus Sci Med. 2015;2(1):e000078.
20. Ruiz-Irastorza G, Crowther M, Branch W, Khamashta MA. Antiphospholipid syndrome. Lancet. 2010;376(9751):1498-509.
21. Zeller CB, Appenzeller S. Cardiovascular disease in systemic lupus erythematosus: the role of traditional and lupus related risk factors. Curr Cardiol Rev. 2008;4(2):116-22.
22. Lewandowski LB, Kaplan MJ. Update on cardiovascular disease in lupus. Curr Opin Rheumatol. 2016;28(5):468-76.
23. Gulinello M, Wen J, Putterman C. Neuropsychiatric Symptoms in Lupus. Psychiatr Ann. 2012;42(9):322-8.
24. Kivity S, Agmon-Levin N, Zandman-Goddard G, Chapman J, Shoenfeld Y. Neuropsychiatric lupus: a mosaic of clinical presentations. BMC Med. 2015;13:43.
25. Mosca M, Tani C, Aringer M, Bombardieri S, Boumpas D, Brey R, et al. European League Against Rheumatism recommendations for monitoring patients with systemic lupus erythematosus in clinical practice and in observational studies. Ann Rheum Dis. 2010;69(7):1269-74.
26. Yu F, Haas M, Glassock R, Zhao MH. Redefining lupus nephritis: clinical implications of pathophysiologic subtypes. Nat Rev Nephrol. 2017;13(8):483-95.
27. Faurschou M, Starklint H, Halberg P, Jacobsen S. Prognostic factors in lupus nephritis: diagnostic and therapeutic delay increases the risk of terminal renal failure. J Rheumatol. 2006;33(8):1563-9.
28. Chen Y, Sun J, Zou K, Yang Y, Liu G. Treatment for lupus nephritis: an overview of systematic reviews and meta-analyses. Rheumatol Int. 2017;37(7):1089-99.
29. Weening JJ, D'Agati VD, Schwartz MM, Seshan SV, Alpers CE, Appel GB, et al. The classification of glomerulonephritis in systemic lupus erythematosus revisited. Kidney Int. 2004;65(2):521-30.
30. Weening JJ, D'Agati VD, Schwartz MM, Seshan SV, Alpers CE, Appel GB, et al. The classification of glomerulonephritis in systemic lupus erythematosus revisited. J Am Soc Nephrol. 2004;15(2):241-50.
31. Lateef A, Petri M. Unmet medical needs in systemic lupus erythematosus. Arthritis Res Ther. 2012;14 Suppl 4:S4.

32. Tunnicliffe DJ, Singh-Grewal D, Kim S, Craig JC, Tong A. Diagnosis, Monitoring, and Treatment of Systemic Lupus Erythematosus: A Systematic Review of Clinical Practice Guidelines. Arthritis Care Res (Hoboken). 2015;67(10):1440-52.
33. Bertsias GK, Tektonidou M, Amoura Z, Aringer M, Bajema I, Berden JH, et al. Joint European League Against Rheumatism and European Renal Association-European Dialysis and Transplant Association (EULAR/ERA-EDTA) recommendations for the management of adult and paediatric lupus nephritis. Ann Rheum Dis. 2012;71(11):1771-82.
34. Touma Z, Gladman DD. Current and future therapies for SLE: obstacles and recommendations for the development of novel treatments. Lupus Sci Med. 2017;4(1):e000239.
35. Yurasov S, Wardemann H, Hammersen J, Tsuiji M, Meffre E, Pascual V, et al. Defective B cell tolerance checkpoints in systemic lupus erythematosus. J Exp Med. 2005;201(5):703-11.
36. Merrill JT, Neuwelt CM, Wallace DJ, Shanahan JC, Latinis KM, Oates JC, et al. Efficacy and safety of rituximab in moderately-to-severely active systemic lupus erythematosus: the randomized, double-blind, phase II/III systemic lupus erythematosus evaluation of rituximab trial. Arthritis Rheum. 2010;62(1):222-33.
37. Rovin BH, Furie R, Latinis K, Looney RJ, Fervenza FC, Sanchez-Guerrero J, et al. Efficacy and safety of rituximab in patients with active proliferative lupus nephritis: the Lupus Nephritis Assessment with Rituximab study. Arthritis Rheum. 2012;64(4):1215-26.
38. Liu Z, Davidson A. BAFF inhibition: a new class of drugs for the treatment of autoimmunity. Exp Cell Res. 2011;317(9):1270-7.
39. Furie R, Nicholls K, Cheng TT, Houssiau F, Burgos-Vargas R, Chen SL, et al. Efficacy and safety of abatacept in lupus nephritis: a twelve-month, randomized, double-blind study. Arthritis Rheumatol. 2014;66(2):379-89.
40. Isenberg DA, Rahman A, Allen E, Farewell V, Akil M, Bruce IN, et al. BILAG 2004. Development and initial validation of an updated version of the British Isles Lupus Assessment Group's disease activity index for patients with systemic lupus erythematosus. Rheumatology (Oxford). 2005;44(7):902-6.
41. Yee CS, Farewell V, Isenberg DA, Rahman A, Teh LS, Griffiths B, et al. British Isles Lupus Assessment Group 2004 index is valid for assessment of disease activity in systemic lupus erythematosus. Arthritis Rheum. 2007;56(12):4113-9.
42. Yee CS, Cresswell L, Farewell V, Rahman A, Teh LS, Griffiths B, et al. Numerical scoring for the BILAG-2004 index. Rheumatology (Oxford). 2010;49(9):1665-9.
43. Block SR, Winfield JB, Lockshin MD, D'Angelo WA, Christian CL. Studies of twins with systemic lupus erythematosus. A review of the literature and presentation of 12 additional sets. Am J Med. 1975;59(4):533-52.
44. Deapen D, Escalante A, Weinrib L, Horwitz D, Bachman B, Roy-Burman P, et al. A revised estimate of twin concordance in systemic lupus erythematosus. Arthritis Rheum. 1992;35(3):311-8.
45. Kuo CF, Grainge MJ, Valdes AM, See LC, Luo SF, Yu KH, et al. Familial Aggregation of Systemic Lupus Erythematosus and Coaggregation of Autoimmune Diseases in Affected Families. JAMA Intern Med. 2015;175(9):1518-26.
46. Alarcon-Segovia D, Alarcon-Riquelme ME, Cardiel MH, Caeiro F, Massardo L, Villa AR, et al. Familial aggregation of systemic lupus erythematosus, rheumatoid

arthritis, and other autoimmune diseases in 1,177 lupus patients from the GLADEL cohort. Arthritis Rheum. 2005;52(4):1138-47.
47. Barcellos LF, May SL, Ramsay PP, Quach HL, Lane JA, Nititham J, et al. High-density SNP screening of the major histocompatibility complex in systemic lupus erythematosus demonstrates strong evidence for independent susceptibility regions. PLoS Genet. 2009;5(10):e1000696.
48. International MHC, Autoimmunity Genetics N, Rioux JD, Goyette P, Vyse TJ, Hammarstrom L, et al. Mapping of multiple susceptibility variants within the MHC region for 7 immune-mediated diseases. Proc Natl Acad Sci U S A. 2009;106(44):18680-5.
49. Bronson PG, Chaivorapol C, Ortmann W, Behrens TW, Graham RR. The genetics of type I interferon in systemic lupus erythematosus. Curr Opin Immunol. 2012;24(5):530-7.
50. Yang Y, Lhotta K, Chung EK, Eder P, Neumair F, Yu CY. Complete complement components C4A and C4B deficiencies in human kidney diseases and systemic lupus erythematosus. J Immunol. 2004;173(4):2803-14.
51. Macedo ACL, Isaac L. Systemic Lupus Erythematosus and Deficiencies of Early Components of the Complement Classical Pathway. Frontiers in Immunology. 2016;7:55.
52. Gunther C, Meurer M, Stein A, Viehweg A, Lee-Kirsch MA. Familial chilblain lupus--a monogenic form of cutaneous lupus erythematosus due to a heterozygous mutation in TREX1. Dermatology. 2009;219(2):162-6.
53. Hom G, Graham RR, Modrek B, Taylor KE, Ortmann W, Garnier S, et al. Association of systemic lupus erythematosus with C8orf13-BLK and ITGAM-ITGAX. N Engl J Med. 2008;358(9):900-9.
54. Graham RR, Hom G, Ortmann W, Behrens TW. Review of recent genome-wide association scans in lupus. J Intern Med. 2009;265(6):680-8.
55. Morris DL, Sheng Y, Zhang Y, Wang Y-F, Zhu Z, Tombleson P, et al. Genome-wide association meta-analysis in Chinese and European individuals identifies ten new loci associated with systemic lupus erythematosus. Nature Genetics. 2016;48:940.
56. Deng Y, Tsao BP. Updates in Lupus Genetics. Current Rheumatology Reports. 2017;19(11):68.
57. Kariuki SN, Kirou KA, MacDermott EJ, Barillas-Arias L, Crow MK, Niewold TB. Cutting edge: autoimmune disease risk variant of STAT4 confers increased sensitivity to IFN-alpha in lupus patients in vivo. J Immunol. 2009;182(1):34-8.
58. Niewold TB, Kelly JA, Flesch MH, Espinoza LR, Harley JB, Crow MK. Association of the IRF5 risk haplotype with high serum interferon-alpha activity in systemic lupus erythematosus patients. Arthritis Rheum. 2008;58(8):2481-7.
59. Morel L. Genetics of human lupus nephritis. Seminars in nephrology. 2007;27(1):2-11.
60. Ramos PS, Brown EE, Kimberly RP, Langefeld CD. Genetic Factors Predisposing to Systemic Lupus Erythematosus and Lupus Nephritis. Seminars in nephrology. 2010;30(2):164-76.
61. Patel DR, Richardson BC. Dissecting complex epigenetic alterations in human lupus. Arthritis Res Ther. 2013;15(1):201.

62. Javierre BM, Fernandez AF, Richter J, Al-Shahrour F, Martin-Subero JI, Rodriguez-Ubreva J, et al. Changes in the pattern of DNA methylation associate with twin discordance in systemic lupus erythematosus. Genome research. 2010;20(2):170-9.
63. H. CS, L. LQ, L. H, J. PM, H. WG, B. S. DNA methylation alterations in the pathogenesis of lupus. Clinical & Experimental Immunology. 2017;187(2):185-92.
64. Zan H, Tat C, Casali P. microRNAs in lupus. Autoimmunity. 2014;47(4):272-85.
65. Deng GM, Tsokos GC. Pathogenesis and targeted treatment of skin injury in SLE. Nat Rev Rheumatol. 2015;11(11):663-9.
66. Casciola-Rosen LA, Anhalt G, Rosen A. Autoantigens targeted in systemic lupus erythematosus are clustered in two populations of surface structures on apoptotic keratinocytes. J Exp Med. 1994;179(4):1317-30.
67. James JA, Kaufman KM, Farris AD, Taylor-Albert E, Lehman TJ, Harley JB. An increased prevalence of Epstein-Barr virus infection in young patients suggests a possible etiology for systemic lupus erythematosus. J Clin Invest. 1997;100(12):3019-26.
68. Nelson P, Rylance P, Roden D, Trela M, Tugnet N. Viruses as potential pathogenic agents in systemic lupus erythematosus. Lupus. 2014;23(6):596-605.
69. Araujo-Fernandez S, Ahijon-Lana M, Isenberg DA. Drug-induced lupus: Including anti-tumour necrosis factor and interferon induced. Lupus. 2014;23(6):545-53.
70. Marcus R. The chemical induction of systemic lupus erythematosus and lupus-like illnesses. Arthritis & Rheumatism. 1981;24(8):1004-9.
71. Botto M, Dell'Agnola C, Bygrave AE, Thompson EM, Cook HT, Petry F, et al. Homozygous C1q deficiency causes glomerulonephritis associated with multiple apoptotic bodies. Nat Genet. 1998;19(1):56-9.
72. Licht R, Dieker JW, Jacobs CW, Tax WJ, Berden JH. Decreased phagocytosis of apoptotic cells in diseased SLE mice. J Autoimmun. 2004;22(2):139-45.
73. Franek F, Dolnikova J. Nucleosomes occurring in protein-free hybridoma cell culture. Evidence for programmed cell death. Febs Lett. 1991;284(2):285-7.
74. Radic M, Marion T, Monestier M. Nucleosomes are exposed at the cell surface in apoptosis. J Immunol. 2004;172(11):6692-700.
75. Kalaaji M, Mortensen E, Jorgensen L, Olsen R, Rekvig OP. Nephritogenic lupus antibodies recognize glomerular basement membrane-associated chromatin fragments released from apoptotic intraglomerular cells. Am J Pathol. 2006;168(6):1779-92.
76. Manson JJ, Isenberg DA. The origin and pathogenic consequences of anti-dsDNA antibodies in systemic lupus erythematosus. Expert Rev Clin Immunol. 2006;2(3):377-85.
77. Ehrenstein MR, Katz DR, Griffiths MH, Papadaki L, Winkler TH, Kalden JR, et al. Human IgG anti-DNA antibodies deposit in kidneys and induce proteinuria in SCID mice. Kidney Int. 1995;48(3):705-11.
78. Kalaaji M, Fenton KA, Mortensen ES, Olsen R, Sturfelt G, Alm P, et al. Glomerular apoptotic nucleosomes are central target structures for nephritogenic antibodies in human SLE nephritis. Kidney Int. 2007;71(7):664-72.

79. Donnelly S, Roake W, Brown S, Young P, Naik H, Wordsworth P, et al. Impaired recognition of apoptotic neutrophils by the C1q/calreticulin and CD91 pathway in systemic lupus erythematosus. Arthritis Rheum. 2006;54(5):1543-56.
80. Kessenbrock K, Krumbholz M, Schonermarck U, Back W, Gross WL, Werb Z, et al. Netting neutrophils in autoimmune small-vessel vasculitis. Nat Med. 2009;15(6):623-5.
81. Lood C, Blanco LP, Purmalek MM, Carmona-Rivera C, De Ravin SS, Smith CK, et al. Neutrophil extracellular traps enriched in oxidized mitochondrial DNA are interferogenic and contribute to lupus-like disease. Nat Med. 2016;22(2):146-53.
82. Kim SJ, Diamond B. Modulation of tolerogenic dendritic cells and autoimmunity. Semin Cell Dev Biol. 2015;41:49-58.
83. Baumann I, Kolowos W, Voll RE, Manger B, Gaipl U, Neuhuber WL, et al. Impaired uptake of apoptotic cells into tingible body macrophages in germinal centers of patients with systemic lupus erythematosus. Arthritis Rheum. 2002;46(1):191-201.
84. Deng GM, Beltran J, Chen C, Terhorst C, Tsokos GC. T cell CD3zeta deficiency enables multiorgan tissue inflammation. J Immunol. 2013;191(7):3563-7.
85. Moulton VR, Tsokos GC. T cell signaling abnormalities contribute to aberrant immune cell function and autoimmunity. J Clin Invest. 2015;125(6):2220-7.
86. Ohl K, Tenbrock K. Regulatory T cells in systemic lupus erythematosus. Eur J Immunol. 2015;45(2):344-55.
87. Tselios K, Sarantopoulos A, Gkougkourelas I, Boura P. CD4+CD25highFOXP3+ T regulatory cells as a biomarker of disease activity in systemic lupus erythematosus: a prospective study. Clin Exp Rheumatol. 2014;32(5):630-9.
88. Craft JE. Follicular helper T cells in immunity and systemic autoimmunity. Nat Rev Rheumatol. 2012;8(6):337-47.
89. Odegard JM, Marks BR, DiPlacido LD, Poholek AC, Kono DH, Dong C, et al. ICOS-dependent extrafollicular helper T cells elicit IgG production via IL-21 in systemic autoimmunity. J Exp Med. 2008;205(12):2873-86.
90. Choi JY, Ho JH, Pasoto SG, Bunin V, Kim ST, Carrasco S, et al. Circulating follicular helper-like T cells in systemic lupus erythematosus: association with disease activity. Arthritis Rheumatol. 2015;67(4):988-99.
91. Wang L, Zhao P, Ma L, Shan Y, Jiang Z, Wang J, et al. Increased interleukin 21 and follicular helper T-like cells and reduced interleukin 10+ B cells in patients with new-onset systemic lupus erythematosus. J Rheumatol. 2014;41(9):1781-92.
92. Wei C, Anolik J, Cappione A, Zheng B, Pugh-Bernard A, Brooks J, et al. A new population of cells lacking expression of CD27 represents a notable component of the B cell memory compartment in systemic lupus erythematosus. J Immunol. 2007;178(10):6624-33.
93. Blair PA, Norena LY, Flores-Borja F, Rawlings DJ, Isenberg DA, Ehrenstein MR, et al. CD19(+)CD24(hi)CD38(hi) B cells exhibit regulatory capacity in healthy individuals but are functionally impaired in systemic Lupus Erythematosus patients. Immunity. 2010;32(1):129-40.
94. Moulton VR, Suarez-Fueyo A, Meidan E, Li H, Mizui M, Tsokos GC. Pathogenesis of Human Systemic Lupus Erythematosus: A Cellular Perspective. Trends Mol Med. 2017;23(7):615-35.

95. Jenks SA, Palmer EM, Marin EY, Hartson L, Chida AS, Richardson C, et al. 9G4+ autoantibodies are an important source of apoptotic cell reactivity associated with high levels of disease activity in systemic lupus erythematosus. Arthritis Rheum. 2013;65(12):3165-75.
96. Suurmond J, Calise J, Malkiel S, Diamond B. DNA-reactive B cells in lupus. Curr Opin Immunol. 2016;43:1-7.
97. Tsokos GC. Systemic lupus erythematosus. N Engl J Med. 2011;365(22):2110-21.
98. Fernandez SA, Lobo AZ, Oliveira ZN, Fukumori LM, AM Pr, Rivitti EA. Prevalence of antinuclear autoantibodies in the serum of normal blood dornors. Rev Hosp Clin Fac Med Sao Paulo. 2003;58(6):315-9.
99. Wandstrat AE, Carr-Johnson F, Branch V, Gray H, Fairhurst AM, Reimold A, et al. Autoantibody profiling to identify individuals at risk for systemic lupus erythematosus. J Autoimmun. 2006;27(3):153-60.
100. Satoh M, Chan EK, Ho LA, Rose KM, Parks CG, Cohn RD, et al. Prevalence and sociodemographic correlates of antinuclear antibodies in the United States. Arthritis Rheum. 2012;64(7):2319-27.
101. Chen Y, Park YB, Patel E, Silverman GJ. IgM antibodies to apoptosis-associated determinants recruit C1q and enhance dendritic cell phagocytosis of apoptotic cells. J Immunol. 2009;182(10):6031-43.
102. Gronwall C, Vas J, Silverman GJ. Protective Roles of Natural IgM Antibodies. Front Immunol. 2012;3:66.
103. Casali P, Notkins AL. CD5+ B lymphocytes, polyreactive antibodies and the human B-cell repertoire. Immunol Today. 1989;10(11):364-8.
104. Greer JM, Panush RS. Incomplete lupus erythematosus. Arch Intern Med. 1989;149(11):2473-6.
105. Aberle T, Bourn RL, Munroe ME, Chen H, Roberts VC, Guthridge JM, et al. Clinical and Serologic Features in Patients With Incomplete Lupus Classification Versus Systemic Lupus Erythematosus Patients and Controls. Arthritis Care Res (Hoboken). 2017;69(12):1780-8.
106. Arbuckle MR, McClain MT, Rubertone MV, Scofield RH, Dennis GJ, James JA, et al. Development of autoantibodies before the clinical onset of systemic lupus erythematosus. N Engl J Med. 2003;349(16):1526-33.
107. Vila LM, Mayor AM, Valentin AH, Garcia-Soberal M, Vila S. Clinical outcome and predictors of disease evolution in patients with incomplete lupus erythematosus. Lupus. 2000;9(2):110-5.
108. Olsen NJ, Karp DR. Autoantibodies and SLE: the threshold for disease. Nat Rev Rheumatol. 2014;10(3):181-6.
109. Pascual V, Farkas L, Banchereau J. Systemic lupus erythematosus: all roads lead to type I interferons. Current Opinion in Immunology. 2006;18(6):676-82.
110. Banchereau J, Pascual V. Type I interferon in systemic lupus erythematosus and other autoimmune diseases. Immunity. 2006;25(3):383-92.
111. de Weerd NA, Nguyen T. The interferons and their receptors-distribution and regulation. Immunology and Cell Biology. 2012;90(5):483-91.
112. Hall JC, Rosen A. Type I interferons: crucial participants in disease amplification in autoimmunity. Nature Reviews Rheumatology. 2010;6(1):40-9.

113. Kotenko SV, Gallagher G, Baurin VV, Lewis-Antes A, Shen ML, Shah NK, et al. IFN-lambda s mediate antiviral protection through a distinct class II cytokine receptor complex. Nature Immunology. 2003;4(1):69-77.
114. Ank N, West H, Bartholdy C, Eriksson K, Thomsen AR, Paludan SR. Lambda interferon (IFN-lambda), a type III IFN, is induced by viruses and IFNs and displays potent antiviral activity against select virus infections in vivo. J Virol. 2006;80(9):4501-9.
115. Jego G, Palucka AK, Blanck JP, Chalouni C, Pascual V, Banchereau J. Plasmacytoid dendritic cells induce plasma cell differentiation through type I interferon and interleukin 6. Immunity. 2003;19(2):225-34.
116. Longhi MP, Trumpfheller C, Idoyaga J, Caskey M, Matos I, Kluger C, et al. Dendritic cells require a systemic type I interferon response to mature and induce CD4+ Th1 immunity with poly IC as adjuvant. J Exp Med. 2009;206(7):1589-602.
117. Le Bon A, Thompson C, Kamphuis E, Durand V, Rossmann C, Kalinke U, et al. Cutting edge: enhancement of antibody responses through direct stimulation of B and T cells by type I IFN. J Immunol. 2006;176(4):2074-8.
118. Farrar MA, Schreiber RD. The molecular cell biology of interferon-gamma and its receptor. Annu Rev Immunol. 1993;11:571-611.
119. Swiecki M, Colonna M. The multifaceted biology of plasmacytoid dendritic cells. Nat Rev Immunol. 2015;15(8):471-85.
120. Naik SH, Sathe P, Park HY, Metcalf D, Proietto AI, Dakic A, et al. Development of plasmacytoid and conventional dendritic cell subtypes from single precursor cells derived in vitro and in vivo. Nat Immunol. 2007;8(11):1217-26.
121. Onai N, Obata-Onai A, Schmid MA, Ohteki T, Jarrossay D, Manz MG. Identification of clonogenic common Flt3+M-CSFR+ plasmacytoid and conventional dendritic cell progenitors in mouse bone marrow. Nat Immunol. 2007;8(11):1207-16.
122. Reizis B. Regulation of plasmacytoid dendritic cell development. Curr Opin Immunol. 2010;22(2):206-11.
123. Onai N, Kurabayashi K, Hosoi-Amaike M, Toyama-Sorimachi N, Matsushima K, Inaba K, et al. A clonogenic progenitor with prominent plasmacytoid dendritic cell developmental potential. Immunity. 2013;38(5):943-57.
124. Cisse B, Caton ML, Lehner M, Maeda T, Scheu S, Locksley R, et al. Transcription factor E2-2 is an essential and specific regulator of plasmacytoid dendritic cell development. Cell. 2008;135(1):37-48.
125. Ghosh HS, Cisse B, Bunin A, Lewis KL, Reizis B. Continuous expression of the transcription factor e2-2 maintains the cell fate of mature plasmacytoid dendritic cells. Immunity. 2010;33(6):905-16.
126. Hacker C, Kirsch RD, Ju XS, Hieronymus T, Gust TC, Kuhl C, et al. Transcriptional profiling identifies Id2 function in dendritic cell development. Nat Immunol. 2003;4(4):380-6.
127. Schmid MA, Kingston D, Boddupalli S, Manz MG. Instructive cytokine signals in dendritic cell lineage commitment. Immunol Rev. 2010;234(1):32-44.
128. Laouar Y, Welte T, Fu XY, Flavell RA. STAT3 is required for Flt3L-dependent dendritic cell differentiation. Immunity. 2003;19(6):903-12.
129. Sathaliyawala T, O'Gorman WE, Greter M, Bogunovic M, Konjufca V, Hou ZE, et al. Mammalian target of rapamycin controls dendritic cell development downstream of Flt3 ligand signaling. Immunity. 2010;33(4):597-606.

130. Chen YL, Chen TT, Pai LM, Wesoly J, Bluyssen HA, Lee CK. A type I IFN-Flt3 ligand axis augments plasmacytoid dendritic cell development from common lymphoid progenitors. J Exp Med. 2013;210(12):2515-22.
131. Lennert K, Remmele W. [Karyometric research on lymph node cells in man. I. Germinoblasts, lymphoblasts & lymphocytes]. Acta Haematol. 1958;19(2):99-113.
132. Siegal FP, Kadowaki N, Shodell M, Fitzgerald-Bocarsly PA, Shah K, Ho S, et al. The nature of the principal type 1 interferon-producing cells in human blood. Science. 1999;284(5421):1835-7.
133. Kim S, Kaiser V, Beier E, Bechheim M, Guenthner-Biller M, Ablasser A, et al. Self-priming determines high type I IFN production by plasmacytoid dendritic cells. Eur J Immunol. 2014;44(3):807-18.
134. Gilliet M, Cao W, Liu YJ. Plasmacytoid dendritic cells: sensing nucleic acids in viral infection and autoimmune diseases. Nat Rev Immunol. 2008;8(8):594-606.
135. Blasius AL, Beutler B. Intracellular toll-like receptors. Immunity. 2010;32(3):305-15.
136. Honda K, Ohba Y, Yanai H, Negishi H, Mizutani T, Takaoka A, et al. Spatiotemporal regulation of MyD88-IRF-7 signalling for robust type-I interferon induction. Nature. 2005;434(7036):1035-40.
137. Guiducci C, Ott G, Chan JH, Damon E, Calacsan C, Matray T, et al. Properties regulating the nature of the plasmacytoid dendritic cell response to Toll-like receptor 9 activation. J Exp Med. 2006;203(8):1999-2008.
138. Hoshino K, Sugiyama T, Matsumoto M, Tanaka T, Saito M, Hemmi H, et al. IkappaB kinase-alpha is critical for interferon-alpha production induced by Toll-like receptors 7 and 9. Nature. 2006;440(7086):949-53.
139. Shinohara ML, Lu L, Bu J, Werneck MB, Kobayashi KS, Glimcher LH, et al. Osteopontin expression is essential for interferon-alpha production by plasmacytoid dendritic cells. Nat Immunol. 2006;7(5):498-506.
140. Cao W, Manicassamy S, Tang H, Kasturi SP, Pirani A, Murthy N, et al. Toll-like receptor-mediated induction of type I interferon in plasmacytoid dendritic cells requires the rapamycin-sensitive PI(3)K-mTOR-p70S6K pathway. Nat Immunol. 2008;9(10):1157-64.
141. Takaoka A, Yanai H, Kondo S, Duncan G, Negishi H, Mizutani T, et al. Integral role of IRF-5 in the gene induction programme activated by Toll-like receptors. Nature. 2005;434(7030):243-9.
142. Sasai M, Linehan MM, Iwasaki A. Bifurcation of Toll-like receptor 9 signaling by adaptor protein 3. Science. 2010;329(5998):1530-4.
143. Blasius AL, Arnold CN, Georgel P, Rutschmann S, Xia Y, Lin P, et al. Slc15a4, AP-3, and Hermansky-Pudlak syndrome proteins are required for Toll-like receptor signaling in plasmacytoid dendritic cells. Proc Natl Acad Sci U S A. 2010;107(46):19973-8.
144. Henault J, Martinez J, Riggs JM, Tian J, Mehta P, Clarke L, et al. Noncanonical autophagy is required for type I interferon secretion in response to DNA-immune complexes. Immunity. 2012;37(6):986-97.
145. Baccala R, Hoebe K, Kono DH, Beutler B, Theofilopoulos AN. TLR-dependent and TLR-independent pathways of type I interferon induction in systemic autoimmunity. Nat Med. 2007;13(5):543-51.

146. Kim T, Pazhoor S, Bao M, Zhang Z, Hanabuchi S, Facchinetti V, et al. Aspartate-glutamate-alanine-histidine box motif (DEAH)/RNA helicase A helicases sense microbial DNA in human plasmacytoid dendritic cells. Proc Natl Acad Sci U S A. 2010;107(34):15181-6.
147. Kumagai Y, Kumar H, Koyama S, Kawai T, Takeuchi O, Akira S. Cutting Edge: TLR-Dependent viral recognition along with type I IFN positive feedback signaling masks the requirement of viral replication for IFN-{alpha} production in plasmacytoid dendritic cells. J Immunol. 2009;182(7):3960-4.
148. Cai X, Chiu YH, Chen ZJ. The cGAS-cGAMP-STING pathway of cytosolic DNA sensing and signaling. Mol Cell. 2014;54(2):289-96.
149. Villadangos JA, Young L. Antigen-presentation properties of plasmacytoid dendritic cells. Immunity. 2008;29(3):352-61.
150. Young LJ, Wilson NS, Schnorrer P, Proietto A, ten Broeke T, Matsuki Y, et al. Differential MHC class II synthesis and ubiquitination confers distinct antigen-presenting properties on conventional and plasmacytoid dendritic cells. Nat Immunol. 2008;9(11):1244-52.
151. Cella M, Facchetti F, Lanzavecchia A, Colonna M. Plasmacytoid dendritic cells activated by influenza virus and CD40L drive a potent TH1 polarization. Nat Immunol. 2000;1(4):305-10.
152. Krug A, Towarowski A, Britsch S, Rothenfusser S, Hornung V, Bals R, et al. Toll-like receptor expression reveals CpG DNA as a unique microbial stimulus for plasmacytoid dendritic cells which synergizes with CD40 ligand to induce high amounts of IL-12. Eur J Immunol. 2001;31(10):3026-37.
153. Yu CF, Peng WM, Oldenburg J, Hoch J, Bieber T, Limmer A, et al. Human plasmacytoid dendritic cells support Th17 cell effector function in response to TLR7 ligation. J Immunol. 2010;184(3):1159-67.
154. Loschko J, Schlitzer A, Dudziak D, Drexler I, Sandholzer N, Bourquin C, et al. Antigen delivery to plasmacytoid dendritic cells via BST2 induces protective T cell-mediated immunity. J Immunol. 2011;186(12):6718-25.
155. Fonteneau JF, Gilliet M, Larsson M, Dasilva I, Munz C, Liu YJ, et al. Activation of influenza virus-specific CD4+ and CD8+ T cells: a new role for plasmacytoid dendritic cells in adaptive immunity. Blood. 2003;101(9):3520-6.
156. Tel J, Schreibelt G, Sittig SP, Mathan TS, Buschow SI, Cruz LJ, et al. Human plasmacytoid dendritic cells efficiently cross-present exogenous Ags to CD8+ T cells despite lower Ag uptake than myeloid dendritic cell subsets. Blood. 2013;121(3):459-67.
157. Hoeffel G, Ripoche AC, Matheoud D, Nascimbeni M, Escriou N, Lebon P, et al. Antigen crosspresentation by human plasmacytoid dendritic cells. Immunity. 2007;27(3):481-92.
158. Guery L, Hugues S. Tolerogenic and activatory plasmacytoid dendritic cells in autoimmunity. Front Immunol. 2013;4:59.
159. Martin-Gayo E, Sierra-Filardi E, Corbi AL, Toribio ML. Plasmacytoid dendritic cells resident in human thymus drive natural Treg cell development. Blood. 2010;115(26):5366-75.
160. Sharma MD, Baban B, Chandler P, Hou DY, Singh N, Yagita H, et al. Plasmacytoid dendritic cells from mouse tumor-draining lymph nodes directly

activate mature Tregs via indoleamine 2,3-dioxygenase. J Clin Invest. 2007;117(9):2570-82.
161. Chen W, Liang X, Peterson AJ, Munn DH, Blazar BR. The indoleamine 2,3-dioxygenase pathway is essential for human plasmacytoid dendritic cell-induced adaptive T regulatory cell generation. J Immunol. 2008;181(8):5396-404.
162. Boasso A, Herbeuval JP, Hardy AW, Anderson SA, Dolan MJ, Fuchs D, et al. HIV inhibits CD4+ T-cell proliferation by inducing indoleamine 2,3-dioxygenase in plasmacytoid dendritic cells. Blood. 2007;109(8):3351-9.
163. Hadeiba H, Lahl K, Edalati A, Oderup C, Habtezion A, Pachynski R, et al. Plasmacytoid dendritic cells transport peripheral antigens to the thymus to promote central tolerance. Immunity. 2012;36(3):438-50.
164. Chappell CP, Giltiay NV, Draves KE, Chen C, Hayden-Ledbetter MS, Shlomchik MJ, et al. Targeting antigens through blood dendritic cell antigen 2 on plasmacytoid dendritic cells promotes immunologic tolerance. J Immunol. 2014;192(12):5789-801.
165. Ivashkiv LB, Donlin LT. Regulation of type I interferon responses. Nat Rev Immunol. 2014;14(1):36-49.
166. Honda K, Takaoka A, Taniguchi T. Type I interferon [corrected] gene induction by the interferon regulatory factor family of transcription factors. Immunity. 2006;25(3):349-60.
167. Honda K, Taniguchi T. IRFs: master regulators of signalling by Toll-like receptors and cytosolic pattern-recognition receptors. Nat Rev Immunol. 2006;6(9):644-58.
168. Means TK, Latz E, Hayashi F, Murali MR, Golenbock DT, Luster AD. Human lupus autoantibody-DNA complexes activate DCs through cooperation of CD32 and TLR9. J Clin Invest. 2005;115(2):407-17.
169. Bao M, Liu YJ. Regulation of TLR7/9 signaling in plasmacytoid dendritic cells. Protein & cell. 2013;4(1):40-52.
170. Cao W, Bover L. Signaling and ligand interaction of ILT7: receptor-mediated regulatory mechanisms for plasmacytoid dendritic cells. Immunol Rev. 2010;234(1):163-76.
171. Tavano B, Galao RP, Graham DR, Neil SJ, Aquino VN, Fuchs D, et al. Ig-like transcript 7, but not bone marrow stromal cell antigen 2 (also known as HM1.24, tetherin, or CD317), modulates plasmacytoid dendritic cell function in primary human blood leukocytes. J Immunol. 2013;190(6):2622-30.
172. Crow MK. Type I interferon in the pathogenesis of lupus. J Immunol. 2014;192(12):5459-68.
173. Kaplan MJ. Role of neutrophils in systemic autoimmune diseases. Arthritis Res Ther. 2013;15(5):219.
174. Eloranta ML, Lovgren T, Finke D, Mathsson L, Ronnelid J, Kastner B, et al. Regulation of the interferon-alpha production induced by RNA-containing immune complexes in plasmacytoid dendritic cells. Arthritis Rheum. 2009;60(8):2418-27.
175. Zhou H, Huang X, Cui H, Luo X, Tang Y, Chen S, et al. miR-155 and its star-form partner miR-155* cooperatively regulate type I interferon production by human plasmacytoid dendritic cells. Blood. 2010;116(26):5885-94.
176. Karrich JJ, Jachimowski LC, Libouban M, Iyer A, Brandwijk K, Taanman-Kueter EW, et al. MicroRNA-146a regulates survival and maturation of human plasmacytoid dendritic cells. Blood. 2013;122(17):3001-9.

177. Seillet C, Laffont S, Tremollieres F, Rouquie N, Ribot C, Arnal JF, et al. The TLR-mediated response of plasmacytoid dendritic cells is positively regulated by estradiol in vivo through cell-intrinsic estrogen receptor alpha signaling. Blood. 2012;119(2):454-64.
178. Yang CH, Murti A, Pfeffer SR, Kim JG, Donner DB, Pfeffer LM. Interferon alpha /beta promotes cell survival by activating nuclear factor kappa B through phosphatidylinositol 3-kinase and Akt. The Journal of biological chemistry. 2001;276(17):13756-61.
179. Chawla-Sarkar M, Leaman DW, Borden EC. Preferential induction of apoptosis by interferon (IFN)-beta compared with IFN-alpha2: correlation with TRAIL/Apo2L induction in melanoma cell lines. Clin Cancer Res. 2001;7(6):1821-31.
180. Badr G, Saad H, Waly H, Hassan K, Abdel-Tawab H, Alhazza IM, et al. Type I interferon (IFN-alpha/beta) rescues B-lymphocytes from apoptosis via PI3Kdelta/Akt, Rho-A, NFkappaB and Bcl-2/Bcl(XL). Cell Immunol. 2010;263(1):31-40.
181. de Weerd NA, Samarajiwa SA, Hertzog PJ. Type I interferon receptors: biochemistry and biological functions. The Journal of biological chemistry. 2007;282(28):20053-7.
182. Gazziola C, Cordani N, Carta S, De Lorenzo E, Colombatti A, Perris R. The relative endogenous expression levels of the IFNAR2 isoforms influence the cytostatic and pro-apoptotic effect of IFNalpha on pleomorphic sarcoma cells. Int J Oncol. 2005;26(1):129-40.
183. Ank N, Iversen MB, Bartholdy C, Staeheli P, Hartmann R, Jensen UB, et al. An important role for type III interferon (IFN-lambda/IL-28) in TLR-induced antiviral activity. J Immunol. 2008;180(4):2474-85.
184. Alase AA, El-Sherbiny YM, Vital EM, Tobin DJ, Turner NA, Wittmann M. IFNlambda Stimulates MxA Production in Human Dermal Fibroblasts via a MAPK-Dependent STAT1-Independent Mechanism. J Invest Dermatol. 2015;135(12):2935-43.
185. Ronnblom LE, Alm GV, Oberg KE. Autoimmunity after alpha-interferon therapy for malignant carcinoid tumors. Ann Intern Med. 1991;115(3):178-83.
186. Ioannou Y, Isenberg DA. Current evidence for the induction of autoimmune rheumatic manifestations by cytokine therapy. Arthritis Rheum. 2000;43(7):1431-42.
187. Okanoue T, Sakamoto S, Itoh Y, Minami M, Yasui K, Sakamoto M, et al. Side effects of high-dose interferon therapy for chronic hepatitis C. J Hepatol. 1996;25(3):283-91.
188. Liu Z, Davidson A. Taming lupus-a new understanding of pathogenesis is leading to clinical advances. Nat Med. 2012;18(6):871-82.
189. Ytterberg SR, Schnitzer TJ. Serum interferon levels in patients with systemic lupus erythematosus. Arthritis Rheum. 1982;25(4):401-6.
190. Bengtsson AA, Sturfelt G, Truedsson L, Blomberg J, Alm G, Vallin H, et al. Activation of type I interferon system in systemic lupus erythematosus correlates with disease activity but not with antiretroviral antibodies. Lupus. 2000;9(9):664-71.
191. Raison CL, Demetrashvili M, Capuron L, Miller AH. Neuropsychiatric adverse effects of interferon-alpha: recognition and management. CNS Drugs. 2005;19(2):105-23.
192. Shiozawa S, Kuroki Y, Kim M, Hirohata S, Ogino T. Interferon-alpha in lupus psychosis. Arthritis Rheum. 1992;35(4):417-22.

193. Anders HJ, Lichtnekert J, Allam R. Interferon-alpha and -beta in kidney inflammation. Kidney Int. 2010;77(10):848-54.
194. Fairhurst AM, Mathian A, Connolly JE, Wang A, Gray HF, George TA, et al. Systemic IFN-alpha drives kidney nephritis in B6.Sle123 mice. Eur J Immunol. 2008;38(7):1948-60.
195. Tucci M, Quatraro C, Lombardi L, Pellegrino C, Dammacco F, Silvestris F. Glomerular accumulation of plasmacytoid dendritic cells in active lupus nephritis: role of interleukin-18. Arthritis Rheum. 2008;58(1):251-62.
196. Yin Z, Dai J, Deng J, Sheikh F, Natalia M, Shih T, et al. Type III IFNs are produced by and stimulate human plasmacytoid dendritic cells. J Immunol. 2012;189(6):2735-45.
197. Zahn S, Rehkamper C, Kummerer BM, Ferring-Schmidt S, Bieber T, Tuting T, et al. Evidence for a pathophysiological role of keratinocyte-derived type III interferon (IFNlambda) in cutaneous lupus erythematosus. J Invest Dermatol. 2011;131(1):133-40.
198. Deng Y, Tsao BP. Genetic susceptibility to systemic lupus erythematosus in the genomic era. Nat Rev Rheumatol. 2010;6(12):683-92.
199. Niewold TB, Hua J, Lehman TJ, Harley JB, Crow MK. High serum IFN-alpha activity is a heritable risk factor for systemic lupus erythematosus. Genes Immun. 2007;8(6):492-502.
200. Sigurdsson S, Nordmark G, Goring HH, Lindroos K, Wiman AC, Sturfelt G, et al. Polymorphisms in the tyrosine kinase 2 and interferon regulatory factor 5 genes are associated with systemic lupus erythematosus. Am J Hum Genet. 2005;76(3):528-37.
201. Graham RR, Kozyrev SV, Baechler EC, Reddy MV, Plenge RM, Bauer JW, et al. A common haplotype of interferon regulatory factor 5 (IRF5) regulates splicing and expression and is associated with increased risk of systemic lupus erythematosus. Nat Genet. 2006;38(5):550-5.
202. Feng D, Stone RC, Eloranta ML, Sangster-Guity N, Nordmark G, Sigurdsson S, et al. Genetic variants and disease-associated factors contribute to enhanced interferon regulatory factor 5 expression in blood cells of patients with systemic lupus erythematosus. Arthritis Rheum. 2010;62(2):562-73.
203. Niewold TB, Kelly JA, Kariuki SN, Franek BS, Kumar AA, Kaufman KM, et al. IRF5 haplotypes demonstrate diverse serological associations which predict serum interferon alpha activity and explain the majority of the genetic association with systemic lupus erythematosus. Ann Rheum Dis. 2012;71(3):463-8.
204. Salloum R, Franek BS, Kariuki SN, Rhee L, Mikolaitis RA, Jolly M, et al. Genetic variation at the IRF7/PHRF1 locus is associated with autoantibody profile and serum interferon-alpha activity in lupus patients. Arthritis Rheum. 2010;62(2):553-61.
205. Cherian TS, Kariuki SN, Franek BS, Buyon JP, Clancy RM, Niewold TB. Brief Report: IRF5 systemic lupus erythematosus risk haplotype is associated with asymptomatic serologic autoimmunity and progression to clinical autoimmunity in mothers of children with neonatal lupus. Arthritis Rheum. 2012;64(10):3383-7.
206. Robinson T, Kariuki SN, Franek BS, Kumabe M, Kumar AA, Badaracco M, et al. Autoimmune disease risk variant of IFIH1 is associated with increased sensitivity to IFN-alpha and serologic autoimmunity in lupus patients. J Immunol. 2011;187(3):1298-303.

207. Leonard D, Svenungsson E, Sandling JK, Berggren O, Jonsen A, Bengtsson C, et al. Coronary heart disease in systemic lupus erythematosus is associated with interferon regulatory factor-8 gene variants. Circ Cardiovasc Genet. 2013;6(3):255-63.
208. Doring Y, Soehnlein O, Drechsler M, Shagdarsuren E, Chaudhari SM, Meiler S, et al. Hematopoietic interferon regulatory factor 8-deficiency accelerates atherosclerosis in mice. Arterioscler Thromb Vasc Biol. 2012;32(7):1613-23.
209. Munz C, Lunemann JD, Getts MT, Miller SD. Antiviral immune responses: triggers of or triggered by autoimmunity? Nat Rev Immunol. 2009;9(4):246-58.
210. Marshak-Rothstein A, Rifkin IR. Immunologically active autoantigens: the role of toll-like receptors in the development of chronic inflammatory disease. Annu Rev Immunol. 2007;25:419-41.
211. Munoz LE, van Bavel C, Franz S, Berden J, Herrmann M, van der Vlag J. Apoptosis in the pathogenesis of systemic lupus erythematosus. Lupus. 2008;17(5):371-5.
212. Barrat FJ, Meeker T, Gregorio J, Chan JH, Uematsu S, Akira S, et al. Nucleic acids of mammalian origin can act as endogenous ligands for toll-like receptors and may promote systemic lupus erythematosus. Journal of Experimental Medicine. 2005;202(8):1131-9.
213. Vollmer J, Tluk S, Schmitz C, Hamm S, Jurk M, Forsbach A, et al. Immune stimulation mediated by autoantigen binding sites within small nuclear RNAs involves Toll-like receptors 7 and 8. J Exp Med. 2005;202(11):1575-85.
214. Lovgren T, Eloranta ML, Kastner B, Wahren-Herlenius M, Alm GV, Ronnblom L. Induction of interferon-alpha by immune complexes or liposomes containing systemic lupus erythematosus autoantigen- and Sjogren's syndrome autoantigen-associated RNA. Arthritis Rheum. 2006;54(6):1917-27.
215. Hung T, Pratt GA, Sundararaman B, Townsend MJ, Chaivorapol C, Bhangale T, et al. The Ro60 autoantigen binds endogenous retroelements and regulates inflammatory gene expression. Science. 2015;350(6259):455-9.
216. Walsh ER, Pisitkun P, Voynova E, Deane JA, Scott BL, Caspi RR, et al. Dual signaling by innate and adaptive immune receptors is required for TLR7-induced B-cell-mediated autoimmunity. Proc Natl Acad Sci U S A. 2012;109(40):16276-81.
217. Le Bon A, Schiavoni G, D'Agostino G, Gresser I, Belardelli F, Tough DF. Type i interferons potently enhance humoral immunity and can promote isotype switching by stimulating dendritic cells in vivo. Immunity. 2001;14(4):461-70.
218. Care MA, Stephenson SJ, Barnes NA, Fan I, Zougman A, El-Sherbiny YM, et al. Network Analysis Identifies Proinflammatory Plasma Cell Polarization for Secretion of ISG15 in Human Autoimmunity. J Immunol. 2016.
219. Ehlers M, Fukuyama H, McGaha TL, Aderem A, Ravetch JV. TLR9/MyD88 signaling is required for class switching to pathogenic IgG2a and 2b autoantibodies in SLE. J Exp Med. 2006;203(3):553-61.
220. Kolumam GA, Thomas S, Thompson LJ, Sprent J, Murali-Krishna K. Type I interferons act directly on CD8 T cells to allow clonal expansion and memory formation in response to viral infection. J Exp Med. 2005;202(5):637-50.
221. Qasim FJ, Mathieson PW, Sendo F, Thiru S, Oliveira DB. Role of neutrophils in the pathogenesis of experimental vasculitis. Am J Pathol. 1996;149(1):81-9.

222. Hotta O, Oda T, Taguma Y, Kitamura H, Chiba S, Miyazawa S, et al. Role of neutrophil elastase in the development of renal necrotizing vasculitis. Clin Nephrol. 1996;45(4):211-6.
223. Garcia-Romo GS, Caielli S, Vega B, Connolly J, Allantaz F, Xu Z, et al. Netting neutrophils are major inducers of type I IFN production in pediatric systemic lupus erythematosus. Sci Transl Med. 2011;3(73):73ra20.
224. Fuchs TA, Abed U, Goosmann C, Hurwitz R, Schulze I, Wahn V, et al. Novel cell death program leads to neutrophil extracellular traps. J Cell Biol. 2007;176(2):231-41.
225. Lande R, Ganguly D, Facchinetti V, Frasca L, Conrad C, Gregorio J, et al. Neutrophils activate plasmacytoid dendritic cells by releasing self-DNA-peptide complexes in systemic lupus erythematosus. Sci Transl Med. 2011;3(73):73ra19.
226. Martinelli S, Urosevic M, Daryadel A, Oberholzer PA, Baumann C, Fey MF, et al. Induction of genes mediating interferon-dependent extracellular trap formation during neutrophil differentiation. The Journal of biological chemistry. 2004;279(42):44123-32.
227. De Paepe B. Interferons as components of the complex web of reactions sustaining inflammation in idiopathic inflammatory myopathies. Cytokine. 2015;74(1):81-7.
228. Wu M, Assassi S. The role of type 1 interferon in systemic sclerosis. Front Immunol. 2013;4:266.
229. Hernandez-Molina G, Leal-Alegre G, Michel-Peregrina M. The meaning of anti-Ro and anti-La antibodies in primary Sjogren's syndrome. Autoimmun Rev. 2011;10(3):123-5.
230. Nguyen CQ, Peck AB. The Interferon-Signature of Sjogren's Syndrome: How Unique Biomarkers Can Identify Underlying Inflammatory and Immunopathological Mechanisms of Specific Diseases. Front Immunol. 2013;4:142.
231. Li H, Ice JA, Lessard CJ, Sivils KL. Interferons in Sjogren's Syndrome: Genes, Mechanisms, and Effects. Front Immunol. 2013;4:290.
232. Bave U, Nordmark G, Lovgren T, Ronnelid J, Cajander S, Eloranta ML, et al. Activation of the type I interferon system in primary Sjogren's syndrome: a possible etiopathogenic mechanism. Arthritis Rheum. 2005;52(4):1185-95.
233. Gottenberg JE, Cagnard N, Lucchesi C, Letourneur F, Mistou S, Lazure T, et al. Activation of IFN pathways and plasmacytoid dendritic cell recruitment in target organs of primary Sjogren's syndrome. Proc Natl Acad Sci U S A. 2006;103(8):2770-5.
234. Kawakami A, Nakashima K, Tamai M, Nakamura H, Iwanaga N, Fujikawa K, et al. Toll-like receptor in salivary glands from patients with Sjogren's syndrome: functional analysis by human salivary gland cell line. J Rheumatol. 2007;34(5):1019-26.
235. Spachidou MP, Bourazopoulou E, Maratheftis CI, Kapsogeorgou EK, Moutsopoulos HM, Tzioufas AG, et al. Expression of functional Toll-like receptors by salivary gland epithelial cells: increased mRNA expression in cells derived from patients with primary Sjogren's syndrome. Clin Exp Immunol. 2007;147(3):497-503.
236. Hjelmervik TO, Petersen K, Jonassen I, Jonsson R, Bolstad AI. Gene expression profiling of minor salivary glands clearly distinguishes primary Sjogren's syndrome patients from healthy control subjects. Arthritis Rheum. 2005;52(5):1534-44.

237. Perez P, Anaya JM, Aguilera S, Urzua U, Munroe D, Molina C, et al. Gene expression and chromosomal location for susceptibility to Sjogren's syndrome. J Autoimmun. 2009;33(2):99-108.
238. Emamian ES, Leon JM, Lessard CJ, Grandits M, Baechler EC, Gaffney PM, et al. Peripheral blood gene expression profiling in Sjogren's syndrome. Genes Immun. 2009;10(4):285-96.
239. Zheng L, Yu C, Zhang Z, Yang C, Cai X. Expression of interferon regulatory factor 1, 3, and 7 in primary Sjogren syndrome. Oral Surg Oral Med Oral Pathol Oral Radiol Endod. 2009;107(5):661-8.
240. Brkic Z, Maria NI, van Helden-Meeuwsen CG, van de Merwe JP, van Daele PL, Dalm VA, et al. Prevalence of interferon type I signature in CD14 monocytes of patients with Sjogren's syndrome and association with disease activity and BAFF gene expression. Ann Rheum Dis. 2013;72(5):728-35.
241. Zhou X, Dimachkie MM, Xiong M, Tan FK, Arnett FC. cDNA microarrays reveal distinct gene expression clusters in idiopathic inflammatory myopathies. Medical science monitor : international medical journal of experimental and clinical research. 2004;10(7):BR191-7.
242. Shrestha S, Wershil B, Sarwark JF, Niewold TB, Philipp T, Pachman LM. Lesional and nonlesional skin from patients with untreated juvenile dermatomyositis displays increased numbers of mast cells and mature plasmacytoid dendritic cells. Arthritis Rheum. 2010;62(9):2813-22.
243. Higgs BW, Liu Z, White B, Zhu W, White WI, Morehouse C, et al. Patients with systemic lupus erythematosus, myositis, rheumatoid arthritis and scleroderma share activation of a common type I interferon pathway. Ann Rheum Dis. 2011;70(11):2029-36.
244. Niewold TB, Kariuki SN, Morgan GA, Shrestha S, Pachman LM. Elevated serum interferon-alpha activity in juvenile dermatomyositis: associations with disease activity at diagnosis and after thirty-six months of therapy. Arthritis Rheum. 2009;60(6):1815-24.
245. Baechler EC, Bauer JW, Slattery CA, Ortmann WA, Espe KJ, Novitzke J, et al. An interferon signature in the peripheral blood of dermatomyositis patients is associated with disease activity. Mol Med. 2007;13(1-2):59-68.
246. Shinohara MM, Davis C, Olerud J. Concurrent antiphospholipid syndrome and cutaneous [corrected] sarcoidosis due to interferon alfa and ribavirin treatment for hepatitis C. J Drugs Dermatol. 2009;8(9):870-2.
247. Balderramo DC, Garcia O, Colmenero J, Espinosa G, Forns X, Gines P. Antiphospholipid syndrome during pegylated interferon alpha-2a therapy for chronic hepatitis C. Dig Liver Dis. 2009;41(7):e4-7.
248. Psarras A, Md Yusof M, El-Sherbiny Y, Hensor E, Wittmann M, Emery P, et al. A9.05 Distinct subsets of interferon-stimulated genes are associated with incomplete and established systemic lupus erythematosus. Annals of the Rheumatic Diseases. 2016;75(Suppl 1):A72.
249. Mohamed AAA, Yusof MYM, El-Sherbiny YM, Cassamoali H, Wittmann M, Hensor E, et al. Increased Interferon activity is associated with progression from Early Incomplete Lupus Erythematosus to SLE. Clinical and Experimental Rheumatology. 2015;33(3):S25-S.

250. Lubbers J, Brink M, de Stadt LAV, Vosslamber S, Wesseling JG, van Schaardenburg D, et al. The type I IFN signature as a biomarker of preclinical rheumatoid arthritis. Annals of the Rheumatic Diseases. 2013;72(5):776-80.
251. Thurlings RM, Boumans M, Tekstra J, van Roon JA, Vos K, van Westing DM, et al. Relationship Between the Type I Interferon Signature and the Response to Rituximab in Rheumatoid Arthritis Patients. Arthritis Rheum-Us. 2010;62(12):3607-14.
252. Raterman H, Vosslamber S, de Ridder S, Nurmohamed M, Lems W, Boers M, et al. The Interferon Type I Signature Towards Prediction of Non-Response to Rituximab in Rheumatoid Arthritis Patients. Annals of the Rheumatic Diseases. 2012;71:195-6.
253. Sekiguchi N, Kawauchi S, Furuya T, Inaba N, Matsuda K, Ando S, et al. Messenger ribonucleic acid expression profile in peripheral blood cells from RA patients following treatment with an anti-TNF-alpha monoclonal antibody, infliximab. Rheumatology. 2008;47(6):780-8.
254. Wright HL, Thomas HB, Moots RJ, Edwards SW. Interferon gene expression signature in rheumatoid arthritis neutrophils correlates with a good response to TNFi therapy. Rheumatology. 2015;54(1):188-93.
255. Kraan TCTMV, Wijbrandts CA, van Baarsen LGM, Voskuyl AE, Rustenburg F, Baggen JM, et al. Rheumatoid arthritis subtypes identified by genomic profiling of peripheral blood cells: assignment of a type I interferon signature in a subpopulation of patients. Annals of the Rheumatic Diseases. 2007;66(8):1008-14.
256. Fuertes MB, Woo SR, Burnett B, Fu YX, Gajewski TF. Type I interferon response and innate immune sensing of cancer. Trends in Immunology. 2013;34(2):67-73.
257. Spaapen RM, Leung MYK, Fuertes MB, Kline JP, Zhang L, Zheng Y, et al. Therapeutic Activity of High-Dose Intratumoral IFN-beta Requires Direct Effect on the Tumor Vasculature. Journal of Immunology. 2014;193(8):4254-60.
258. Bosinger SE, Hosiawa KA, Cameron MJ, Persad D, Rang LS, Xu LL, et al. Gene expression profiling of host response in models of acute HIV infection. Journal of Immunology. 2004;173(11):6858-63.
259. Amsler L, Verweij MC, DeFilippis VR. The Tiers and Dimensions of Evasion of the Type I Interferon Response by Human Cytomegalovirus. J Mol Biol. 2013;425(24):4857-71.
260. Snell LM, Brooks DG. New insights into type I interferon and the immunopathogenesis of persistent viral infections. Current Opinion in Immunology. 2015;34:91-8.
261. Sandler NG, Bosinger SE, Estes JD, Zhu RTR, Tharp GK, Boritz E, et al. Type I interferon responses in rhesus macaques prevent SIV infection and slow disease progression. Nature. 2014;511(7511):601-+.
262. Boshuizen MCS, de Winther MPJ. Interferons as Essential Modulators of Atherosclerosis. Arterioscl Throm Vas. 2015;35(7):1579-88.
263. Crow YJ, Manel N. Aicardi-Goutieres syndrome and the type I interferonopathies. Nature Reviews Immunology. 2015;15(7):429-40.
264. Rice GI, Kasher PR, Forte GMA, Mannion NM, Greenwood SM, Szynkiewicz M, et al. Mutations in ADAR1 cause Aicardi-Goutieres syndrome associated with a type I interferon signature. Nature Genetics. 2012;44(11):1243-8.

265. Rice GI, Forte GMA, Szynkiewicz M, Chase DS, Aeby A, Abdel-Hamid MS, et al. Assessment of interferon-related biomarkers in Aicardi-Goutieres syndrome associated with mutations in TREX1, RNASEH2A, RNASEH2B, RNASEH2C, SAMHD1, and ADAR: a case-control study. Lancet Neurology. 2013;12(12):1159-69.
266. Crow YJ, Chase DS, Schmidt JL, Szynkiewicz M, Forte GMA, Gornall HL, et al. Characterization of Human Disease Phenotypes Associated with Mutations in TREX1, RNASEH2A, RNASEH2B, RNASEH2C, SAMHD1, ADAR, and IFIH1. American Journal of Medical Genetics Part A. 2015;167(2):296-312.
267. Kuznik A, Bencina M, Svajger U, Jeras M, Rozman B, Jerala R. Mechanism of Endosomal TLR Inhibition by Antimalarial Drugs and Imidazoquinolines. Journal of Immunology. 2011;186(8):4794-804.
268. Sacre K, Criswell LA, McCune JM. Hydroxychloroquine is associated with impaired interferon-alpha and tumor necrosis factor-alpha production by plasmacytoid dendritic cells in systemic lupus erythematosus. Arthritis Research & Therapy. 2012;14(3).
269. Li J, Wang XH, Zhang FC, Yin H. Toll-like receptors as therapeutic targets for autoimmune connective tissue diseases. Pharmacol Therapeut. 2013;138(3):441-51.
270. Wu YW, Tang W, Zuo JP. Toll-like receptors: potential targets for lupus treatment. Acta Pharmacologica Sinica. 2015;36(12):1395-407.
271. De Bosscher K, Vanden Berghe W, Haegeman G. The interplay between the glucocorticoid receptor and nuclear factor-kappa B or activator protein-1: Molecular mechanisms for gene repression. Endocr Rev. 2003;24(4):488-522.
272. Guiducci C, Gong M, Xu ZH, Gill M, Chaussabel D, Meeker T, et al. TLR recognition of self nucleic acids hampers glucocorticoid activity in lupus. Nature. 2010;465(7300):937-U10.
273. Merrill JT, Wallace DJ, Petri M, Kirou KA, Yao YH, White WI, et al. Safety profile and clinical activity of sifalimumab, a fully human anti-interferon alpha monoclonal antibody, in systemic lupus erythematosus: a phase I, multicentre, double-blind randomised study. Annals of the Rheumatic Diseases. 2011;70(11):1905-13.
274. Petri M, Wallace DJ, Spindler A, Chindalore V, Kalunian K, Mysler E, et al. Sifalimumab, a Human AntiInterferon- Monoclonal Antibody, in Systemic Lupus Erythematosus: A Phase I Randomized, Controlled, Dose-Escalation Study. Arthritis Rheum-Us. 2013;65(4):1011-21.
275. Tcherepanova I, Curtis M, Sale M, Miesowicz F, Nicolette C. RESULTS OF A RANDOMIZED PLACEBO CONTROLLED PHASE IA STUDY OF AGS-009, A HUMANIZED ANTI-INTERFERON-alpha MONOCLONAL ANTIBODY IN SUBJECTS WITH SYSTEMIC LUPUS ERYTHEMATOSUS. Annals of the Rheumatic Diseases. 2012;71:536-7.
276. Yao YH, Richman L, Higgs BW, Morehouse CA, de los Reyes M, Brohawn P, et al. Neutralization of Interferon-alpha/beta-Inducible Genes and Downstream Effect in a Phase I Trial of an Anti-Interferon-alpha Monoclonal Antibody in Systemic Lupus Erythematosus. Arthritis Rheum-Us. 2009;60(6):1785-96.
277. Khamashta M, Merrill JT, Werth VP, Furie R, Kalunian K, Illei GG, et al. Safety and Efficacy of Sifalimumab, an Anti IFN-Alpha Monoclonal Antibody, in a Phase 2b Study of Moderate to Severe Systemic Lupus Erythematosus (SLE). Arthritis & Rheumatology. 2014;66(12):3530-1.
278. Kalunian KC, Merrill JT, Maciuca R, McBride JM, Townsend MJ, Wei XH, et al. A Phase II study of the efficacy and safety of rontalizumab (rhuMAb interferon-alpha)

in patients with systemic lupus erythematosus (ROSE). Annals of the Rheumatic Diseases. 2016;75(1):196-202.
279. Wang B, Higgs BW, Chang L, Vainshtein I, Liu Z, Streicher K, et al. Pharmacogenomics and translational simulations to bridge indications for an anti-interferon-alpha receptor antibody. Clin Pharmacol Ther. 2013;93(6):483-92.
280. Peng L, Oganesyan V, Wu H, Dall'Acqua WF, Damschroder MM. Molecular basis for antagonistic activity of anifrolumab, an anti-interferon-alpha receptor 1 antibody. MAbs. 2015;7(2):428-39.
281. Brohawn P, Santiago L, Morehouse C, Higgs B, Illei G, Ranade K. Target Modulation of a Type I Interferon Gene Signature and Pharmacokinetics of Anifrolumab in a Phase IIb Study of Patients with Moderate to Severe Systemic Lupus Erythematosus. Arthritis & Rheumatology. 2015;67.
282. Rowland SL, Riggs JM, Gilfillan S, Bugatti M, Vermi W, Kolbeck R, et al. Early, transient depletion of plasmacytoid dendritic cells ameliorates autoimmunity in a lupus model. J Exp Med. 2014;211(10):1977-91.
283. Zhan Y, Carrington EM, Ko HJ, Vikstrom IB, Oon S, Zhang JG, et al. Bcl-2 antagonists kill plasmacytoid dendritic cells from lupus-prone mice and dampen interferon-alpha production. Arthritis Rheumatol. 2015;67(3):797-808.
284. Ichikawa HT, Conley T, Muchamuel T, Jiang J, Lee S, Owen T, et al. Beneficial effect of novel proteasome inhibitors in murine lupus via dual inhibition of type I interferon and autoantibody-secreting cells. Arthritis Rheum. 2012;64(2):493-503.
285. Pellerin A, Otero K, Czerkowicz JM, Kerns HM, Shapiro RI, Ranger AM, et al. Anti-BDCA2 monoclonal antibody inhibits plasmacytoid dendritic cell activation through Fc-dependent and Fc-independent mechanisms. EMBO Mol Med. 2015;7(4):464-76.
286. Lauwerys BR, Hachulla E, Spertini F, Lazaro E, Jorgensen C, Mariette X, et al. Down-regulation of interferon signature in systemic lupus erythematosus patients by active immunization with interferon alpha-kinoid. Arthritis Rheum. 2013;65(2):447-56.
287. Bennett L, Palucka AK, Arce E, Cantrell V, Borvak J, Banchereau J, et al. Interferon and granulopoiesis signatures in systemic lupus erythematosus blood. J Exp Med. 2003;197(6):711-23.
288. Kirou KA, Lee C, George S, Louca K, Peterson MG, Crow MK. Activation of the interferon-alpha pathway identifies a subgroup of systemic lupus erythematosus patients with distinct serologic features and active disease. Arthritis Rheum. 2005;52(5):1491-503.
289. Crow MK, Kirou KA, Wohlgemuth J. Microarray analysis of interferon-regulated genes in SLE. Autoimmunity. 2003;36(8):481-90.
290. Baechler EC, Batliwalla FM, Karypis G, Gaffney PM, Ortmann WA, Espe KJ, et al. Interferon-inducible gene expression signature in peripheral blood cells of patients with severe lupus. Proc Natl Acad Sci U S A. 2003;100(5):2610-5.
291. Feng X, Wu H, Grossman JM, Hanvivadhanakul P, FitzGerald JD, Park GS, et al. Association of increased interferon-inducible gene expression with disease activity and lupus nephritis in patients with systemic lupus erythematosus. Arthritis Rheum. 2006;54(9):2951-62.

292. Petri M, Singh S, Tesfasyone H, Dedrick R, Fry K, Lal P, et al. Longitudinal expression of type I interferon responsive genes in systemic lupus erythematosus. Lupus. 2009;18(11):980-9.
293. Landolt-Marticorena C, Bonventi G, Lubovich A, Ferguson C, Unnithan T, Su J, et al. Lack of association between the interferon-alpha signature and longitudinal changes in disease activity in systemic lupus erythematosus. Ann Rheum Dis. 2009;68(9):1440-6.
294. Chiche L, Jourde-Chiche N, Whalen E, Presnell S, Gersuk V, Dang K, et al. Modular transcriptional repertoire analyses of adults with systemic lupus erythematosus reveal distinct type I and type II interferon signatures. Arthritis Rheumatol. 2014;66(6):1583-95.
295. Han GM, Chen SL, Shen N, Ye S, Bao CD, Gu YY. Analysis of gene expression profiles in human systemic lupus erythematosus using oligonucleotide microarray. Genes Immun. 2003;4(3):177-86.
296. Becker AM, Dao KH, Han BK, Kornu R, Lakhanpal S, Mobley AB, et al. SLE peripheral blood B cell, T cell and myeloid cell transcriptomes display unique profiles and each subset contributes to the interferon signature. PLoS One. 2013;8(6):e67003.
297. Jin Z, Fan W, Jensen MA, Dorschner JM, Vsetecka DM, Amin S, et al. Single cell interferon signatures in lupus patient monocytes reveal a differential impact of interferon signaling between monocyte subtypes. Cytokine. 2015;76(1):102-3.
298. Absher DM, Li X, Waite LL, Gibson A, Roberts K, Edberg J, et al. Genome-wide DNA methylation analysis of systemic lupus erythematosus reveals persistent hypomethylation of interferon genes and compositional changes to CD4+ T-cell populations. PLoS Genet. 2013;9(8):e1003678.
299. Coit P, Jeffries M, Altorok N, Dozmorov MG, Koelsch KA, Wren JD, et al. Genome-wide DNA methylation study suggests epigenetic accessibility and transcriptional poising of interferon-regulated genes in naive CD4+ T cells from lupus patients. J Autoimmun. 2013;43:78-84.
300. El-Sherbiny Y, Yusof MM, Hensor E, Psarras A, Mohamed A, Wittmann M, et al. A9.06 Analysis of cell-specific interferon response in systemic lupus erythematosus using a novel flow cytometric assay. Annals of the Rheumatic Diseases. 2016;75(Suppl 1):A72.
301. Li QZ, Zhou J, Lian Y, Zhang B, Branch VK, Carr-Johnson F, et al. Interferon signature gene expression is correlated with autoantibody profiles in patients with incomplete lupus syndromes. Clin Exp Immunol. 2010;159(3):281-91.
302. El-Sherbiny YM, Psarras A, Md Yusof MY, Hensor EMA, Tooze R, Doody G, et al. A novel two-score system for interferon status segregates autoimmune diseases and correlates with clinical features. Sci Rep. 2018;8(1):5793.
303. Kuhn RM, Haussler D, Kent WJ. The UCSC genome browser and associated tools. Brief Bioinform. 2013;14(2):144-61.
304. Dobin A, Davis CA, Schlesinger F, Drenkow J, Zaleski C, Jha S, et al. STAR: ultrafast universal RNA-seq aligner. Bioinformatics. 2013;29(1):15-21.
305. Karolchik D, Hinrichs AS, Furey TS, Roskin KM, Sugnet CW, Haussler D, et al. The UCSC Table Browser data retrieval tool. Nucleic Acids Res. 2004;32(Database issue):D493-6.

306. Okonechnikov K, Conesa A, Garcia-Alcalde F. Qualimap 2: advanced multi-sample quality control for high-throughput sequencing data. Bioinformatics. 2016;32(2):292-4.
307. Adiconis X, Borges-Rivera D, Satija R, DeLuca DS, Busby MA, Berlin AM, et al. Comparative analysis of RNA sequencing methods for degraded or low-input samples. Nat Methods. 2013;10(7):623-9.
308. Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, Homer N, et al. The Sequence Alignment/Map format and SAMtools. Bioinformatics. 2009;25(16):2078-9.
309. Robinson JT, Thorvaldsdottir H, Winckler W, Guttman M, Lander ES, Getz G, et al. Integrative genomics viewer. Nat Biotechnol. 2011;29(1):24-6.
310. Liao Y, Smyth GK, Shi W. The Subread aligner: fast, accurate and scalable read mapping by seed-and-vote. Nucleic Acids Res. 2013;41(10):e108.
311. Anders S, Huber W. Differential expression analysis for sequence count data. Genome Biol. 2010;11(10):R106.
312. Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol. 2014;15(12):550.
313. Kolde R, Laur S, Adler P, Vilo J. Robust rank aggregation for gene list integration and meta-analysis. Bioinformatics. 2012;28(4):573-80.
314. Yu G, Wang LG, Han Y, He QY. clusterProfiler: an R package for comparing biological themes among gene clusters. OMICS. 2012;16(5):284-7.
315. Yu G, He QY. ReactomePA: an R/Bioconductor package for reactome pathway analysis and visualization. Mol Biosyst. 2016;12(2):477-9.
316. Kanehisa M, Goto S. KEGG: kyoto encyclopedia of genes and genomes. Nucleic Acids Res. 2000;28(1):27-30.
317. Luo W, Brouwer C. Pathview: an R/Bioconductor package for pathway-based data integration and visualization. Bioinformatics. 2013;29(14):1830-1.
318. Kuhn A, Wozniacka A, Szepietowski JC, Glaser R, Lehmann P, Haust M, et al. Photoprovocation in cutaneous lupus erythematosus: a multicenter study evaluating a standardized protocol. J Invest Dermatol. 2011;131(8):1622-30.
319. Ruland V, Haust M, Stilling RM, Metze D, Amler S, Ruzicka T, et al. Updated analysis of standardized photoprovocation in patients with cutaneous lupus erythematosus. Arthritis Care Res (Hoboken). 2013;65(5):767-76.
320. Wang F, Flanagan J, Su N, Wang LC, Bui S, Nielson A, et al. RNAscope: a novel in situ RNA analysis platform for formalin-fixed, paraffin-embedded tissues. J Mol Diagn. 2012;14(1):22-9.
321. Psarras A, Emery P, Vital EM. Type I interferon-mediated autoimmune diseases: pathogenesis, diagnosis and targeted therapy. Rheumatology (Oxford). 2017;56(10):1662-75.
322. El-Sherbiny YM, Psarras A, Yusof MYM, Hensor EMA, Tooze R, Doody G, et al. A novel two-score system for interferon status segregates autoimmune diseases and correlates with clinical features. Sci Rep. 2018;8(1):5793.
323. Md Yusof MY, Psarras A, El-Sherbiny YM, Hensor EMA, Dutton K, Ul-Hassan S, 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;77(10):1432-9.
324. Bave U, Magnusson M, Eloranta ML, Perers A, Alm GV, Ronnblom L. Fc gamma RIIa is expressed on natural IFN-alpha-producing cells (plasmacytoid dendritic cells)

and is required for the IFN-alpha production induced by apoptotic cells combined with lupus IgG. J Immunol. 2003;171(6):3296-302.
325. Rodero MP, Decalf J, Bondet V, Hunt D, Rice GI, Werneke S, et al. Detection of interferon alpha protein reveals differential levels and cellular sources in disease. J Exp Med. 2017;214(5):1547-55.
326. Gall A, Treuting P, Elkon KB, Loo YM, Gale M, Jr., Barber GN, et al. Autoimmunity initiates in nonhematopoietic cells and progresses via lymphocytes in an interferon-dependent autoimmune disease. Immunity. 2012;36(1):120-31.
327. Cella M, Jarrossay D, Facchetti F, Alebardi O, Nakajima H, Lanzavecchia A, et al. Plasmacytoid monocytes migrate to inflamed lymph nodes and produce large amounts of type I interferon. Nat Med. 1999;5(8):919-23.
328. Gary-Gouy H, Lebon P, Dalloul AH. Type I interferon production by plasmacytoid dendritic cells and monocytes is triggered by viruses, but the level of production is controlled by distinct cytokines. J Interferon Cytokine Res. 2002;22(6):653-9.
329. Jin O, Kavikondala S, Sun L, Fu R, Mok MY, Chan A, et al. Systemic lupus erythematosus patients have increased number of circulating plasmacytoid dendritic cells, but decreased myeloid dendritic cells with deficient CD83 expression. Lupus. 2008;17(7):654-62.
330. Blanco P, Palucka AK, Gill M, Pascual V, Banchereau J. Induction of dendritic cell differentiation by IFN-alpha in systemic lupus erythematosus. Science. 2001;294(5546):1540-3.
331. Kwok SK, Lee JY, Park SH, Cho ML, Min SY, Park SH, et al. Dysfunctional interferon-alpha production by peripheral plasmacytoid dendritic cells upon Toll-like receptor-9 stimulation in patients with systemic lupus erythematosus. Arthritis Res Ther. 2008;10(2):R29.
332. Banchereau J, Pascual V. Type I interferon in systemic lupus erythematosus and other autoimmune diseases. Immunity. 2006;25(3):383-92.
333. Liao X, Li S, Settlage RE, Sun S, Ren J, Reihl AM, et al. Cutting Edge: Plasmacytoid Dendritic Cells in Late-Stage Lupus Mice Defective in Producing IFN-alpha. J Immunol. 2015;195(10):4578-82.
334. Zhou Z, Ma J, Xiao C, Han X, Qiu R, Wang Y, et al. Phenotypic and functional alterations of pDCs in lupus-prone mice. Sci Rep. 2016;6:20373.
335. Ah Kioon MD, Tripodo C, Fernandez D, Kirou KA, Spiera RF, Crow MK, et al. Plasmacytoid dendritic cells promote systemic sclerosis with a key role for TLR8. Sci Transl Med. 2018;10(423).
336. Vermi W, Bonecchi R, Facchetti F, Bianchi D, Sozzani S, Festa S, et al. Recruitment of immature plasmacytoid dendritic cells (plasmacytoid monocytes) and myeloid dendritic cells in primary cutaneous melanomas. J Pathol. 2003;200(2):255-68.
337. Gerlini G, Urso C, Mariotti G, Di Gennaro P, Palli D, Brandani P, et al. Plasmacytoid dendritic cells represent a major dendritic cell subset in sentinel lymph nodes of melanoma patients and accumulate in metastatic nodes. Clin Immunol. 2007;125(2):184-93.
338. Conrad C, Gregorio J, Wang YH, Ito T, Meller S, Hanabuchi S, et al. Plasmacytoid dendritic cells promote immunosuppression in ovarian cancer via ICOS costimulation of Foxp3(+) T-regulatory cells. Cancer Res. 2012;72(20):5240-9.

339. Vincent IE, Zannetti C, Lucifora J, Norder H, Protzer U, Hainaut P, et al. Hepatitis B virus impairs TLR9 expression and function in plasmacytoid dendritic cells. PLoS One. 2011;6(10):e26315.
340. Lo CC, Schwartz JA, Johnson DJ, Yu M, Aidarus N, Mujib S, et al. HIV delays IFN-alpha production from human plasmacytoid dendritic cells and is associated with SYK phosphorylation. PLoS One. 2012;7(5):e37052.
341. Zhang H, Gregorio JD, Iwahori T, Zhang X, Choi O, Tolentino LL, et al. A distinct subset of plasmacytoid dendritic cells induces activation and differentiation of B and T lymphocytes. Proc Natl Acad Sci U S A. 2017;114(8):1988-93.
342. Alculumbre SG, Saint-Andre V, Di Domizio J, Vargas P, Sirven P, Bost P, et al. Diversification of human plasmacytoid predendritic cells in response to a single stimulus. Nat Immunol. 2018;19(1):63-75.
343. Miyara M, Amoura Z, Parizot C, Badoual C, Dorgham K, Trad S, et al. Global natural regulatory T cell depletion in active systemic lupus erythematosus. J Immunol. 2005;175(12):8392-400.
344. Comte D, Karampetsou MP, Kis-Toth K, Yoshida N, Bradley SJ, Kyttaris VC, et al. Brief Report: CD4+ T Cells From Patients With Systemic Lupus Erythematosus Respond Poorly to Exogenous Interleukin-2. Arthritis Rheumatol. 2017;69(4):808-13.
345. Kammer GM. Altered regulation of IL-2 production in systemic lupus erythematosus: an evolving paradigm. J Clin Invest. 2005;115(4):836-40.
346. Kobayashi K, Hernandez LD, Galan JE, Janeway CA, Jr., Medzhitov R, Flavell RA. IRAK-M is a negative regulator of Toll-like receptor signaling. Cell. 2002;110(2):191-202.
347. Agrawal A, Agrawal S, Cao JN, Su H, Osann K, Gupta S. Altered innate immune functioning of dendritic cells in elderly humans: a role of phosphoinositide 3-kinase-signaling pathway. J Immunol. 2007;178(11):6912-22.
348. Duramad O, Fearon KL, Chan JH, Kanzler H, Marshall JD, Coffman RL, et al. IL-10 regulates plasmacytoid dendritic cell response to CpG-containing immunostimulatory sequences. Blood. 2003;102(13):4487-92.
349. Shodell M, Siegal FP. Circulating, interferon-producing plasmacytoid dendritic cells decline during human ageing. Scand J Immunol. 2002;56(5):518-21.
350. Stout-Delgado HW, Yang X, Walker WE, Tesar BM, Goldstein DR. Aging impairs IFN regulatory factor 7 up-regulation in plasmacytoid dendritic cells during TLR9 activation. J Immunol. 2008;181(10):6747-56.
351. Bokov A, Chaudhuri A, Richardson A. The role of oxidative damage and stress in aging. Mech Ageing Dev. 2004;125(10-11):811-26.
352. LaFleur DW, Nardelli B, Tsareva T, Mather D, Feng P, Semenuk M, et al. Interferon-kappa, a novel type I interferon expressed in human keratinocytes. The Journal of biological chemistry. 2001;276(43):39765-71.
353. Stannard JN, Reed TJ, Myers E, Lowe L, Sarkar MK, Xing X, et al. Lupus Skin Is Primed for IL-6 Inflammatory Responses through a Keratinocyte-Mediated Autocrine Type I Interferon Loop. J Invest Dermatol. 2017;137(1):115-22.
354. Sarkar MK, Hile GA, Tsoi LC, Xing X, Liu J, Liang Y, et al. Photosensitivity and type I IFN responses in cutaneous lupus are driven by epidermal-derived interferon kappa. Ann Rheum Dis. 2018;77(11):1653-64.

355. Karrich JJ, Jachimowski LC, Uittenbogaart CH, Blom B. The plasmacytoid dendritic cell as the Swiss army knife of the immune system: molecular regulation of its multifaceted functions. J Immunol. 2014;193(12):5772-8.
356. Kadowaki N, Antonenko S, Lau JY, Liu YJ. Natural interferon alpha/beta-producing cells link innate and adaptive immunity. J Exp Med. 2000;192(2):219-26.
357. Guillerey C, Mouries J, Polo G, Doyen N, Law HK, Chan S, et al. Pivotal role of plasmacytoid dendritic cells in inflammation and NK-cell responses after TLR9 triggering in mice. Blood. 2012;120(1):90-9.
358. Bekeredjian-Ding IB, Wagner M, Hornung V, Giese T, Schnurr M, Endres S, et al. Plasmacytoid dendritic cells control TLR7 sensitivity of naive B cells via type I IFN. J Immunol. 2005;174(7):4043-50.
359. Karrich JJ, Jachimowski LC, Nagasawa M, Kamp A, Balzarolo M, Wolkers MC, et al. IL-21-stimulated human plasmacytoid dendritic cells secrete granzyme B, which impairs their capacity to induce T-cell proliferation. Blood. 2013;121(16):3103-11.
360. Cervantes-Barragan L, Lewis KL, Firner S, Thiel V, Hugues S, Reith W, et al. Plasmacytoid dendritic cells control T-cell response to chronic viral infection. Proc Natl Acad Sci U S A. 2012;109(8):3012-7.
361. Vermi W, Soncini M, Melocchi L, Sozzani S, Facchetti F. Plasmacytoid dendritic cells and cancer. J Leukoc Biol. 2011;90(4):681-90.
362. Dzionek A, Sohma Y, Nagafune J, Cella M, Colonna M, Facchetti F, et al. BDCA-2, a novel plasmacytoid dendritic cell-specific type II C-type lectin, mediates antigen capture and is a potent inhibitor of interferon alpha/beta induction. J Exp Med. 2001;194(12):1823-34.
363. Cao W, Rosen DB, Ito T, Bover L, Bao M, Watanabe G, et al. Plasmacytoid dendritic cell-specific receptor ILT7-Fc epsilonRI gamma inhibits Toll-like receptor-induced interferon production. J Exp Med. 2006;203(6):1399-405.
364. Fuchs A, Cella M, Kondo T, Colonna M. Paradoxic inhibition of human natural interferon-producing cells by the activating receptor NKp44. Blood. 2005;106(6):2076-82.
365. Ozenci V, Kouwenhoven M, Huang YM, Kivisakk P, Link H. Multiple sclerosis is associated with an imbalance between tumour necrosis factor-alpha (TNF-alpha)- and IL-10-secreting blood cells that is corrected by interferon-beta (IFN-beta) treatment. Clin Exp Immunol. 2000;120(1):147-53.
366. Cantaert T, Baeten D, Tak PP, van Baarsen LG. Type I IFN and TNFalpha cross-regulation in immune-mediated inflammatory disease: basic concepts and clinical relevance. Arthritis Res Ther. 2010;12(5):219.
367. Palucka AK, Blanck JP, Bennett L, Pascual V, Banchereau J. Cross-regulation of TNF and IFN-alpha in autoimmune diseases. Proc Natl Acad Sci U S A. 2005;102(9):3372-7.
368. Villani AC, Satija R, Reynolds G, Sarkizova S, Shekhar K, Fletcher J, et al. Single-cell RNA-seq reveals new types of human blood dendritic cells, monocytes, and progenitors. Science. 2017;356(6335).
369. Almoallim H, Al-Ghamdi Y, Almaghrabi H, Alyasi O. Anti-Tumor Necrosis Factor-alpha Induced Systemic Lupus Erythematosus(). The open rheumatology journal. 2012;6:315-9.
370. Williams EL, Gadola S, Edwards CJ. Anti-TNF-induced lupus. Rheumatology (Oxford). 2009;48(7):716-20.

371. Cavanagh LL, Boyce A, Smith L, Padmanabha J, Filgueira L, Pietschmann P, et al. Rheumatoid arthritis synovium contains plasmacytoid dendritic cells. Arthritis Res Ther. 2005;7(2):R230-40.