The role of invariant Natural Killer T cells in SLE patients with atherosclerosis

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Declaration

I, Edward Charles Smith, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

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Abstract

Accelerated atherosclerosis is a complication of the rheumatic disease systemic lupus erythematosus (SLE). I questioned the role of invariant Natural Killer T (iNKT) cells in this process, since they are known to be defective in SLE but also promote atherosclerosis in response to CD1d-mediated lipid presentation. SLE patients with asymptomatic plaque (SLE-P) had an altered iNKT cell phenotype to those without plaque (SLE-NP), characterised by differences in activation marker expression and increased IL-4. This SLE-P iNKT cell phenotype correlated with differences in serum lipids including VLDL and coud be recapitulated *in vitro* by culturing healthy PBMCs with serum from SLE-P patients, an effect that was inhibited in the presence of anti-CD1d. Whilst differences in CD1d and lipid raft co-localisation and recycling were found in SLE patients, no difference was observed between SLE-NP and SLE-P patients. Isolation of phospholipids from SLE-P patients confirmed that differences in the lipids being presented were driving the anti-inflammatory iNKT cell phenotype in SLE-P patients. The finding that healthy iNKT cells, differentiated in the presence of healthy monocytes and serum from SLE-P patients, could induce THP-1 macrophage polarisation towards an anti-inflammatory M2-like phenotype suggested a protective role for iNKT cells in SLE patients with subclinical atherosclerosis. This was confirmed by studying a group of SLE patients who had suffered a cardiovascular event, where this protective iNKT cell phenotype seen in asymptomatic patients was lost.

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Abbreviations

ABCA1 - ATP Binding Cassette A1
ACR – American College of Rheumatology
ADMA – asymmetric dimethylarginine
ANE - di-4-ANEPPDHQ
α-GalCer – alpha galactosylceramide
AGL – altered glycolipid ligands
AP(2) – adaptor protein (2)
Apo(A1) – Apolipoprotein(A1)
APC – allophycocyanin
APCs – antigen presenting cells
APL – anti-phospholipid
APS – anti-phospholipid syndrome
ATG5 – autophagy protein 5
βGalCer – β galactosylceramide
βGlcCer - β-glucosylceramide
βLacCer - β-lactosylceramide
BAFF – B cell activation factor
BCR – B cell receptor
BDS – Bright detail similarity (feature)
BILAG – British Isles Lupus Activity Group
BMI – body mass index
Bcl-2 – B cell lymphoma 2
BP – blood pressure
Breg – Regulatory B cell
BSA – bovine serum albumin
BV – Brilliant violet
CAC – Coronary artery calcification
cAMP - Cyclic adenosine monophosphate
CCA – common carotid artery
CFA – common femoral artery
CD – Cluster of differentiation
cDNA – complementary DNA
CDR – complementary determinant region
CRP – C reactive protein
CTB – Cholera toxin B subunit
CTL – cytotoxic lymphocyte
CTLA-4 - cytotoxic T-lymphocyte-associated protein 4
CVD – cardiovascular disease
DAG - diacylglycerol
DC – dendritic cell
DEC205 – dendritic cell 205
DMARD – disease modifying anti-rheumatic drug
DMSO – dimethyl sulfoxide
DNA – deoxyribonucleic acid
DP – double positive
DsDNA – double stranded deoxyribonucleic acid
EAE - experimental autoimmune encephalomyelitis
EEA1 – early endosome antigen 1

EDTA - Ethylenediaminetetraacetic acid
ERK - Extracellular signal-regulated kinases
FCS – fetal calf serum
FDA – US Food and Drug Administration
FSC – forward scatter
FITC – Fluorescein isothiocyanate
Foxp3 – Forkhead box protein 3
GATA-3 - Guanine adenine thymine adenine sequence-binding protein 3
GD(3) – Disaloganglioside (3)
GM(1) – Monosialotetrahexosylganglioside (1)
GSL – glycosphingolipid
GSM – grey scale median
GVHD – graft versus host disease
HDL – high density lipoprotein
HLA – human leukocyte antigen
HsCRP – high sensitivity CRP
LDL(R) – low density lipoprotein (receptor)
ICOS(L) - inducible costimulatory (ligand)
Ig – immunoglobulin
iGb3 - Isoglobotrihexosylceramide
IFN – interferon
IL – interleukin
iNOS – inducible nitric oxide synthase
IRF – interferon regulatory factor
IMT – intima-media thickness
iNKT cell – invariant Natural Killer T cell
iTCR – invariant T cell receptor
i(Treg) – inducible (Treg)
JAK – Janus kinase
JNK - c-Jun N-terminal kinases
LAT - linker for activation of T cells
LAP – latency associated peptide
Lck - lymphocyte-specific protein tyrosine kinase
LDL(R) – low density lipoprotein (receptor)
LFA-1 – lymphocyte function associated antigen 1
LOX-1 - Lectin-like oxidized low-density lipoprotein (LDL) receptor-1
LLT1 - lectin-like transcript 1
LPC - lyso-phosphatidylcholine
LXR – Liver X receptor
Lyn – Lck/Yes novel tyrosine kinase
MAP kinase – mitogen associated protein kinase
MAIT – mucosal associated invariant T
MCP – monocytic chemotactic protein
MDSC – myeloid derived suppressor cell
MDDC – monocyte-derived dendritic cell
MBL – mannose binding lectin
MβCD - methyl-β-cyclodextrin
mDC – myeloid dendritic cell
Mg - milligram
MHC – major histocompatibility complex
MI – myocardial infarction

MIP – monocyte inflammatory protein
mRNA – messenger ribonucleic acid
miRNA – micro ribonucleic acid
MMP – matrix metalloproteinase
MSR1 – macrophage scavenger receptor 1
mTOR – mechanistic/mammalian target of rapamycin
MTP – microsomal triglyceride transfer protein
ng - nanograms
NETs – neutrophil extracellular traps
NFAT – nuclear factor of activated T cells
NF-kB - nuclear factor kappa-light-chain-enhancer of activated B cells
NK cell – natural killer cell
NKT – natural killer T cell
NO – nitric oxide
NOD – not obese or diabetic
NPC2 - Niemann-Pick Disease Type C2 Protein
n(Treg) – natural (Treg)
NU-αGalCer – naphthylurea-α-GalCer
OVA - ovalbumin
oxLDL – oxidized low density lipoprotein
PAI – plasminogen activator inhibitor
PAR – protease activated receptor
PBMCs – peripheral blood mononuclear cells
PBS – phosphate buffered saline
PC – phosphatidylcholine
pDC – plasmacytoid dendritic cell
PDK-1 - phosphoinositide dependent protein kinase-1
PD-1 – programmed death 1
Pen/strep – penicillin and streptomycin
PE – Phycoerythrin
PE – phosphatidylethanolamine
PFA – paraformaldehyde
PKA – protein kinase A
PLA2 - phospholipase A2
PLE – phospholipase E
PLZF - Promyelocytic leukaemia zinc finger protein
PMA – phorbol-12-myrisate 13-acetate
PPAR-γ - Peroxisome proliferator-activated receptor γ
PPBP – Pro-platelet basic protein
PTPN22 – protein tyrosine phosphatase, non-receptor type 22
RA – rheumatoid arthritis
RAG1 – recombination activating gene 1
RAR – retinoic acid receptor
Raptor – regulatory-associated protein of MTOR
rh – recombinant human
RNA – ribonucleic acid
ROR-(γt) – RAR-related orphan receptor (γt)
ROS – reactive oxygen species
SHP-1 - Src homology region 2 domain-containing phosphatase-1
SLAM - signaling lymphocyte activation molecule
SLE – Systemic Lupus Erythematosus

SLEDAI – Systemic Lupus Erythematosus Disease Activity Index
SLE-NP – SLE patients without plaque
SLE-P – SLE patients with plaque
SLE-CV –SLE patients who have suffered a cardiovascular event
SNP – single nucleotide polymorphism
SOCS – suppressor of cytokine signalling
SR-A – scavenger receptor A
SS – systemic sclerosis
SSC – side scatter
STAT – signal transducer and activator of transcription
TCR – T cell receptor
T-bet – T-box transcription factor TBX21
TGF – transforming growth factor
Th1 – T helper type 1
Th2 – T helper type 2
Th17 – T helper 17
TLR – Toll-like receptor
TNF – tumour necrosis factor
Treg – regulatory T cell
US – Ultrasound
V-CAM - vascular cell adhesion molecule
VDR – Vitamin D receptor
VEGF – vascular endothelial growth factor
VLDL – very low density lipoprotein
ZAP-70 – Zeta-chain-associated protein kinase 70

CHAPTER I: Introduction

Introduction

1.1 Systemic lupus erythematosus and cardiovascular risk: overview

1.1.1 Introduction to SLE

Systemic Lupus Erythematosus (SLE) is a highly heterogeneous autoimmune disorder, currently of unknown etiology. It the United Kingdom it has a variable prevalence depending on ethnicity, affecting between 40 (Caucasian) and 200 (Afro-Caribbean) in every 100,000 people [1]. It is associated with considerable morbidity and mortality through severe inflammation and organ damage, and is significantly more common in women of child bearing age who are 8-15 times more likely than men to develop SLE [2, 3]. Despite significant improvements in mortality rate from a 50% 5 year survival in 1955 to an 85% 10 year survival in recent times [4], SLE still remains difficult to control with the the therapeutics currently available meaning that patients' quality of life is substantially diminished [5]. This issue is highlighted in a recent study, which demonstrated that only 15% of SLE patients attending an academic clinic sustained good to excellent disease control over the course of one year [6]. The improved lifespan for SLE patients has lead to the emergence of several co-morbidities including an increased risk of cardiovascular disease and recurrent infections, of which patients with high disease activity are thought to be at greater risk [7, 8]. Accelerated atherosclerosis is particularly prevalent in SLE patients, where between 30 and 40% of patients are affected in studies, which have used vascular ultrasound [9].

As a systemic autoimmune disorder, SLE exhibits a range of clinical manifestations including arthritis, pleurisy, pericarditis, skin rashes, renal damage, blood disorders and neurological disorders [10, 11]. Such complications are a result of chronic inflammation, which occurs as a consequence of defective apoptosis, and T and B cell hyper-responsiveness to self-antigen such as dsDNA. Subsequent production of autoantibodies causes tissue damage directly or indirectly through a type III hypersensitivity reaction, resulting in excessive immune complex formation. These immune complexes become trapped in the microvasculature, resulting in a chronic inflammatory response which damages organs such as the skin, heart and kidneys and leads to the development of distinguishing clinical features such as the butterfly rash.

1.1.2 Etiopathogenesis of SLE

Whilst the etiology of SLE remains unclear, a combination of genetic, environmental and hormonal factors has been shown to be associated with the disease. Genetics factors have been implicated in the development of SLE for over 40 years. This is evident from the finding that the prevalence of SLE is approximately 1 in 100 in first-degree relatives of SLE patients, or approximately 20 times greater than the general population [12]. Moreover, increased concordance rates have been observed amongst monozygotic twins compared to dizygotic twins – rates of approximately 25% and 2% respectively [13]; together suggesting a large genetic basis for the disease.

However, disease inheritance does not follow traditional Mendelian rules of genetics, suggesting that SLE is a result of a combination of multiple genetic variations; some of which may be common in the general population. So far, over 10 genome-wide association studies have identified over 50 genes associated with SLE in the form of single nucleotide polymorphisms (SNPs), polymorphisms leading to alternative splicing, and polymorphisms in the 3' untranslated region such as miRNAs, which influence gene expression [14].

Genetic polymorphisms include those that influence lymphocyte function such as Signal Transducer and Activator of Transcription (STAT) 4 [15], Protein tyrosine phosphatase, non-receptor type 22 (PTPN22) [16], Lyn [17] and interleukin (IL)-10 [18], which have all been linked to defective B cell function. Other polymorphisms include interferon regulatory factor (IRF)-5, -7 and -8 [19-21], and Toll-like receptor (TLR)-7 and -9 [22, 23], which are involved in innate immune signaling, as well as Autophagy protein 5 (ATG5) and DNase1 [24, 25], which are involved in immune complex clearance.

Of the genetic factors which predispose to SLE, female gender is probably the strongest, for reasons which are not yet completely understood. One explanation is that estrogen contributes to disease development; a theory which has been proven in animal models, where estrogen treatment worsened disease [26]. But the presence of SLE in children and postmenopausal women suggests that other female-specific factors play a role, such as differences in the immune system between men and women. This has been hypothesized

to be a result of inappropriate activation of normally-silenced immune genes located on the X chromosome due to DNA demethylation [27, 28].

Environmental factors, notably ultra-violet light exposure may also contribute to SLE development [29]. Despite many large-scale epidemiological studies only silica dust exposure [30], and to a lesser extent smoking [31, 32], have been identified as possible factors which contribute to SLE by acting as a trigger and influencing DNA methylation. Certain drugs such as procainamide and hydralazine have previously been shown to induce SLE-like immune features but rarely cause full-blown SLE [33, 34], whilst low vitamin D levels have been hypothesised to play a role in SLE development [35].

1.1.3 Current approaches in SLE diagnosis and treatment

A widely used set of classification criteria are the 1997 Revised American College of Rheumatology (ACR) Criteria. Individuals must display at least four of the 11 clinical and laboratory classification criteria (Table 1.1) [36]. Patients' disease activity varies substantially over time and may be assessed through a variety of indices. Probably the best known are the Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) and British Isles Lupus Assessment Group (BILAG) [37]. In particular, the BILAG index scores disease activity in 9 distinct organs or systems [38]. Scores ranging from A (most active) to E (never active) are converted numerically (A=12, B=8, C=1, D=0, E=0) and combined to give a global score [38, 39]. Laboratory tests used to reflect disease activity include the measurement of complement protein C3, lymphocyte count and anti-dsDNA antibodies in the blood [40].

SLE is treated according to both organ involvement and disease severity. Therapeutic strategies include the use of hydroxychloroquine, steroids and immunosuppressive agents such as methotrexate and mycophenolate [41]. More recently several biological therapies have been developed, which target various aspects of B cell and T cell dysfunction in SLE. These include abatacept, which binds cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) on T cells to inhibit interactions with antigen presenting cells [42], belimumab, which neutralizes B cell activation Factor (BAFF) to prevent of B cell over-activation [43], and rituximab, which binds CD20 to deplete B cells [44]. Successful results have been observed in clinical trials for belimumab [45], which has since been approved for use in SLE by the US Food and Drug Administration (FDA) [46]. Promising results have been observed for rituximab, which has been used to treat over 700 SLE patients in nearly 30 trials [47]. Whilst the majority of patients demonstrated improvement in both clinical features of the disease and biomarkers such as anti-dsDNA levels [48, 49], two major trials (EXPLORER and LUNAR) failed to meet their primary endpoints [44]. However, this was likely a consequence of poor trial design and specifically the excessive use of concomitant steroids and immunosuppressives [50]. Despite improvements in patient life expectancy and quality of life, it is unknown whether current therapeutic intervention could be contributing to other complications in SLE patients such as increased cardiovascular risk, the focus of this study [9].

Symptom	Description
Malar rash	Also known as the butterfly rash, characterized by red or purple scaly skin
Discoid rash	Affects the face and scalp; characterized by red scaly patches which clear in places to leave scarring
Photosensitivity	Skin rash as a result of unusual reaction to sunlight
Oral ulcers	Oral or nasopharyngeal ulceration, usually painless
Arthritis	Non-erosive arthritis involving two or more peripheral joints, characterised by tenderness or swelling
Serositis	Pleuritis: history of pleuritic pain or rub heard by a physician or evidence of plural effusion or pericarditis documented by electrocardiogram
Renal disorder	Persistent proteinuria greater than 0.5 grams per day or greater than 3+ if quantification not performed or cellular casts may be red cell, haemoglobin, granular, tubular or mixed.
Neurological disorder	Seizures in the absence of offending drugs or known metabolic derangements; e.g. uraemia, ketoacidosis, or electrolyte imbalance or psychosis
Hematological disorder	Hemolytic anaemia with reticulocytosis, leukopenia (less than 4000mm^3 on two or more occasions) or lymphopenia (less than 1500mm^3 on two or more occasions) or thrombocytopenia (less than 100000mm^3)
Immunologic disorder	Anti-DNA antibody to native DNA in abnormal titre or anti-Sm presence of antibody to Sm nuclear antigen or positive finding of antiphospholipid antibodies based on: 1. An abnormal serum level 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 titer 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 Diagnostic criteria for SLE

1.1.4 SLE and Cardiovascular Risk

As a major cause of death in SLE patients, the relationship between SLE and cardiovascular disease is well known and is thought to be independent of traditional risk factors. In 1976 Urowitz et al. first identified an association between SLE and death by myocardial infarction (MI) in a cohort of SLE patients in Toronto, with 6 out of 11 deaths occurring within a year of diagnosis [51]. Furthermore, patients were calculated to have a five-fold greater risk of MI compared to the general population, the average age for which occurred at 49, compared to 65-74 in the general population. Since then multiple studies have evaluated the link between SLE and cardiovascular disease with emphasis on atherosclerosis, which is responsible for around 30% of deaths in SLE patients [52].

The first major study to analyse the association between SLE and cardiovascular events was carried out by Manzi et al. where 498 women with SLE were assessed for MI and angina over a period of 13 years [53]. Data were compared to 2208 age-matched healthy women from the Framingham Offspring Study [54]. They observed a disturbingly higher incidence of cardiovascular events in younger SLE patients, particularly those under 55 years who normally would be unlikely to suffer a cardiovascular event [53]. Based on this cohort, they calculated that the risk of MI in female SLE patients aged 35-44 years was over 50 times greater than the general population [53].

The association between SLE and cardiovascular disease has since been confirmed by numerous studies, most notably by Roman et al. which used a case-control approach and assessed atherosclerotic plaque by ultrasound [55]. Whilst atherosclerosis in SLE patients was not found to be associated disease activity in this study, associations with longer disease duration, a higher damage-index score and less aggressive immunosuppressive therapy suggested that inflammation was responsible for the development of atherosclerosis in these patients [55]. However, specific disease-related factors which influence the rate of atherosclerosis progression in SLE patients remain elusive. Whilst several studies have implicated age of SLE onset as a determinant of atherosclerosis [56, 57], studies which adjusted for patient age have shown this not to be the case [58, 59]. The LASER study suggested that persistently high disease activity was a major contributing factor towards cardiovascular disease in SLE patients, which could not be prevented by therapeutics such as corticosteroids and azathioprine [60]. However, the extent to which this is true remains

under question since Kiani et al. for example found that SLE disease activity (quantified by SLEDAI and anti-dsDNA) did not predict subclinical atherosclerosis in SLE patients [58].

1.1.5 Traditional cardiovascular risk factors in SLE patients

In the general population cardiovascular risk factors play a large role in the development of atherosclerosis and include environmental factors such as diet, physical activity and smoking in combination with genetic factors [61]. In particular the combination of obesity, diabetes and hypertension (termed metabolic syndrome) has been shown to greatly enhance cardiovascular disease risk [62]. Such risk factors have repeatedly been identified by the Framingham Heart Study as being significantly associated with cardiovascular disease mortality [63].

However, there is a lack of consensus on the importance of cardiovascular risk factors in SLE patients, and the extent to which they contribute to the development of atherosclerosis in patients. Evidence suggests that SLE patients consistently carry more Framingham cardiovascular risk factors compared to healthy controls including dyslipidemia, hypertension and diabetes mellitus [53, 64]. Moreover, several studies have observed an increased incidence of metabolic syndrome in SLE compared to the general population. Parker et al. observed an incidence of 30% in SLE patients compared to 20% in a control population [65], whilst Sabio et al. observed an incidence of 20% compared to 13% in healthy controls [66]. A study on a Mexican cohort of SLE patients found that the absence of traditional cardiovascular risk factors was associated with a lack of subclinical myocardial ischemia [67], highlighting the importance of cardiovascular risk factors as major determinants of cardiovascular disease in SLE patients. It is unknown however whether metabolic syndrome manifests itself as a result of SLE itself, or due to preventable risk factors such as smoking, which has previously been shown to be associated with metabolic syndrome in SLE patients [68].

Most studies agree that whilst important in predicting cardiovascular disease in SLE patients, the increased prevalence of atherosclerosis cannot be explained by traditional Framingham risk factors alone. This is evident from large population based studies including Roman et al, who found SLE presence to be the most important correlate of atherosclerosis

other than age [55]. Similarly, Asanuma et al. reported that the presence of atherosclerosis could not be accounted for through traditional cardiovascular risk factors, indicating that the presence of SLE alone is enough to significantly increase an individual's risk of cardiovascular disease [69]. This is supported by Esdaile et al. who found that the increased risk of coronary heart disease and stroke in 263 SLE patients could not be explained by Framingham risk factors alone [70]. Studies have therefore focused on identifying non-traditional cardiovascular risk factors which predict atherosclerosis development, as well as other biomarkers which can identify patients most at risk [69]. A summary of traditional and non-traditional cardiovascular risk factors in SLE patients is shown in Table 1.2.

Traditional Risk Factors	Non-traditional Risk Factors
Hypertension	Corticosteroid use
Hypercholesterolemia	Endothelial cell apoptosis
Hypertriglyceridemia	Anti-heat shock protein autoantibodies
Low HDL cholesterol	Increased oxidised LDL (oxLDL)
High body mass index (BMI)	High levels of immune complexes
Insulin resistance	High levels of ADMA
Diabetes mellitus	Increased levels of proinflammatory cytokines
Renal impairment	Dendritic cell CD86 overexpression
Early menopause	T cell CD40L upregulation
Increased C reactive protein	
Smoking	
Family history of cardiovascular disease	

Table 1.2 Traditional and non-traditional cardiovascular risk factors in SLE patients

Table adapted from Westerweel et al [71]. HDL – high density lipoprotein; BMI-body mass index; LDL – low density lipoprotein; ADMA - asymmetric dimethylarginine

1.1.6 Other determinants of cardiovascular disease in SLE patients

In order to explain the increased cardiovascular risk in SLE patients, studies have investigated a wide range of non-traditional cardiovascular risk factors including genetics, inflammatory biomarkers associated with SLE and patient medication. Several genetic polymorphisms are associated with increased cardiovascular disease risk in SLE including interferon regulatory factor 8 (IRF8) [72], matrix metalloproteinase (MMP)-2 [73], plasminogen activator inhibitor (PAI)-1 [74] and mannose binding lectin (MBL)-2 genes [75]. These polymorphisms are associated with altered immune cell function in SLE, which has been shown to contribute to and the development of atherosclerosis in these patients.

Oxidative stress is known to play a major role in endothelial dysfunction, which precedes many of the latter arterial changes in atherosclerosis. SLE patients have higher levels of oxidative stress compared to their healthy counterparts [76], which may occur as a consequence of increased traditional cardiovascular risk factors such as smoking, hypertension and insulin resistance [77]. This leads to the formation of advanced glycation end products which are toxic to endothelial cells [78], whilst the consequential oxidation of lipids induces endothelial secretion of monocyte chemotactic protein 1 (MCP-1), IL-6 and IL-8 which promote inflammation of the vasculature [64].

In support of this, several cytokines have been associated with the presence of atherosclerosis in SLE patients. For example, Zhu et al. observed elevated IL-6 and IL-17 and together with decreased IL-10 levels in SLE patients with carotid plaque, which was correlated with a T helper 17 (Th17)/ T regulatory cell (Treg) imbalance [79]. Additionally, Ruiz-Limon et al. demonstrated that both cardiovascular events and presence of atherosclerosis in SLE patients were correlated with several inflammatory biomarkers including MCP-1, monocyte inflammatory protein (MIP)-1α and tissue factor (TF) [80].

Interferon (IFN)-α is a cytokine associated with active disease in SLE patients [81] that has been found to play a role in the development of atherosclerosis. Specifically, IFN-α has been shown to induce platelet aggregation, vascular thrombosis, immune cell activation and the secretion of cytokines and MMPs by plasmacytoid dendritic cells (pDCs) [82]. IFN-α also mediates endothelial progenitor cell death, thus inhibiting many of the repair processes within the endothelium [83].

Autoantibodies associated with SLE may also contribute to cardiovascular disease risk, with those against phospholipids, apoA1, oxidised LDL (oxLDL) and annexin V hypothesised to play a role in plaque development. Approximately 20 to 40% of SLE patients have anti-phospholipid (APL) antibodies [84], which are thought to contribute the early stages of endothelial cell dysfunction through interactions with β2 glycoprotein 1 [9]. However, clinical studies have shown variable results since higher APL levels have been shown to correlate with an increased risk of MI in otherwise healthy men [85, 86] yet patients with primary antiphospholipid syndrome (APS), which occurs in up to 70% of SLE patients [84], do not have significantly increased endothelial cell dysfunction in comparison [87, 88].

Anti-apoA1 autoantibodies are present in 10-35 % of SLE patients, the presence of which is associated with SLE disease activity [89, 90]. Due to the anti-inflammatory role of the apoA1-component of HDL, it is thought that that anti-apoA1 autoantibodies inhibit the atheroprotective effects of apoA1 and HDL [9]. Recently however, Croca et al. found no association between apoA1 and SLE patients who had suffered a cardiovascular event, suggesting that it may not pay a large role in the development of atherosclerosis in SLE patients [90].

Autoantibodies against oxLDL play a pathogenic role in SLE patients with high disease activity, and often appear following detection of antilipoprotein lipase autoantibodies [91]. The underlying mechanism is unclear, but it is believed that the presence of oxLDL complexes in the subendothelial space coupled with defective clearance of immune complexes may play a role in plaque development [9]. Interestingly, IgG anti-oxLDL autoantibodies appear to contribute to atherosclerosis, whilst IgM anti-oxLDL antibodies may even be considered protective [92].

SLE patients show elevated levels of anti-annexin-V antibodies [93], which inhibit the atheroprotective interactions between annexin-V and the phospholipid bilayer of the endothelium [94]. Autoantibodies can also induce the formation of neutrophil extracellular traps (NETs) [95], which induce IFN-α production by pDCs as well as endothelial cell apoptosis [96].

Other serum factors postulated to promote atherosclerosis in SLE patients include homocysteine and adipokines. Homocysteine levels are higher in SLE patients, which may

be due to renal dysfunction [97, 98]. Studies show that homocysteine can induce endothelial cell death [99], decrease nitric oxide bioavailability [100] and promote thickening of the intimal-medial wall [101]. Leptin is an adipokine which, in addition to its role as an appetite suppressant, may contribute to atherosclerosis development [102]. Increased leptin levels have been observed in the serum of both juvenile and adult SLE patients [103, 104], where it may play a role in plaque development. In contrast, one study found another adipokine, adiponectin to be protective against cardiovascular disease in both healthy individuals and SLE patients [105]. Data however is currently limited and the mechanisms as such remain unknown.

C reactive protein (CRP) is another biomarker that may be useful in predicting cardiovascular risk in otherwise healthy individuals, however it appears to be less effective in SLE patients. Whilst in the general population CRP levels have been shown to strongly correlate with atherosclerosis, in SLE data are inconsistent and despite high levels of other inflammatory markers, CRP levels are generally low [106]. In the LUMINA study as well as a smaller study in Sweden, high CRP concentrations were associated with a minimal 1.5 to 3.3 fold increase in cardiovascular risk in SLE patients [107, 108].

SLE patient medication is another factor which could significantly increase cardiovascular disease risk. Bessant et al. observed a significant association between cardiovascular disease risk and steroid treatment [109], whilst no association was observed for azathioprine and cyclophosphamide. Conversely, other forms of medication for SLE patients such as hydroxychloroquine have been shown to reduce cardiovascular risk [109, 110]; however this is likely dependent on patient responsiveness to treatment in terms of disease activity.

1.1.7 Current treatment of cardiovascular disease in SLE patients

Primary prevention of cardiovascular disease involves the management of pre-existing conditions included in the metabolic syndrome [111]. These include abdominal (central) obesity, high blood pressure, high serum triglycerides and low serum high density cholesterol (HDL) levels; treatment for which, reduces the likelihood of a cardiovascular event. However, there is increasingly more emphasis on recognising the initial stages of atherosclerosis development, which may precede the development of conditions such as dyslipidemia and hypertensison. Methods to diagnose early atherosclerosis in asymptomatic patients include vascular imaging by ultrasound scan, which is sensitive enough to detect plaques at the preclinical stage [112], as well as surrogate measures such as flow mediated dilation to detect subtle changes in arterial function [113]. Such methods could prove beneficial in diseases such as SLE where analysis of serum biomarkers may not always be as useful compared to the general population.

In the general population dyslipidaemia is treated by changes in diet followed by statins, where patients' lipids are difficult to control. Despite studies identifying SLE and dyslipidaemia as significant co-morbidities [114], not all SLE patients are considered dyslipideamic since clinical lipid measures often fall within normal ranges, which may be attributable to the various therapeutics available for SLE [115, 116]. Statins are therefore not routinely prescribed to patients due to greater clinical emphasis on treating the root cause of SLE – the patient's disease activity. Statins have numerous effects on the immune system, which make them suitable as a way of reducing cardiovascular risk in SLE patients. These include the inhibition of cholesterol biosynthesis, reduced inflammatory cytokine production and upregulation of nitric oxide (NO) synthesis [117]. Statin treatment has been shown clinically to decrease cardiovascular mortality by reducing inflammation within atherosclerotic plaques, through inhibition of monocyte-endothelial cell interactions and decreased expression of TF and MMPs [118, 119]. For example, fluvastatin has recently been shown to reduce the thrombotic tendency of monocytes from anti-phospholipid patients, accompanied by a reduction in protease activated receptor (PAR) and vascular endothelial growth factor (VEGF) expression [120].

Several clinical trials have explored the potential for statin use in SLE patients with mixed results. Ruiz-limon et al. observed several positive benefits of statin treatment in SLE

patients including downregulation of several inflammatory markers [80]. For instance, several members of the nuclear factor kappa B (NFκB) signalling pathway including protein kinase A (PKA), Rho Kinase and cyclic adenosine monophosphate (cAMP) showed diminished expression following Fluvastatin treatment, supporting the notion that statins inhibit *NFκB* signalling in healthy individuals. Fluvastatin also downregulated *IκBKG* expression in SLE patients, which regulates *NFκB* signalling, as well as *NFκBIA*, which inhibits nuclear import of *NFκB* [80]. A similar effect was seen for *ETS1*, a transcription factor regulating MCP-1 and phospholipase expression. Furthermore, treatment of SLE patients with fluvastatin upregulated genes involved in mitochondrial biogenesis resulting in greater numbers of mitochondria with a lower oxidative potential [80]. Since the redox potential of lipids can influence atherosclerosis in SLE, this indicates that statin treatment may be beneficial in reducing cardiovascular risk in SLE patients.

In a different study, Rosuvastatin was found to reduce levels of high sensitivity CRP (hsCRP), homocysteine and several endothelial markers in stable SLE patients with subclinical atherosclerosis [121]. Moreover, low doses (10mg/day) were found to significantly decrease LDL cholesterol and hsCRP after 12 months therapy. This was associated with a reduction in P-selectin and thrombomodulin in patients with very low disease activity (SLEDAI <2).

Whilst it is clear statins may be beneficial in SLE, other reports are less convincing [122, 123]. For example, the Lupus Atheroclerosis Prevention Study (LAPS), a randomised control trial of 200 patients who were treated with 20mg atorvastatin over a 2 year period observed no effect of statin treatmaent on atherosclerosis development [123]. Similarly, Schanberg et al. found no significant difference in SLE disease activity or atherosclerosis risk following treatment with atorvastatin in a cohort of 221 juvenile SLE patients [124]. Possible explanations for these differences include the variable sample sizes and follow-up periods in these studies, suggesting that further clinical trials are needed [122].

1.2 Systemic lupus erythematosus and atherosclerosis: immune mechanisms

1.2.1 SLE: immune mechanisms overview

As an autoimmune disease, SLE occurs as a result of innate and adaptive immune dysregulation, and is characterised by a loss of tolerance and hyper-responsiveness to self-antigens such as double- stranded DNA (dsDNA) (Figure 1.1). A range of genetic and immunopathological defects have been identified in cellular processes including apoptotic cell clearance, the complement cascade and lipid metabolism [125-127].

Under normal homeostatic conditions, cell death occurs through apoptosis – a programmed cell death where intracellular components are retained within apoptotic vesicles (rather than released into the extracellular milieu), before being engulfed by phagocytes and macrophages, which rapidly clear apoptotic debris [128]. During apoptosis, phosphatidylserine is exposed on the external membrane, along with the expression of modified or “cryptic” self-antigens not normally exposed to the immune system [129]. Such antigens are thought to play a role in recognition by macrophages, resulting in a tolerogenic response characterised by release of transforming growth factor β (TGFβ) and IL-10, which prevents inflammation [128].

In SLE however, monocyte-derived macrophages show impaired adhesion and phagocytic activity resulting in secondary necrosis whereby the lysis of apoptotic vesicles releases intracellular contents into the extracellular environment [128, 130]. This gives rise to an abundance of nuclear antigens not normally present in healthy individuals, which are subsequently recognised as foreign by the immune system through activation of nucleic acid-sensing TLR3, TLR7, TLR8, and TLR9 on dendritic cells (DCs) [131]. In particular this drives IFN-α production by pDCs, which exerts a range of immunological effects including B cell differentiation, immunoglobulin class switching and production of autoantibodies, as well as T cell and B cell survival (Figure 1.2) [132]. Alternatively, self-antigen or immune complexes may also be presented by myeloid DCs (mDCs) directly to T and B cells in the presence of co-stimulatory signals, thus promoting an autoimmune response as opposed to the tolerogenic response seen under normal homeostatic conditions [133]. This is attributable to T and B cell dysfunction whereby lymphocytes have a lower threshold for

activation resulting in hypersensitivity, which is well described in SLE [134]. Inflammation may also be perpetuated through an imbalance in $CD4^+$ T cell subsets, with an increase in T helper 17 (Th17) cells [135], which are associated with autoimmunity and a reduction in Tregs, which antagonise other T cell subsets to induce tolerance [136]. In addition to their role in B cell activation, autoreactive T cells infiltrate sites of inflammation, secrete cytokines and chemokines, and perpetuate the immune response by inducing recruitment of other immune cell types.

Due to their importance in autoantibody production, B cells are major players in the pathogenesis of SLE. They can either be activated classically by T lymphocytes, or alternatively through autoantigen recognition via TLR3, 7, and 9, resulting in the production of autoantibodies against a plethora of nuclear, cytoplasmic and surface antigens. Defective B cell responses are maintained through polyclonal B cell activation and expansion, mediated through B cell activating factor (BAFF) which is increased in SLE patients [137]. The finding that autoantibodies are of high affinity and IgG class-switched indicate a major role for T cells in providing B cell help; supported by the finding of increased CD40 ligand (CD40L) expression on T cells [138], and the demonstration of nucleosome specific T cells in patient blood [48].

Importantly, the presence of autoantibodies in SLE patients contributes to SLE disease pathogenesis by inducing the formation of large immune complexes with the potential to cause tissue damage. This is worsened by defects in the complement cascade, as a result of C1q deficiency or low C2 and C4, and leads to reduced agglutination, opsonisation and impaired uptake by antigen presenting cells. These large immune complexes may become trapped in the microvasculature, resulting in direct organ damage to the skin, joints or the kidneys in the form of a flare. Alternatively, tissue damage may occur indirectly through a type III hypersensitivity reaction whereby immune complex recognition and phagocytosis is FcγR-mediated, i.e. the immune response becomes directed against the autoantibodies themselves. This results in the activation of proinflammatory genes such as NFκB, and the secretion of vast amounts of cytokines and inflammatory mediators, resulting in systemic inflammation.

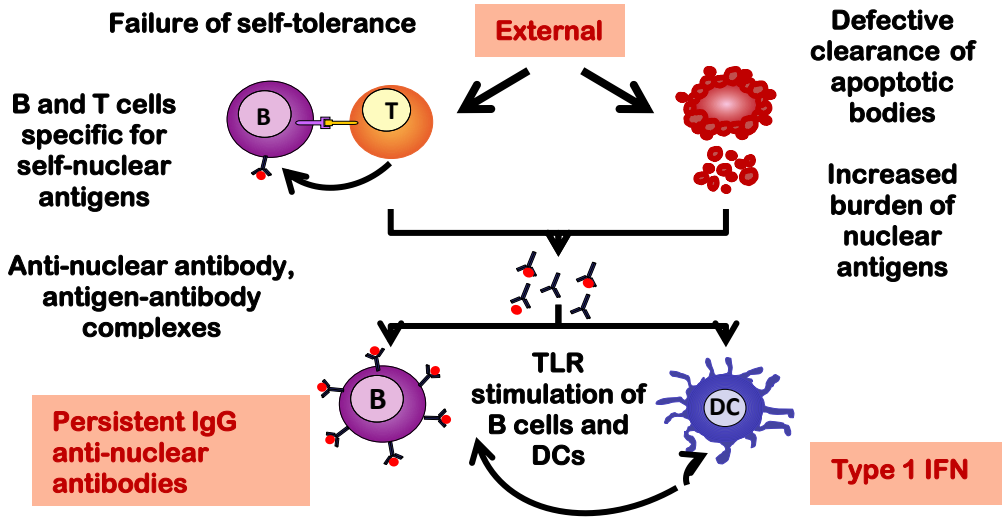


Figure 1.1 SLE immunopathogenesis

SLE is characterized by both a failure in B cell and T cell self-tolerance, and defects in apoptotic cell clearance, which both occur as a result of unknown external triggers. Nuclear antigens, which are exposed as a result of defective cell death, are recognized by B cell autoantibodies to form antigen-antibody complexes. Subsequent interactions between T cells and B cells results in class switching to IgG, whilst recognition of nuclear antigens through TLRs present on B cells and DCs induces type I IFN production. Such cellular interactions perpetuate disease through inflammatory cytokine production and further immune complex formation.

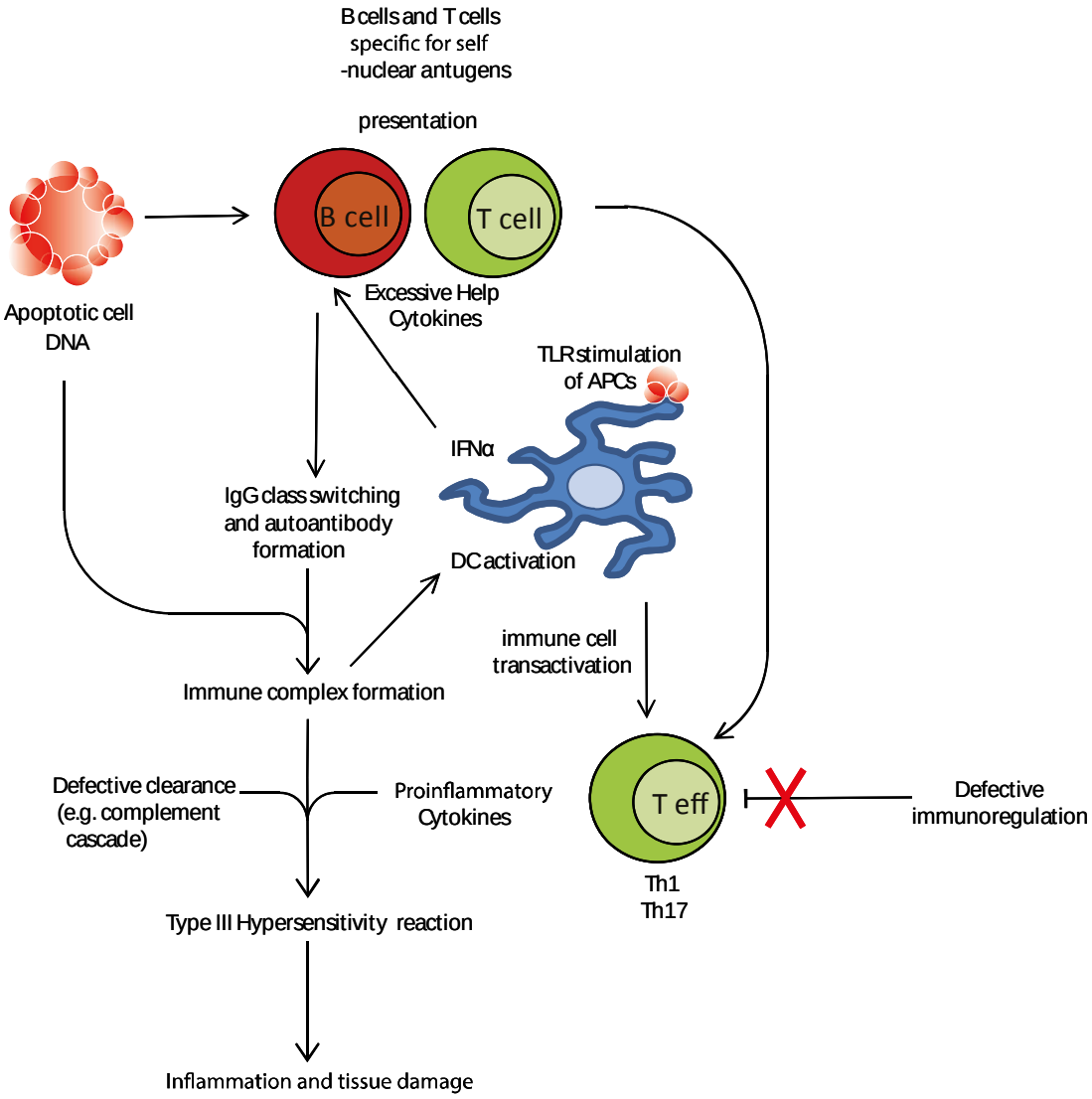


Figure 1.2 Immune mechanisms of SLE

SLE is characterised by aberrant immune responses to antigen present in apoptotic cells, which upon presentation to T cells results in excessive proinflammatory cytokine release by Th1 and Th17 effector T cells. These autoreactive T cells provide B cell help, which enables class switching and release of autoantibodies which are reactive against double stranded DNA and RNA. Excessive autoantibody release results in immune complex formation, followed by dendritic cell activation and the release of IFN-α, which perpetuates the process by supporting IgG class switching and B cell survival. Poor immunoregulation (e.g. by Tregs) combined with defective immune complex clearance (e.g. by complement) results in a type III hypersensitivity reaction, mediated by persistent immune complexes and ultimately resulting in chronic inflammation and tissue damage.

1.2.2 Atherosclerosis: immune mechanisms overview

Atherosclerosis is an inflammatory disorder characterised by the desposition apolipoprotein B (ApoB)-containing lipoproteins within the vasculature and subsequent uptake by immune cells within the intimal arterial layers. The deposition and oxidation of lipids initiates an inflammatory cascade resulting in vascular swelling and arterial occlusion, which can ultimately result in a stroke or MI (Figure 1.3) [139]. Particular regions of the arterial tree such as the aorta are more prone to this, as they are exposed to high shear stress blood flow [139]. This previously described "response to retention" model of atherosclerosis pins this root cause as the ApoB lipoproteins themselves [139]; modified lipoproteins which now act as antigens within susceptible areas of the vasculature.

A causal relationship between lipoprotein and subendothelial matrix interactions and the development of atherosclerosis has previously been demonstrated in genetically engineered mice [140, 141]. This is supported by epidemiological studies, demonstrating the link between high circulating concentrations of cholesterol and atherosclerotic cardiovascular disease [92]. In the blood cholesterol is transported by low density lipoprotein (LDL) in complexes comprised of an esterified cholesterol and triglyceride core, surrounded by a phospholipid and ApoB shell [139].

The initial stage of atherosclerotic plaque development is characterised by the "fatty streak", which is comprised of subendothelial lipids and cholesterol-laden foam cells formed upon excessive lipid uptake by macrophages [92]. The mechanisms involved in foam cell formation have yet to be fully characterised but likely combines a range of cellular processes. These include macropinocytosis, receptor-mediated endocytosis, scavenger receptor-mediated uptake and direct uptake via dendritic processes, which protrude through the endothelium [142]. Once engulfed by macrophages lipoproteins are processed within the endoplasmic reticulum and free cholesterol is re-esterified, resulting in lipid droplet formation, characteristic of foam cells [143]. This process is perpetuated through persistent dyslipidaemia, characterised by an abundance of LDL, which undergoes oxidation to form the pro-atherogenic oxLDL. The activation of endothelial cells by oxLDL results in enhanced expression of adhesion molecules E-selectin and vascular cell adhesion protein 1 (VCAM) on endothelial cells, which facilitates transendothelial migration of monocytes, DCs and T cells [92]. The abundance of trapped oxLDL within the intima results in a localised immune

response characterised by influx of immune cells, and permanent structural changes within the arteries.

Vulnerable plaques, which are liable to rupture, are characterised by a large necrotic core, fibrous cap and decreased smooth muscle and extracellular matrix content [144]. This loss of plaque stability is often a result of smooth muscle cell apoptosis, which exacerbates inflammation and is associated within fibrous cap thinning and an expanded necrotic core [145]. Loss of smooth muscle and cell matrix occurs through several mechanisms, including secretion of MMP-7 from macrophages [146], and tryptase and chymase release from mast cells; all of which act directly on smooth muscle cells leading to defects in inducible nitric oxide synthase (iNOS) production and cell adhesion [147].

Immunocytochemistry and flow cytometry of atherosclerotic specimens with antibodies specific for a range of cell types has confirmed the presence of macrophages, DCs, $CD4^+$ T cells, $CD8^+$ T cells, B lymphocytes, mast cells and occasionally neutrophils within the atherosclerotic plaque [148]. This range of cell types has subsequently been shown to contribute to all stages of plaque development and will be explored in subsequent sections [92].

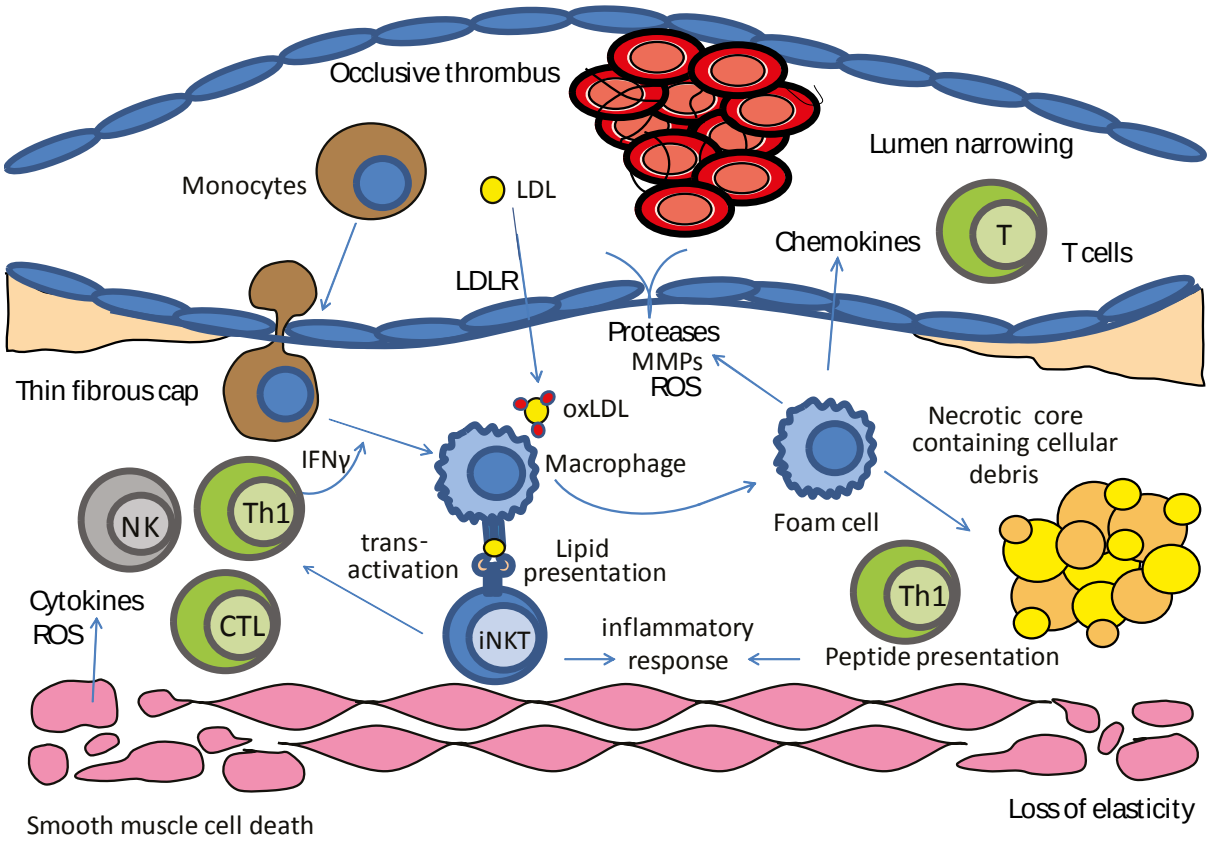


Figure 1.3 Immunopathogenesis of atherosclerosis

Atherosclerosis is initiated as a result of dyslipidemia, in particular by oxidised LDL which is endocytosed by macrophages via scavenger receptor activation. This results in the release of proteases, matrix metalloproteinases and reactive oxygen species, which contribute to remodelling of the endothelium as well as increased lipid oxidation. Moreover, chemokine release by macrophages combined with increased endothelial cell adhesion molecule expression results in increased monocyte transendothelial migration into the intima. The presence of lipid-reactive T cells (such as iNKT cells) combined with the abundance of lipid antigen results in activation of adaptive immune cells (NK cells, Th1 cells and CTLs) which act in a positive feedback manner to induce macrophage differentiation through IFN-γ, as well as contributing to the inflammatory response. Progression of atherosclerotic plaques is characterised by foam cell formation and formation of a necrotic core, observed in advanced lesions, where macrophages are unable to process endocytosed lipid antigen. This results in the release of peptide antigen, which exacerbates the inflammatory response, resulting in tissue damage to smooth muscle cells and narrowing of the lumen.

1.2.3 Antigen presenting cells: overview

An antigen presenting cell (APC) is a cell that is capable of presenting antigen complexed with major histocompatibility complex (MHC) to T cells. Whilst many cells in the human body function as APCs professional APCs include dendritic cells, monocytes/macrophages and B cells. With the exception of B cells, APCs are considered part of the innate immune system, which is characterised by its ability to process and present antigen in a non-specific manner to T cells to generate an adaptive immune response.

In contrast to non-professional APCs which are mostly concerned with presenting endogenous antigen (such as self-antigen or viral antigen during infection), professional APCs are extremely efficient at internalising exogenous antigen through phagocytosis or receptor-mediated endocytosis. One method of antigen recognition occurs through activation of scavenger receptors, which recognise commonly occuring ligands including lipoproteins, apoptotic cells, phospholipids and proteoglycans in response to inflammation [149]. In addition to facilitating receptor-mediated endocytosis, scavenger receptors also induce various signalling cascades resulting in activation of multiple inflammatory genes [150] (Figure 1.4).

One particularly abundant class of scavenger receptor are class A scavenger receptors (SR-A), which are involved in recognising modified or oxidised LDL particles and as a result have been implicated in the development of atherosclerosis [149]. This is apparent due to the finding that SR-A is upregulated on endothelial cells upon exposure to oxidative stress, whilst SR-A activation in macrophage is thought to induce maturation to foam cells. Following recognition, SR-A undergoes internalization via clathrin dependent or independent endocytosis.

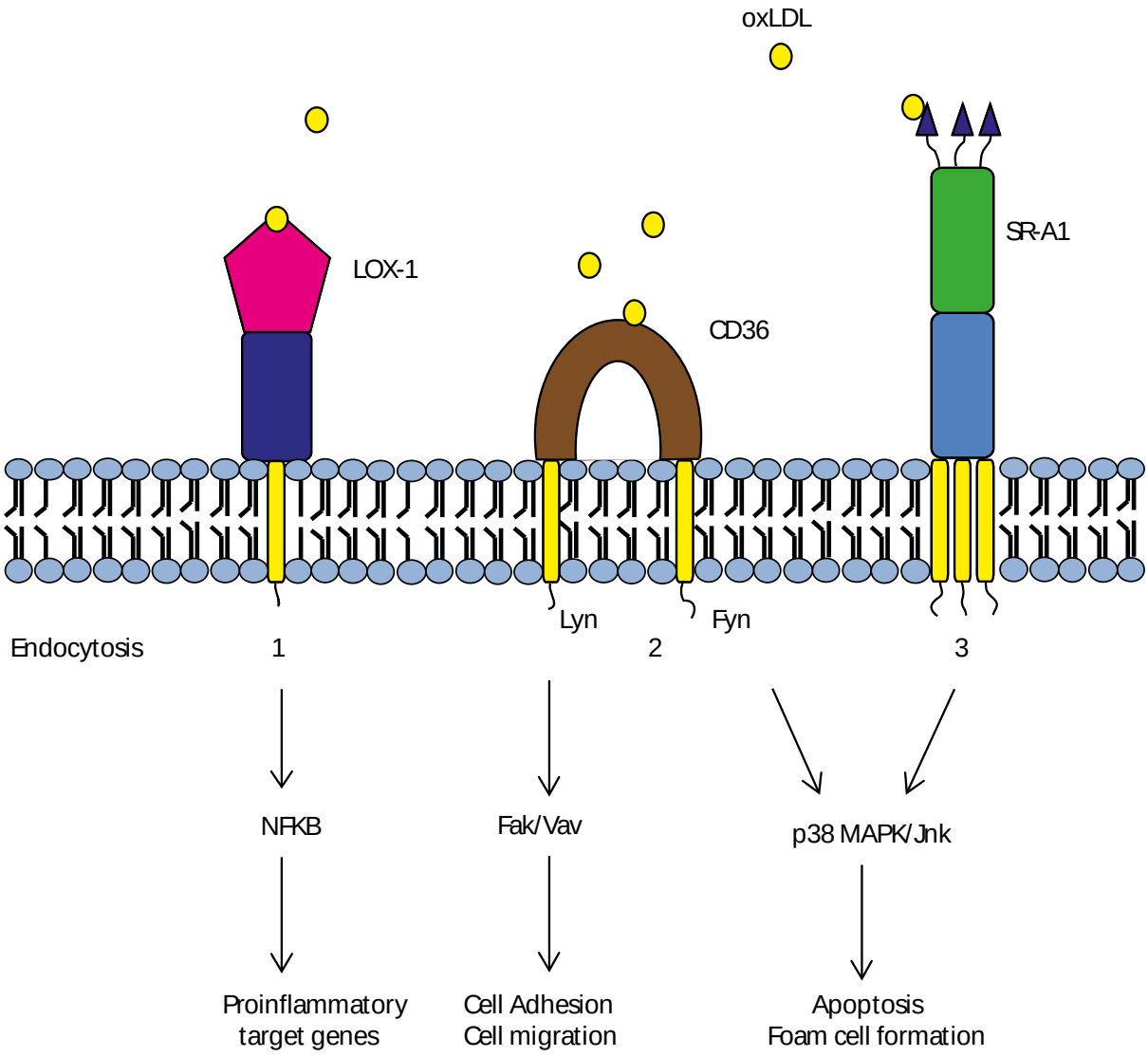


Figure 1.4 Lipid uptake by scavenger receptors

Overview of the signalling pathways activated upon oxLDL recognition by scavenger receptors LOX-1, CD36 and SRA-1. Upon binding of oxLDL, differential endocytosis pathways are activated including (1) caveolae-mediated uptake, (2) lipid-raft dependent uptake, and (3) clathrin-independent pathway. Figure based on Zani et al. [150] LOX-1 - Lectin-like oxidized low-density lipoprotein (LDL) receptor-1

CD36 (also known as SR-B2) recognises oxidised phospholipids and lipoproteins, as well as long chain fatty acids and apoptotic cells [151], and is upregulated on macrophages where it functions as an “eat me” signal to induce further uptake of oxLDL and subsequent foam cell formation [152]. Moreover, CD36 expression is closely linked to lipid metabolism since it is upregulated through several mechanisms including peroxisome proliferator-activated receptor γ (PPARγ) signalling and de novo ceramide synthesis [153, 154]. Other roles of CD36 include the recognition of cells undergoing apoptosis via a modified phosphatidylcholine/serine ligand [155, 156], as well as angiogenesis and oxidative stress, which all contribute to atherosclerosis [157].

Lectin-like oxidised low density lipoprotein receptor (LOX-1) recognises a variety of ligands including oxLDL, bacterial components, and phosphatidylserine which is present on apoptotic cells [158]. It is upregulated on macrophages in response to the proinflammatory cytokines IFN-γ and tumour necrosis factor (TNF)-α [159], as well as in response to the hypertensive-related stimuli angiotensin-II and endothelin-1 [160]. Importantly, LOX-1 expression is markedly increased within atherosclerotic lesions compared to healthy individuals, where it plays roles in atherosclerosis progression [161].

The low density lipoprotein receptor (LDLR) family of receptors mediate uptake of low density lipoprotein (LDL), intermediate density lipoprotein (IDL) or very low density lipoprotein (VLDL), and play a crucial role in the clearance of ApoE-containing lipoprotein. The LDLR is a major determinant of plasma cholesterol levels due to its role in removing cholesterol from the blood through receptor-mediated endocytosis [162]. Its expression is regulated by the *liver x receptor (LXR)* genes, which control lipid biosynthesis [162].

Not much is known about the role of scavenger receptors on other immune cells including B cells, T cells and monocytes or whether their functions are altered in SLE patients. A study by Chen et al. observed that SLE patients produce autoantibodies against SR-A, which may prevent the phagocytosis of apoptotic cells and thus result in the loss of self-tolerance in SLE patients [163]. In addition, Reiss et al. previously showed that SLE patient serum upregulated scavenger receptor CD36 expression in THP-1 human monocytes to a much greater extent than serum from healthy donors [164], which could result in increased lipid uptake by macrophages and thus a greater propensity for developing atherosclerosis. This is supported by the finding that IFN-α can upregulate SR-A in human monocytes and

macrophages [165], which potentially results in increased lipid uptake and foam cell formation - factors which could influence cardiovascular risk in inflammatory diseases such as SLE.

1.2.4 Monocytes in SLE and atherosclerosis

Monocytes originate from monocyte-DC precursors within the bone marrow, from which the classical, intermediate and non-classical monocytes arise, which can be characterised based mainly on CD14 and CD16 expression (Table 1.3).

Classical or $CD14^{++}$ monocytes are analogous to $Ly6C^{hi}$ monocytes in mice, and have been shown to upregulate MHC class II upon extravasation [166]. Remarkably, $Ly6c^{high}$ and $Ly6c^{low}$ monocytes demonstrate plasticity in response to a range of stimuli, polarisation of which is driven according to the balance between Th1 and Th2 cytokines, which results in the generation of M1 or M2 macrophages [166].

Several studies have shown that monocytes play major roles in the initiation and development of atherosclerotic plaque [17, 18]. In mice proinflammatory $Ly6C^{high}$ monocytes (which are analogous to $CD14^{+}CD16^{+}$ monocytes in humans) expressing TNF-α, MMPs and cathepsins are recruited initially following MI in mice. This is followed by the predominance of $Ly6C^{low}$ monocytes which express high levels of VEGF and are thought to play a role in tissue repair [167]. In humans, numbers of $CD16^{+}$ (intermediate) monocytes have been found to correlate with traditional cardiovascular risk factors such as body mass index and intima-media thickness (IMT) [168-170]. Intermediate monocytes are also thought to produce higher amounts of reactive oxygen species (ROS), TNF-α and IL-1β, which contribute to plaque development [171]. Data on the frequency and functions of different monocyte subsets in SLE is confusing due to conflicting reports [172-174], which is thought to be due to differences in gating strategy [175]. Interestingly, intermediate and non-classical monocytes have been shown to have higher expression of TLRs and co-stimulatory molecules indicating a role in antigen presentation, whilst high expression of scavenger receptors have been observed on classical monocytes supporting a role for these cells in phagocytosis [175].

	Inflammatory/Classical	Intermediate	Resident/Nonclassical
Surface markers (human monocytes)	$CD14^{++}CD16^{-}$ $CX3CR1^{lo}$ $CD62L^{+}$ $CD115^{+}$	$CD14^{+}CD16^{+}$ $CX3CR1^{hi}$ $CD62L^{-}$ $CD115^{+}$	$CD14^{+}CD16^{+}$ $CCR2^{-}$ $VCAM^{hi}$ $CD64^{lo}$
Recruitment	Early in acute inflammation	Early in acute inflammation	Late in acute inflammation
Reservoirs	Spleen	Unknown	Unknown
Acute Inflammation	Accumulate in inured myocardium and perform inflammatory and proteolytic functions	Inflammation?	Assumed role in granulation tissue formation and angiogenesis, later mobilization
Chronic Inflammation	Accumulate in atherosclerotic plaque	Given scavenger receptor (CD163 and CD204) expression, accumulation in plaque is likely.	Accumulate in plaque via CCR5 and CX3CR1.

Table 1.3 Characteristics of different monocyte subsets in humans

Human monocytes can be divided into classical (inflammatory), intermediate and non-classical (tissue resident) subpopuations based on CD14 and CD16 expression other markers. Each population play various roles during different stages of atherosclerosis. Figure adapted from Ghattas et al. 2013 [176].

There is evidence implicating that monocytes are defective in SLE patients in terms of cytokine secretion; indeed it is recognized that mouse models of SLE are characterised by increased IFN-α production by monocytes in response to immune complexes [177, 178]. Moreover, monocytes from SLE patients have impaired IL-12p70 (T cell stimulating factor) production, which is compensated for by increased IL-10 production [12]. IL-10 subsequently acts in a pathogenic manner by inducing anti-dsDNA release [13]. A study by Korman et al. suggests that monocytes from SLE patients are programmed to differentiate into macrophages with increased pathogenic potential upon entering sites of vascular inflammation, thus promoting atherosclerosis progression [179]. Moreover, several signal transduction genes were found to be differentially regulated in SLE patients during monocyte to macrophage differentiation. These included Janus kinase 2 (JAK2), STAT6, TLR8, TLR2, VEGFB, macrophage scavenger receptor 1 (MSR1) and CD163, which have previously been shown to be involved in the pathogenesis of atherosclerosis [180-182].

Interestingly, monocytes can also differentiate into cells with DC-like properties in the presence of serum from SLE patients, and could subsequently stimulate conventional $CD4^+$ T cell proliferation [183]. The finding that this could be blocked by IFN-α neutralising antibody suggests that the ability of SLE serum to induce T cell expansion via DC differentiation is an IFN-α-dependent process. Moreover, the addition of IFN-α to autologous healthy serum was able to mimic the T cell expansion observed with SLE serum treatment [183]. The ability of monocytes from SLE patients to differentiate into DCs could therefore act as a mechanism to compensate for the lack of pDCs in SLE patients [183]. These monocyte-derived dendritic cells (MDDCs) in SLE patients are believed to play a pathogenic role by efficiently capturing apoptotic cells and nucleosomes and presenting them to T cells [184]**.**

1.2.5 Macrophages in SLE and atherosclerosis

Monocytes circulate in the blood and only become macrophages upon infiltration into the tissues and activation. They participate in both innate and adaptive immune cells as APCs, and play other roles such as the clearance of apoptotic cells and reverse cholesterol transport. It has been proposed that defective clearance of apoptotic bodies by macrophages in SLE may contribute to the development of atherosclerosis by providing a source for autoantigen production and inducing local inflammation through natural

degradation and enzyme release [185]. Macrophages can be categorised according to their *in vitro* and *in vivo* functions, as either proinflammatory, M1 cells or anti-inflammatory, M2 cells [186]. Importantly, polarisation towards M1 and M2 phenotypes is not dependent upon T cells, as has been demonstrated using recombination activating gene 1 (RAG1) knockout mice (which lack T cells) [187].

M1 macrophages can be induced in vitro by IFN-γ and through TLR stimulation, in particular by lipopolysaccharide (LPS) [186]. Characterised by CD68 and CXCR2, they are proinflammatory in nature and express iNOS, TNFα, IL-1β, IL-6, IL-12 as well as proteolytic enzymes [186]. In contrast, M2 macrophages are induced by T helper 2 (Th2) cytokines such as IL-4 and IL-13, and are characterised by expression of CD163 and Mannose receptor I (CD206) [186]. They are considered anti-inflammatory as they are major producers of TGFβ, IL-1RA and IL-10. However, it is important to note that there are inconsistencies in the literature regarding the markers used for M1 and M2 macrophages, which must be considered when comparing different studies.

Vulnerable plaques are generally characterised by M1 macrophage polarisation, however the role of M2 macrophages remains less clear [188]. One recent study observed that M1 macrophages were found almost exclusively in plaques from symptomatic patients with unstable plaques, whereas M2 macrophages were found in plaques from both symptomatic and asymptomatic patients and were associated with increased plaque stability [189]. Studies using mouse models of atherosclerosis suggest that there could be differences in M1 and M2 macrophage polarisation depending upon the stages of plaque development since M2 macrophages infiltrate atherosclerotic lesions during the early stages of disease in atherosclerosis-susceptible ApoE knockout mice, and convert to the M1 phenotype with plaque progression - a finding associated with a switch from IFN-γ to IL-4 production [190].

Moreover, evidence suggests that M1 and M2 macrophages may be confined to particular regions of the plaque. For example, M1 macrophages are abundant in rupture-prone areas, whilst M2 macrophages are found within the vascular adventitia, with no dominant phenotype within the fibrous cap [191]. It is therefore possible that macrophages from patients with unstable plaque could be more polarised towards the M1 phenotype, whilst those with stable plaque could exhibit more polarisation towards the M2 phenotype. M1 macrophages have also been shown to contribute to SLE and are associated with increased

disease activity; conversely adoptive transfer of M2 macrophages was found to be protective in a mouse model of SLE [192]. This pathogenic role for M1 macrophages in SLE is also supported by Iwata et al. where increased M1 macrophage polarisation was observed in a mouse model of Lupus nephritis [193].

1.2.6 Dendritic cells in SLE and atherosclerosis

Dendritic cells (DCs) are identified according to their major histocompatibility complex (MHC) class II molecules and integrin CD11c expression, as well as their ability to migrate to lymphoid organs and activate T cells. Classical dendritic cells (cDCs) reside within most tissues of the body including the skin, gastrointestinal and respiratory tracts. Upon uptake of self or foreign antigen, they migrate to lymph nodes to present antigen to T cells in the context of MHC. Under normal physiological conditions they exist in an inactivated state, only acquiring an activated surface phenotype such as the upregulation of costimulatory molecules following stimulation by TLRs. Activated cDCs secrete IFN-α, IL-12 and IL-23.

In contrast, pDCs circulate in the blood and lymphoid organs where they form the first line of defense against viral infections through high expression of TLR7 and TLR9 within endosomal compartments[194]. Upon recognition of nucleic acids pDCs contribute to the inflammatory response through secretion of IFN-α [194], or alternatively can play a tolerogenic role through the induction of IL-10-secreting natural Tregs (nTregs) [195]. SLE patients have been shown to have lower numbers of HLA-DR^{+}CD11c^{-}CD123^{+} pDCs compared to both healthy controls and other autoimmune diseases such as juvenile diabetes mellitus [183].

1.2.7 B lymphocytes in SLE and atherosclerosis

B lymphocytes (along with T lymphocytes) are cells of the adaptive immune system, meaning that they orchestrate very specific responses to a particular antigen, often following activation by cells of the innate immune system (e.g. APCs), resulting in long term memory formation. B cells may require T cell help for activation upon encountering antigens, which enables them to differentiate into antibody-producing effector plasma cells [196]. Alternatively, B cells can be activated in the absence of T cells through TLR signalling, for example upon recognizing microbial viral components, commonly through TLR4 and TLR9 [197]. B cells are diverse in terms of the cytokines they produce following activation which include IL-4, IL-6, IL-10, IL-21, IL-23, TNF and lymphotoxin [196-198].

Importantly B cells also function as APCs due to the expression of MHC class I and II which enables them to interact with the T cell receptor, necessary for the presentation of protein. B cells are thought to present lower doses of peptide antigen compared to dendritic cells or macrophages. To ensure optimal T cell activation, costimulatory molecules on B cells such as CD80, CD86 and OX40L must bind their co-receptors on T cells [199, 200]. B cells can be divided into various subsets based on expression of surface markers CD24 and CD38. The $CD19^{+}CD24^{hi}CD38^{hi}$ population are referred to as immature B cells, which include regulatory B cells (Bregs), characterized by high IL-10 production. The suppressive capacity of Bregs has been demonstrated both *in vitro* and *in vivo*, where they have been shown to inhibit Th17 responses [166, 167, 201]. Current evidence on whether they can support or enhance Treg function remains inconclusive.

The role of B cells in autoimmunity extends beyond autoantibody production since B cells can exert positive and negative effects on immune balance [189, 194, 195]. Unlike T cell subsets, Bregs express no definitive transcription factor, and are instead functionally defined through their high IL-10 production following stimulation. These cells express a similar surface phenotype to immature B cells, and are characterized by $CD19^{+}CD24^{hi}CD38^{hi}$ expression. Interestingly, Bregs produce higher levels of IL-10 in untreated rheumatoid arthritis (RA), systemic sclerosis (SS) and SLE patients compared to healthy controls [202].

Autoreactive B cells have been identified in both SLE patients and mouse models of the disease, and are thought to play a key role in SLE pathogenesis [203]. Such B cells have a

lower threshold for activation, upon which they produce excessive amounts of autoantibodies leading to immune complex formation throughout the body [203]. Evidence shows that B cells from SLE patients have several molecular defects, which contribute to B cell autoreactivity. These include the Fcγ receptors, the B cell receptor (BCR), TLR and Lyn signalling [203]. Such cellular and molecular abnormalities make B cells attractive therapeutic targets for SLE with drugs such as belimumab and rituximab currently in use [43, 50].

Whilst B cells have been identified within atherosclerotic plaque, their role in the development of atherosclerosis remains unclear. In addition to their function as APCs, interactions between B cells and Th2 cells are responsible for antibody class switching, mediated by the Th2 signature cytokine IL-4, which is present in preclinical atherosclerotic plaques [204]. This protective role for B cells is supported by Major et al. who demonstrated that adoptive transfer of B cells could attenuate atherosclerosis in $LDLR^{-/-}$ mice [205]. This was later supported by Doran et al. in B cell deficient $ApoE^{-/-}$ mice, which had reduced atherosclerosis compared to ApoE single knockouts [206]. In contrast, knockout of BAFFR (present on follicular and marginal zone B cells) in combination with ApoE reduced atherosclerosis, suggesting a pathogenic role for B cells in the disease [207]. This pathogenic role for B cells is supported by the finding that B cells in humans produce IgM antibodies against modified lipid antigens in the blood [208]. The discrepancy on the role of B cells in atherosclerosis could be due to differences in the stage of plaque development under investigation, or due to differences in B cell subsets whereby the B-1 subset, for example, has been shown to be atheroprotective [209].

1.2.8 T lymphocytes in SLE and atherosclerosis

T lymphocytes, and specifically $CD4^+$ T helper cells are key players in the adaptive immune system due to their importance in regulating B cell humoral responses, as well as $CD8^+$ cytotoxic T cell responses (Figure 1.5). Upon peptide recognition by MHC class II, naïve T cells become activated and differentiate into T effector cells with clonal expansion ensuring long lasting memory to that particular antigen. During this process $CD4^+$ T cells differentiate into several distinct T helper lineage according to factors such as the particular antigen

encountered, the strength of the signal, the cytokine milieu and the presence of co-stimulatory molecules [66].

Th1 cells are characterised by the signature cytokine IFN-γ, and the expression of transcription factor T-bet (TBX21). They can be induced in the presence of IL-12, which is associated with viral infections, during which they provide help to $CD8^+$ cytotoxic T cells. In contrast Th2 cells are characterised by IL-4 production, and expression of transcription factor GATA-3. Th2 cells are essential in providing help to B cells to induce a humoral immune response characterised by antibody class switching and plasma cell formation.

More recently Th17 cells have been described, characterised by production of IL-17, and expression of transcription factor RORγt. Th17 cells are induced in the presence of TGFβ and IL-21, IL-6 and IL-23, or IL-1β and have been shown to play roles in the immune response to extracellular pathogens, as well as in autoimmune diseases [65, 67, 210]. Whilst TGF-β and IL-21 induce naïve $CD4^+$ T cells to become Th17 cells, recent evidence suggests that IL-23 is important for the inflammatory potential and survival of Th17 cells [163, 165, 210]. Despite the lack of surface markers on Th17 cells, it has been shown that human Th17 cells express high levels of CCR6, CD161 and CD49d [129, 149, 151, 164].

The presence of T cells within atherosclerotic plaque was first reported 30 years ago by Hansson et al. where they can be detected from as early as the fatty streak stage [211, 212], reaching up to 10-20% of leukocytes in advanced human lesions [213]. The majority of T cells within atherosclerotic lesions are $CD4^+$ T helper cells, and are primarily of the Th1 lineage, although other subsets have been identified [214]. The importance of T cells to atherosclerosis is highlighted by animal models, where adoptive transfer of $CD4^+$ T cells aggravates the disease [215]. Whilst atherosclerosis is classically considered as a Th1 mediated disease, data for Th2 cell involvement is somewhat confusing due to the fact that both pro- and anti-atherogenic roles have been reported.

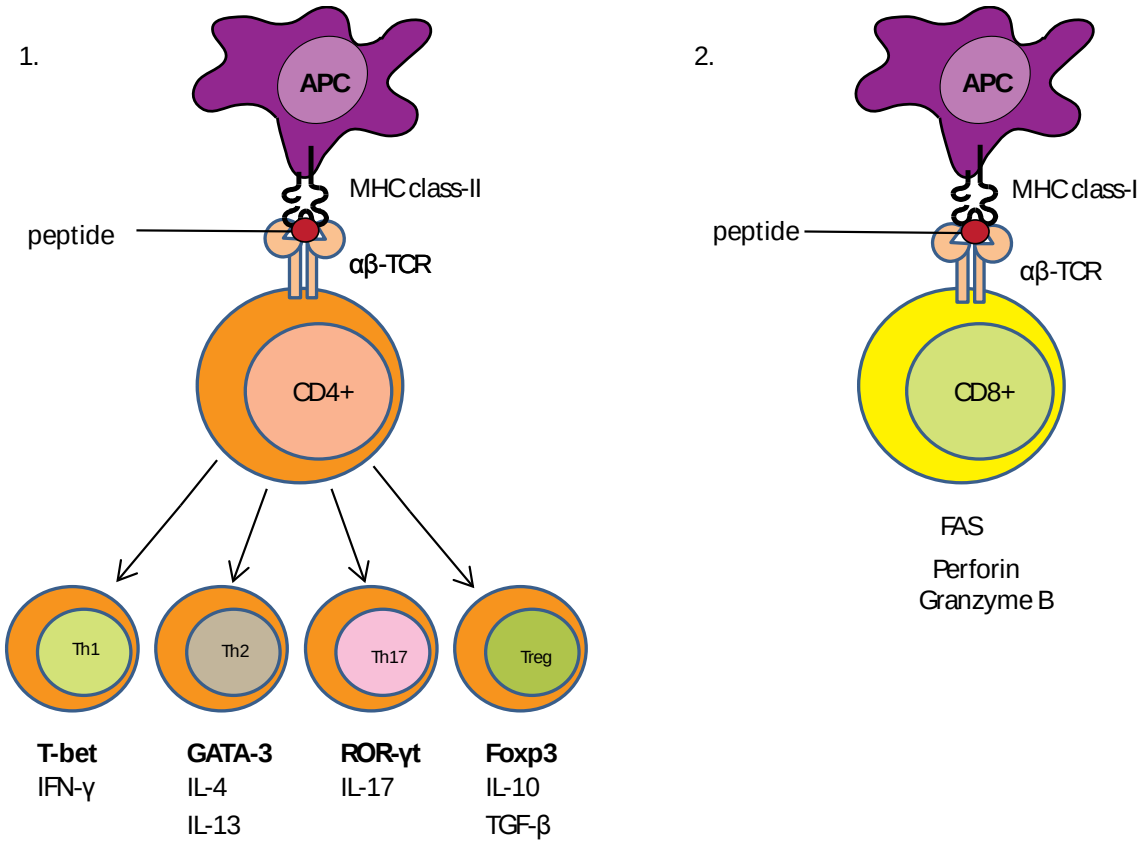


Figure 1.5 Conventional αβ T cell subsets in humans

Conventional T cells express an αβ T cell receptor formed through V, D, J recombination, which results in a multitude of possibilities for TCR gene rearrangements. (1) CD4+ T cells (or T helper cells are reactive to peptide antigen presented via MHC class II on APCs. Activation of naïve T cells results in the acquisition of various transcription factors, which drives T helper cells towards a particular subset and dictates their cytokine profile. This enables them to communicate with other cells of the immune system to elicit the appropriate response to the particular antigen. (2) CD8+ T cells or cytotoxic lymphocytes are reactive to peptide antigen presented via MHC class I, which is present on every cell in the body. Upon recognition of foreign antigen, CD8+ T cells exert multiple killing mechanisms including secretion of perforin and Granzyme B as well as the induction of apoptosis through FAS-FASL signalling.

The role of regulatory T cells (Tregs) in homeostasis and the prevention of autoimmune responses has been well described [210, 216, 217] and are generally characterised as $CD4^+CD25^+CD127^-$ T cells. Importantly, CD25 (IL-2-α receptor subunit) plays a role in sequestering IL-2 and preventing clonal expansion of other potentially autoreactive T cells [218, 219]. The transcription factor *Forkhead box protein 3 (Foxp3)*, which is often used to identify Tregs, is a critical factor for Treg development and function, although this has since been challenged [220, 221]. Tregs can be subdivided into natural (nTregs) and induced Tregs (iTregs). However, since both subtypes are phenotypically and functionally similar in the periphery, it is unclear what percentage of each contributes to the total Treg pool. Differentiation of other T cell subsets into iTregs can be recreated *ex vivo* in response to TGF-β signalling which induces *Foxp3* expression [222, 223]. In addition IL-2 and all-trans retinoic acid may act to enhance iTreg development and function [224-226]. Interestingly, iTregs have been reported to be more stable than nTregs in inflammatory conditions, which may make them a better therapeutic target for treating cancer or autoimmune diseases.

Tregs are viewed as protective in atherosclerosis and have been identified within atherosclerotic plaques in both humans and mice; albeit in low numbers [227, 228]. However, it is thought that the balance between Tregs and other T helper cell subsets may be more important in determining plaque progression. This is evident from a study by Mengya et al. which showed that the balance between Treg and Th17 function may play a role in the development of atherosclerosis in SLE patients [79]. Moreover, SLE patients with plaque had fewer *Foxp3*$^+$ T cells, with reduced suppressive capacity, as well as an increase in expression of the Th17 transcription factor *RORγt*, compared to plaque negative patients and healthy controls [79]. This was further associated with increased serum IL-6; a cytokine known for its Th17-inducing properties [79].

Presentation of peptides via MHC class I on APCs results in CD8+ T cell activation, clonal expansion and initiation of cytotoxic T lymphocyte (CTL)-mediated killing, directed towards cells expressing the particular peptide antigen [135]. Two pathways are involved in the killing of target cells by CTLs; these are the release of the cytolytic enzymes perforin and granzyme B, and the induction of the Fas signalling pathway, which culminates in the apoptosis of target cells [135]. Interestingly patients with SLE, multiple sclerosis (MS) and Sjogren's syndrome have increased perforin and granzyme B expression [135-137]. In SLE this was associated with a $CCR7^-CD45RA^+$ differentiated effector phenotype, and increased disease activity [136]. Furthermore, SLE patients have been reported to have increased membrane

TNFα on CTLs, which is believed to be important in increased apoptosis and the generation of autoantibodies [138]. Similarly $CD8^+$ T cells play a pathogenic role in atherosclerosis; as the dominant lymphocyte type in advanced plaques they are known to target vascular endothelial and smooth muscle cells for apoptosis, and play a role in the development of the necrotic core in advanced lesions [229, 230].

Several unconventional T cell populations exist in the immune system, which possess a specialised T cell receptor in contrast to the diverse αβ TCR present on conventional T cells (Figure 1.6). Among those with a limited repertoire diversity are γδ T cells [130], Natural Killer T cells [133], and mucosal associated invariant T (MAIT) cells [134]. All these populations are considered highly conserved and tissue specific, and play a role at the interface between innate and adaptive immunity. Interestingly, NKT cell levels percentages found to correlate with MAIT cell percentages in SLE patients but not RA patients, indicating a general defect in innate T cell lymphocytes in SLE [132].

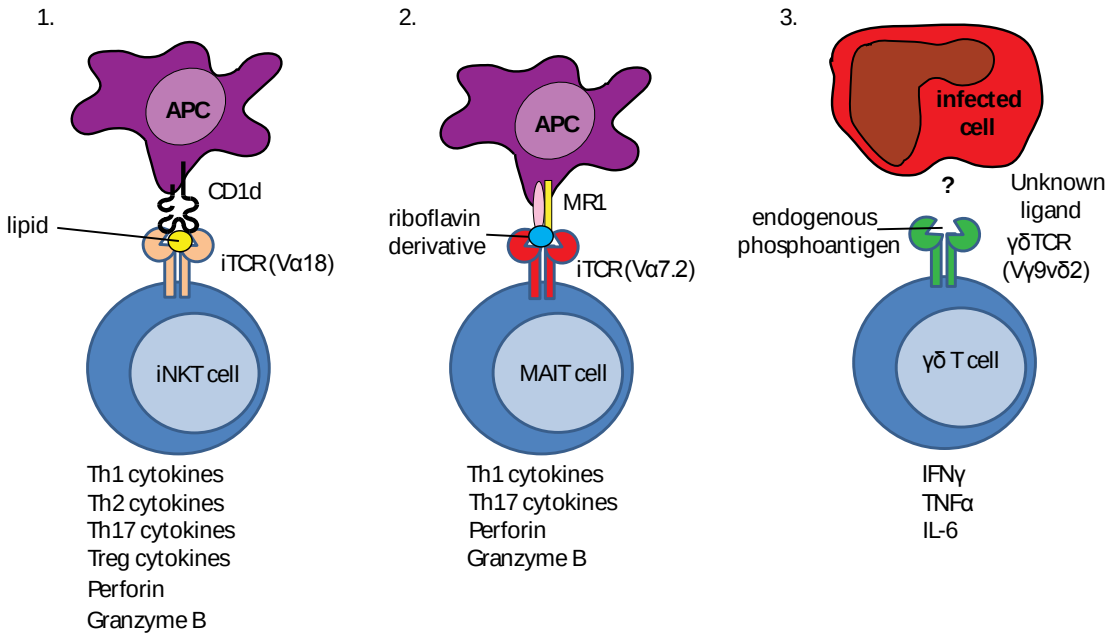


Figure 1.6 Unconventional T cells in humans

Unconventional T cells include iNKT cells, MAIT cells and γδ T cells. (1) iNKT cells are responsive to lipid antigen presented via CD1d and (2) MAIT cells are responsive to a riboflavin derivative presented via MR1 , whilst (3) γδ T cells respond to an unknown ligand. All unconventional T cell types are innate-like meaning that they respond rapidly to antigen, yet have poor memory unlike conventional T cell subsets.

Essentially, many of these unconventional T cell lineages are thought to be important in immunoregulation where they are capable of mediating a balance between activation and tolerance in the same individual. It is therefore unsurprising that these cell types are often deficient in autoimmune diseases. MAIT cells, which are important in mucosal immunity, have been shown to be numerically and functionally deficient in SLE patients, whereby they produce less IFN-γ upon stimulation compared to healthy controls [132]. Similarly, a particular subset of Vδ1 T cells, expressing CD25, CD27 and *Foxp3* has been shown to be substantially decreased in SLE patients [131].

SLE patients also have numerical and functional deficiencies in Natural Killer T (NKT) cells, which specifically recognize and respond to lipid antigens [231-233]. This seemingly protective or tolerogenic role for NKT cells in autoimmunity is in contrast to their pathogenic role reported in animal models of atherosclerosis [234]. Hence a dichotomy exists on their role in humans, in particular for SLE patients who go on to develop atherosclerosis. The role of iNKT cells and how they are defective in SLE patients with atherosclerosis will be explored in more detail in the following sections.

1.3 Invariant natural killer T cells and their role in SLE and atherosclerosis

1.3.1 iNKT cells: overview

Natural killer T cells are a small population of specialized immune cells, which straddle the gap between innate and adaptive immunity due to their ability to undergo rapid activation without the requirement for co-stimulation. They were first identified in mice in 1987 by three independent research groups, but it was not until 1995 that the term “NKT cell” was coined [235-238]. Although they constitute less than 1% of peripheral blood mononuclear cells (PBMCs), NKT cells have an important role in immune homeostasis and diseases such as cancer, bacterial, viral and fungal infection. Unusually however, they are restricted in terms of antigen recognition by CD1d, a major histocompatibility complex (MHC) class I-like molecule. Indeed, the initial observation that they were seemingly activated through CD1d alone lead many to believe that NKT cells were autoreactive [239].

Similar to conventional T cells NKT cells express an αβ TCR, yet instead of responding to peptide antigen in the context of MHC class I or II, NKT cells have evolved to exclusively recognize and respond to lipid antigens in the context of CD1d [240]. Two types of NKT cells exist in humans, categorised based on their αβ TCR expression. Type I or iNKT cells, are the predominant class in humans and express an invariant T cell receptor (iTCR) formed of an alpha chain comprised of Vα24 and Jα18 and a beta chain comprised of Vβ11 [241], which is homologous to the semi-invariant TCR in mice formed of Vα14, Jα18 and Vβ8.2/Vβ7/Vβ2 chains [234]. In contrast, type II NKT cells express diverse αβ TCRs yet relatively little is known about them due to their lack of distinct surface markers or functional characteristics. As a result, far fewer studies have looked at the role of type II NKT cells in disease, particularly in humans [240]. Here, the focus of this project is the type I or iNKT cells and their role in SLE and atherosclerosis.

In terms of gene expression, iNKT cells are a complete hybrid between NK cells and T cells and are no closer to either lineage in terms of transcriptional profile [242]. This enables them to orchestrate a multitude of innate and adaptive immune responses, with the specificity of T cells and rapid nature of NK cells. Existing in a “poised effector state”, they rapidly downregulate their iTCR expression upon activation, whilst secreting copious amounts of

both pro- and anti-inflammatory cytokines [243]. Indeed, iNKT cells have a strong immunomodulatory potential; they are capable of influencing both activation and tolerance in different health and disease states. Despite their potency however, very little is known about the mechanisms which regulate their different effector responses, particularly in autoimmune diseases where iNKT cells are known to be defective.

1.3.2 iNKT cell development

Innate-like T cells are defined by expression of the transcription factor *promyelocytic leukaemia zinc finger (PLZF)*, which is evolutionarily conserved amongst Vγ1-Vδ6.3 T cells, MAIT cells and iNKT cells and involved in iNKT cell development [244] (Figure 1.7). iNKT cells are first selected for in the thymus, specifically in the presence of $CD4^+CD8^+$ thymocytes (rather than thymic epithelial cells), which present glycolipid antigens in the context of CD1d to NKT precursors [245]. These double positive thymocytes also deliver crucial signals for iNKT cell development through expression of signaling lymphocyte activation molecule (SLAM) and the glycoprotein Ly108 [246]. This thymic education is extremely important in promoting self-tolerance, whilst preventing autoimmunity and may therefore be important in determining the balance between the tolerogenic and pathogenic functions of iNKT cells in diseases such as SLE and atherosclerosis.

Following selection, iNKT cell thymocytes undergo elevated TCR signalling through Ras/MAP kinase and the calcineurin/NFAT pathways, resulting in elevated early growth response protein (Egr)1 and Egr2 expression compared to conventional T cells. This is known as stage 0 or the $CD69^+CD24^{high}$ transitional stage, where iNKT cell development branches off from that of conventional T cells [247-249]. During this stage Egr2 is expressed by 100% of NKT thymocytes and promotes cell survival through *Bcl2* and *FasL* induction. Egr2 binds to a site proximinal to *PLZF*, thus inducing its expression which is required for the acquisition of effector properties [244, 249]. In the absence of *PLZF* however, iNKT cells revert to naïve-like cells and produce IL-2, rather than IL-4 or IFN-γ [244]. The importance of *PLZF* to iNKT cell function has further been demonstrated by expression in transgenic mice under the CD4 promoter. *PLZF* was found to induce an iNKT cell-like phenotype, where CD44 and LFA-1 expression were induced, CD62L was downregulated and cells were able to simultaneously produce both IL-4 and IFN-γ [250]. Despite its requirement in iNKT cell

development, iNKT cells lacking *PLZF* have recently been described, indicating that *PLZF* expression may not necessarily be a defining feature of all iNKT cells [251].

iNKT cell development has mainly been studied in mice, where it is divided into stages or checkpoints based on CD24 and CD44 expression. For example, thymocytes deficient in the transcription factor *PLZF* are blocked at stage 1 ($CD44^{low}NK1.1^{+}$) and show properties of naïve T cells such as ineffective expansion and preferential localization to lymph nodes [252-254]. At stage 2 $CD44^{hi}$ $NK1.1^{-}$ cells migrate from the thymus, and preferentially localize to the liver, spleen and bone marrow [252]. Constitutive expression of activation markers CD69 and CD44 is a distinct feature of naïve iNKT cells compared to naïve T cells [255]. These markers are normally found on mature T cells which have previously been exposed to antigen, suggesting that iNKT cells may already be specific for certain lipid antigens upon leaving the thymus. Other factors regulating iNKT cell "responsiveness from birth" include the tyrosine kinase Fyn, NF-κB transcription factors and IL-15, which in conventional T cells are more important for secondary immune responses [256].

Importantly, distinct signaling pathways are required for the development of mature iNKT cells. For example, iNKT cells fail to mature in the absence of *phosphoinositide dependent protein kinase-1 (PDK-1)*, *mechanistic/mammalian target of rapamycin* (*mTOR*), *myc* and *Fyn* signalling, despite normal development of conventional αβ T cells [257-262]. Recent studies have also suggested that iNKT cell development is under the control of miR181a [263]. The high proliferative capacity of iNKT cells during early thymic development is thought to be linked to activation of *mTOR* and *myc*, which are key regulators of T cell proliferation and metabolism [262]. In addition, these signaling molecules have also been proposed to play a role in nuclear translocation of *PLZF* during iNKT cell development [264].

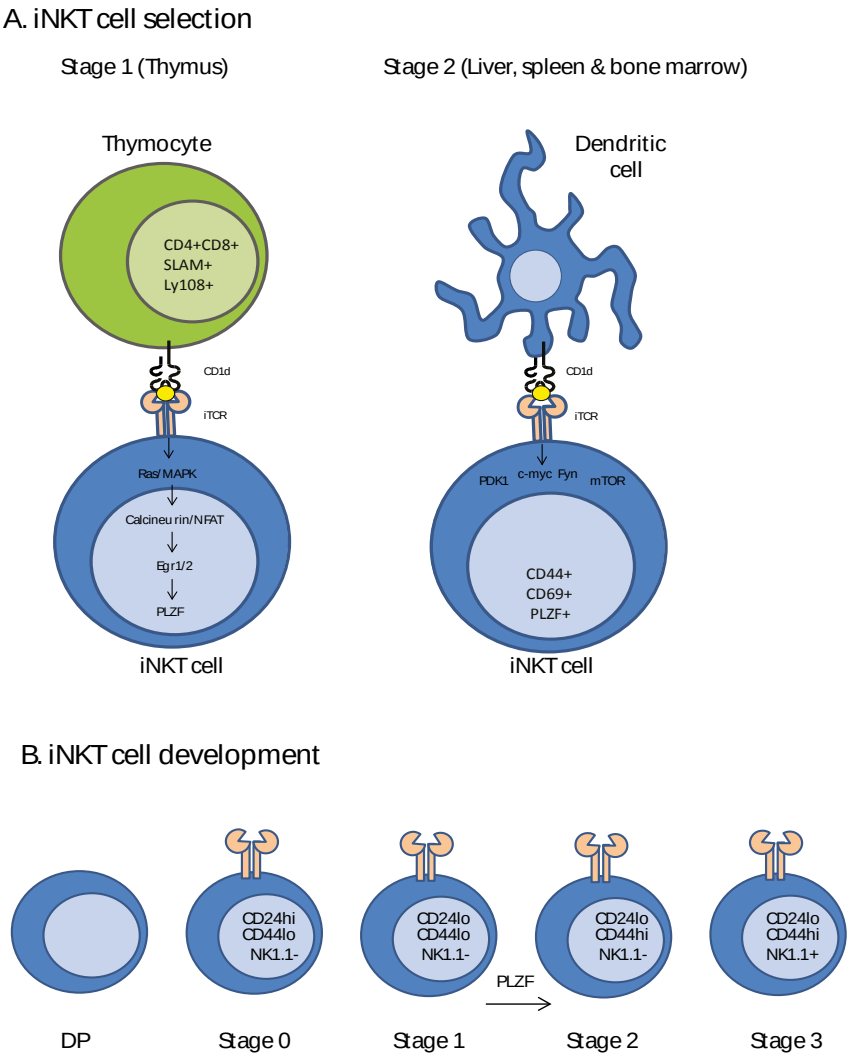


Figure 1.7 iNKT cell development

A. Following acquisition of CD24 during stage 0, the iNKT cell lineage diverges from that of conventional T cells, where presentation of self-lipids in the thymus (stage 1) initiates several unique signaling events resulting in the induction of PLZF expression. Further signaling events in the periphery (stage 2-3) result in upregulation of CD69 and NK cell receptors upon recognition of CD1d and lipid antigen.

B. Linear model of iNKT cell development based on Chandra and Kronenberg [265]. Stage 1 occurs in the thymus where a decrease in CD24 expression upon self-lipid recognition is followed by the induction of *PLZF* and an increase in CD44 expression. Stage 2 thymic emigrants subsequently upregulate NK1.1 (in mice) upon recognition of CD1d-lipid antigen complexes, resulting in stage 3 quiescent iNKT cells.

Under normal conditions iNKT cell responsiveness is regulated to prevent autoimmunity. For example, iNKT cells have been shown to upregulate the inhibitor SHP-1 and reduce iTCR responsiveness upon encountering DCs [266]. This functional education ensures that iNKT cell reactivity is fine-tuned towards stronger stimulatory signals normally seen during inflammation. The importance of *PLZF* expression in iNKT cells is highlighted by the finding that $CD8^+$ iNKT cells expressing *PLZF* are reduced in patients with autoimmune disease, suggesting that a defect in *PLZF* may be responsible for improper iNKT cell development in these patients [267].

1.3.3 CD1d-dependent iNKT cell activation

The most widely accepted mechanism of iNKT cell activation is through CD1d-mediated lipid antigen presentation, although other mechanisms have been published including cytokines alone and activation of natural killer receptors which will be discussed in later sections. CD1d is a member of the CD1 family of glycoproteins of which five isotypes exist in humans (CD1a-e); all of which involved in the presentation of lipid antigens [268]. They are expressed by a range of immune cells including monocytes, macrophages, B cells, dendritic cells (DCs) and thymocytes (but not mature T cells) [269], as well as cells outside of the hematopoietic system e.g. epithelial and vascular smooth muscle cells [270]. The CD1 molecule is structurally homologous to major histocompatibility complex (MHC) class I molecules due to their antigen-binding groove being defined by two α helices on an antiparallel β sheet (Figure 1.8). CD1a-d are involved in the presentation of lipid antigens to T cells at the cell membrane, whilst CD1e functions intracellularly to regulate glycolipid processing and loading of other CD1 isotypes [268]. CD1a-d can further be divided into group I (CD1a-c) and group II (CD1d) and characterized by differences in the size of the binding groove and the species which express each isoform (Table 1.3).

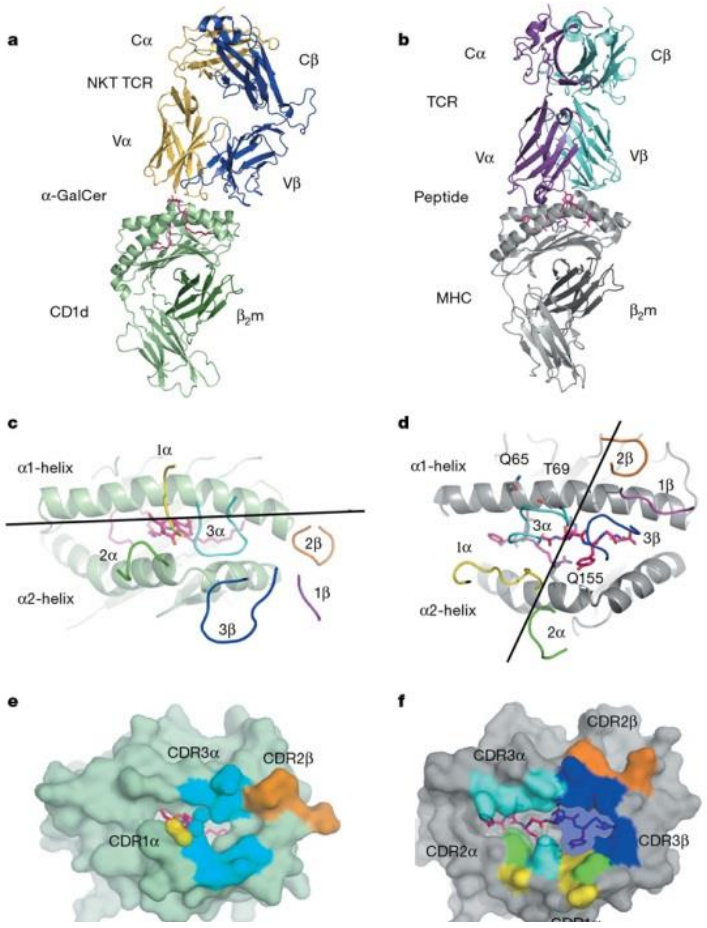


Figure 1.8 Interaction between CD1d and iTCR in humans

The way lipids interact with the CD1d binding groove and the iTCR, compared to how peptides are presented to a typical T cell receptor (LC13) via MHC class I. (a) iNKT TCR–α-GalCer – CD1d complex. (b) TCR-peptide-MHC class I complex. (c) Parallel docking mode of iNKT iTCR CDR loops onto human CD1d–α-GalCer. (d) Diagonal docking mode of a typical TCR interaction with pMHC-I. (e) Footprint of the iNKT cell iTCR on the surface of human CD1d–α-GalCer. (f) Footprint of LC13 on the surface of HLA-B8-FLR. Figure obtained from Borg et al. Nature 2007 [271].

CD1 family	Species expressed in	Binding groove size
CD1a	humans	Small
CD1b	humans	Large
CD1c	humans	Intermediate
CD1d	humans, mice, rats	Intermediate

Table 1.3 CD1 expression shows cross-species variation, with each isoform displaying differences in groove size and as a result, lipid-binding specificity [268].

CD1d is the most extensively studied of the CD1 molecules and in particular is highly expressed by CD11c+ DCs and marginal zone B cells. It is the only CD1 isoform present in mice and rats, and as a result the mechanisms involved in the processing and presentation of lipids via CD1d are extremely well characterised compared to the other isoforms. Early research showed that CD1d contained 2 deep hydrophobic pockets [272], suggesting that it may be involved in lipid antigen presentation. This was later confirmed in 1997 when the iNKT cell ligand α-galacytosylceramide (α-GalCer) was isolated from marine sponge [273]; the potency of which has rendered it an invaluable tool in studying the uptake of lipid antigens and presentation via CD1d [274].

The capture and internalization of lipid antigens by APCs occurs through a number of different mechanisms including the uptake of exogenous lipids by scavenger receptors which is described in Section 1.2.3 [275]. This is necessary for CD1d-mediated antigen presentation, with αGalCer uptake mainly being controlled by SR- A [276]. Due to their hydrophobic nature, lipids require the binding of proteins or lipoproteins for extracellular transport [276]. This may occur through the binding of ApoE to lipid complexes, and subsequent internalization via the LDLR [275]. Alternatively, B cells have been reported to internalize lipid antigens directly via the B cell receptor (BCR)[277]; a concept which may prove useful in the design of vaccines containing lipid-bound adjuvants [278].

Lipid antigen processing and presentation is controlled by a number of chaperones, which prevent inappropriate binding of lipids to the CD1d molecule (Figure 1.9). During this process, CD1d undergoes internalization and vesicular trafficking to endosomal and lysosomal compartments, where CD1d exchanges its ligand with lipids which are either endogenous to the cell, or obtained from exogenous sources through endocytosis [279]. CD1d then returns to the cell surface to present the lipid to the iTCR present on iNKT cells. Whilst the pathways involved in CD1d-mediated lipid antigen presentation have been well characterized, some of the mutant mouse models used develop associated lysosomal storage disorders, meaning that there are potential inaccuracies with the current theories [245].

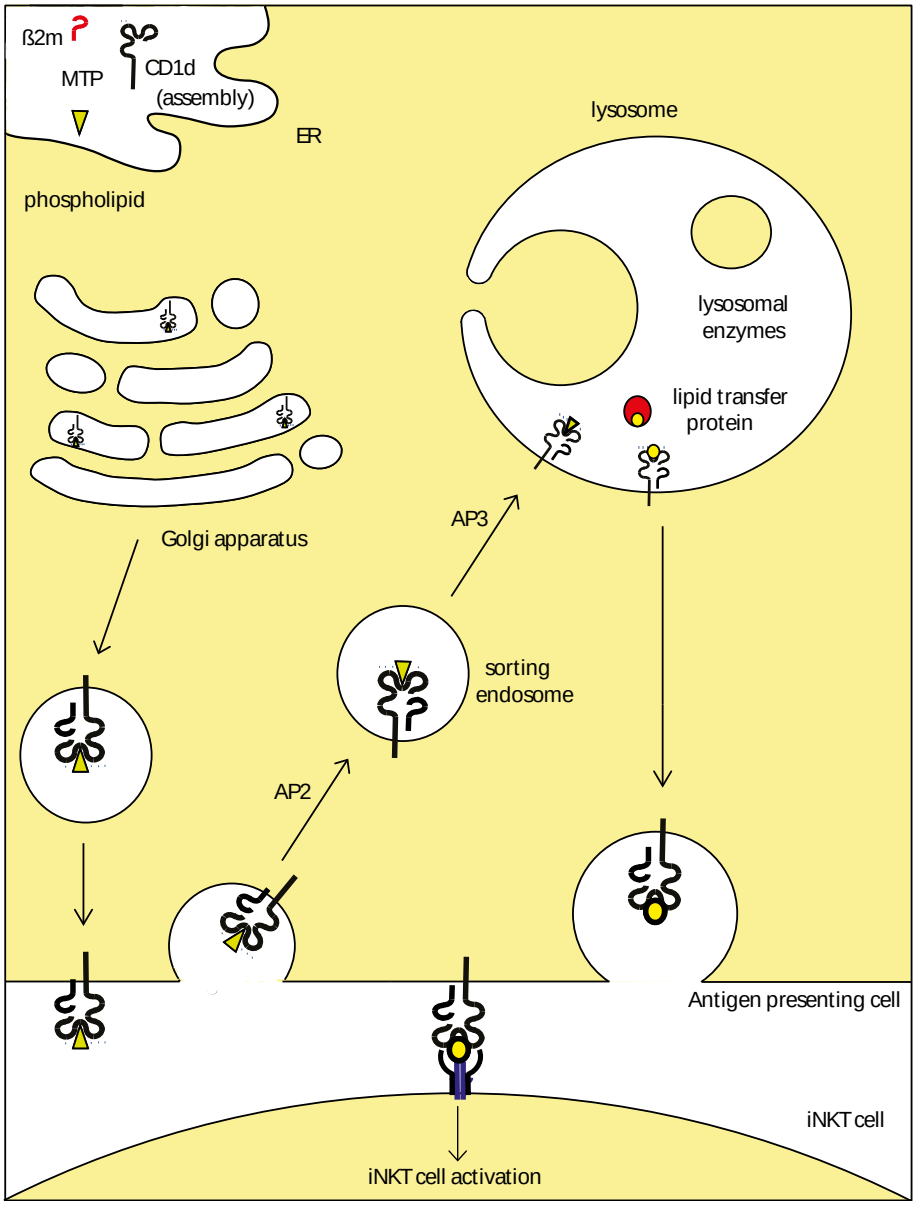


Figure 1.9 CD1d recycling and lipid loading

Assembly of CD1d takes place in the endoplasmic reticulum, where it forms a complex with β2m. Subsequently lipid ligands such as glycosylphosphatidylinositol are loaded onto CD1d, facilitated by microsomal triglyceride transfer protein. These complexes are trafficked to the cell surface through the Golgi network, before being internalized through the endosomes and lysosomes under the control of adaptor protein 2 (AP2) and AP3. Within the lysosomes, ER-derived lipids are exchanged for lysosomal lipid antigens, mediated by saposins and Niemann-pick type C2 protein (NCP2). CD1d then traffics back to the cell membrane, where presentation of lipid antigens to the invariant T cell receptor occurs [245].

Lipid antigens presented via CD1 are known as lipid-linked antigens. This is because, with the exception of mycolic acid, the hydrophilic head groups interact with the binding pockets of CD1 and are presented. Mass spectrometry has identified a range of chemical moieties that fit the CD1d binding groove, which are mainly categorized as sphingolipids and glycerolipids with one, two or four aycl chains [268] (Figure 1.10).

Most functional studies on iNKT cells have used the NKT cell agonist α-GalCer which differs from other activating lipids because it normally induces iNKT cell anergy following antigen presentation, whereas phenyl-containing analogues do not – even following repeated administration [280]. This suggests that the response to α-GalCer is very unique, and uncharacteristic of normal physiological antigens. α-GalCer induced iNKT cell anergy has been shown to occur through the egr2/3 pathway, which upregulates PD-1 and cbl-b in NKT cells [280]. α-GalCer can also induce other effects in vivo such as the expansion of myeloid derived suppressor cells (MDSCs) in mice, as well as upregulating the death receptors TRAIL and FASL and stimulating IL-33 secretion from Kupffer cells in the liver [280].

The potency of α-GalCer makes it a suitable candidate for a vaccine adjuvant to enhance immunity to cancers [281] and infections such as malaria [282] and is most effective when delivered mucosally to DCs [283]. This is due to the ability of iNKT cells to bridge the gap between innate and adaptive immunity, resulting in maturation of DCs by upregulation of CD40, CD80 and CD86 and secretion of proinflammatory cytokines such as IL-12 and TNFα [284]. Subsequent presentation of peptide antigen such as irradiated sporozoites or tumour antigen results in the activation of $CD4^+$ and $CD8^+$ T cells, which are specific for either Plasmodium or the particular tumour [281, 282]. In the case of malaria the use of α-GalCer adjuvants (such as KRN7000) can enhance immunogenicity by 1000 fold compared to just an anti-malarial adjuvant alone, for example[285]. The challenge now is to optomise the structure of iNKT cell ligands to generate a potent Th1-biasing cytokine response in the case of cancer and infections [283], whereas Th2-biasing ligands could prove more suitable for treating autoimmune diseases.

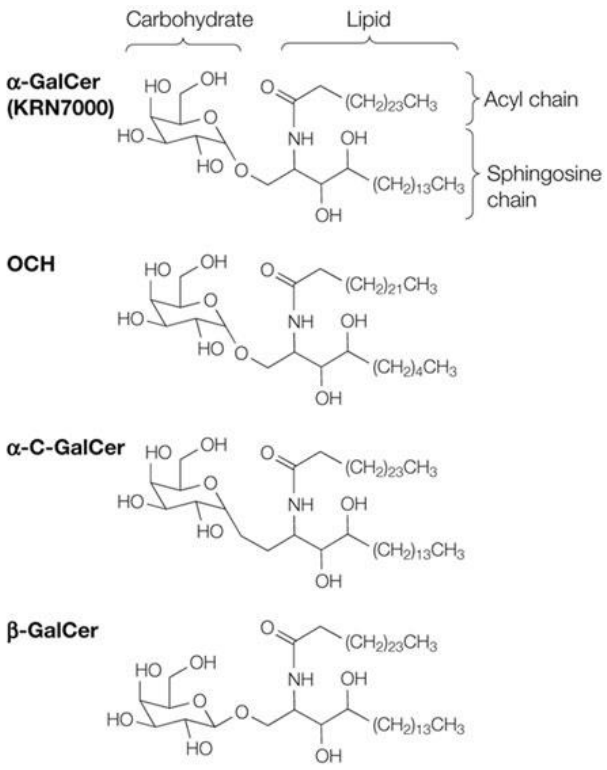


Figure 1.10 The chemical structure of α-GalCer and similar derivative analogues.

These molecules are glycosphingolipids characterised by a galactose carbohydrate linked to a ceramide lipid with variable length acyl and sphingosine chains. Figure from Van Kaer et al, Nature Immunology Reviews [274].

1.3.4 CD1d-independent iNKT cell activation

In addition to CD1d-iTCR interactions, iNKT cells can be activated indirectly through cytokines such as IL-12 which may be produced following TLR stimulation on APCs [286]. For example, Tyznik et al. showed that APCs unable to produce IL-12 fail to induce iNKT cell activation suggesting that in certain situations CD1d-iTCR interactions might only play a minor role [287]. Furthermore, activation of iNKT cells by IL-12 appears to be more effective in combination with small amounts of either IL-18 or type I IFN, and results in the secretion of mainly IFN-γ [288].

Whilst CD1d-iTCR interaction is still generally required for activation, it is thought that iNKT cells may be activated through cytokines alone if they've experienced previous iTCR engagement. This is due to the finding that infections with microbes lacking foreign lipid antigens such as *S. Typhimurium* were unable to induce expression of a reporter gene associated with iTCR-mediated activation [289]. Moreover, transfer of iNKT cells to a CD1d-deficient mouse or deletion of the iTCR α chain of iNKT cells has shown that "former" iNKT cells are still able to respond to inflammatory stimuli in the absence of CD1d-mediated lipid antigen presentation [289, 290]. iNKT cell activation through cytokines alone couold therefore represent an innate-like mechanism to reactivate iNKT cells during times of infection.

Activation of iNKT cells can also occur through the NK receptors CD161 and NKG2D; even in the absence of CD1d [291, 292]. CD161 recognizes lectin-like transcript 1 (LLT1), a marker present on activated leukocytes and DCs [222], whilst NKG2D recognizes ligands associated with MHC class-I, which are altered due to cellular stress [292]. Interestingly, iNKT cell activation via NKG2D has been shown to induce perforin and granzyme-mediated cytolytic effector functions, as well as functioning as a costimulatory molecule to enhance signaling downstream of the iTCR [293].

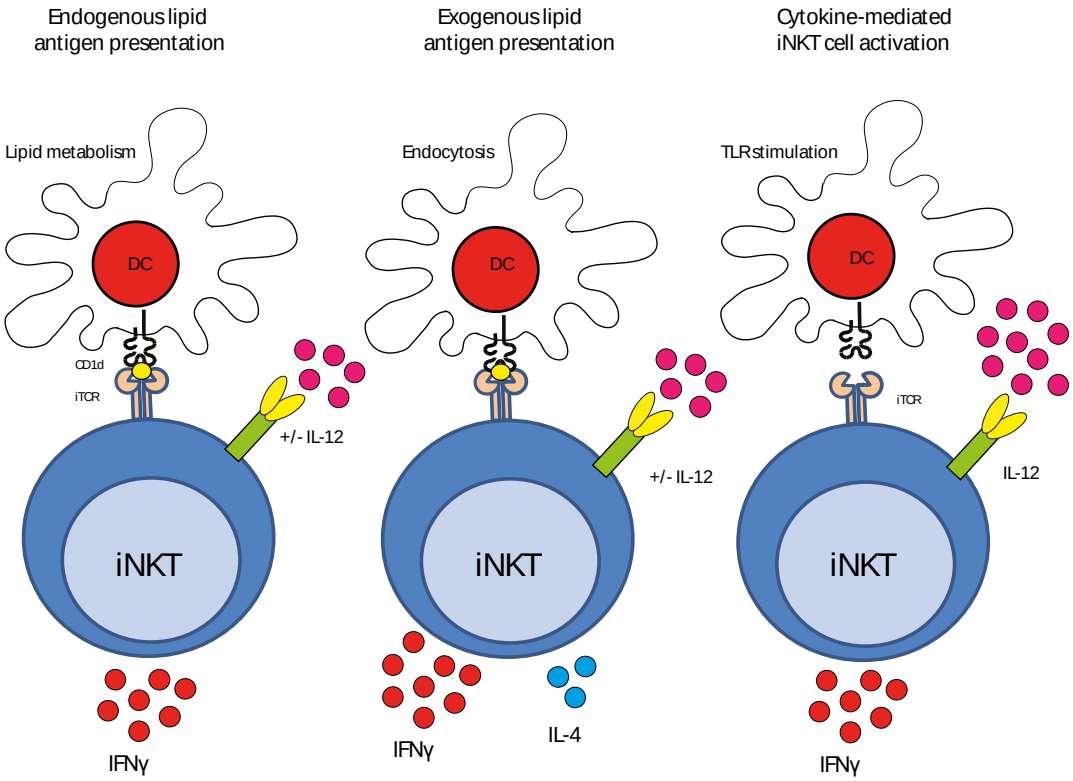


Figure 1.11 Different mechanisms of iNKT cell activation

iNKT cells can be activated either through presentation of endogenous or exogenous lipid antigens via CD1d, as well as through IL-12. Endogenous lipid presentation is the presentation of self-lipids, which may be altered through changes in lipid metabolism during times of cellular stress, and is associated with IFN-γ secretion. Alternatively, exogenous antigen can be endocytosed and presented directly, leading to both IFN-γ and IL-4 secretion. Finally, stimulation of TLRs on antigen presenting cells can result in the production of IL-12, which induces IFN-γ secretion by iNKT cells.

1.3.5 Exogenous lipid antigens

One would assume by nature that the lack of diversity in the iTCR restricts the possibilities of lipid antigens capable of recognition. However, this could not be further from the truth as the iTCR has been reported to bind a vast array of chemically distant antigens [294]. These include α-GalCer itself as well as analogues with the potential to skew the immune response towards a Th1 or Th2 response [295, 296]. These synthetic analogues known as "altered glycolipid ligands" are formed through minor alterations in the head group, acyl chain or sphingosine chain [285, 297-299].

The ability of iNKT cells to recognise different activating lipids can be seen through studying those of bacterial origin. Such foreign lipids can act in either a direct manner to induce iNKT cell activation, or through inducing the expression of endogenous iNKT cell ligands [300]. Microbes capable of directly activating iNKT cells include the Sphingomonadaceae family of Gram-negative bacteria, which express GSL-1 on their outer membrane [301]. GSL-1 is comprised of a mono- or oligo-saccharide bound to a ceramide lipid, with an α linkage on the 1' carbon, which strikingly resembles α-GalCer in structure [302]. However, GSL-1 is not as strong an antigen as α-GalCer due to the presence of a glucuronosyl head group in GSL-1 instead of a galactosyl head group, which alters its affinity for CD1d [303]. Another bacterium, *Bacteroides fragilis* produces a GSL named α-GalCerBF or GSLBf-717, which resembles α-GalCer apart from minor differences in their fatty acid and sphingosine bases, but unlike α-GalCer is thought to play an immunoregulatory role [304]. Recently, *Aspergillus fumigatus* was identified as the first fungal antigen for iNKT cells. Its antigen aperamide B was found to have a β-linked sugar (instead of α-linked), with modified acyl and sphingosine chains compared to α-GalCer, and has been implicated in promoting airway hypersensitivity [305].

Diacylglycerols (DAG), such as BbGL-II from *Borrelia burgdorferi*, have also been reported to activate iNKT cells [306]. There are several different forms of DAG with different affinities for CD1d, which is determined by the length and saturation of the acyl chain [307]. Compared to GSLs, fewer hydrogen bonds with the F' pocket of CD1d are required for DAG to bind, which enables more flexible arrangements [268]. This results in various orientations of the carbohydrate head groups, and therefore alternate levels of antigenicity [307].

Despite being largely uncharacterized, phosphotidylinositols have been shown to activate iNKT cells, but not through the conventional means of occupying both A' and F' pockets of CD1d due to the lack of a hexose sugar and two acyl groups. For example, during *Helicobacter pylori* infection, the host cell synthesizes a cholesterol-galactoside antigen, which can activate both human and mouse iNKT cells [308]. The LPPG antigen from the protozoan *Entamoeba histolytica* can also activate iNKT cells, the antigen moiety consisting of a diacylated phosphoinositol [309]. However, it is unknown whether the LPPG antigen activates iNKT cells directly, or through TLR2 stimulation and the subsequent induction of self-antigen.

1.3.6 Endogenous lipid antigen

During iNKT cell development, iNKT cells become activated in the thymus in the absence of foreign antigen, suggesting that there are iNKT cell-specific self-antigens in humans. Self-lipids also play a role during infection where they are produced due to external stimuli such as TLR activation. Although the identity of self-lipids is contentious, such iNKT cell antigens are thought to include isoglobotrihexosylceramide (iGb3), β-glucosylceramide (β-GlcCer) and β-galactosylceramide (β-GalCer).

iGb3 was first identified as a self-antigen for iNKT cells in mice as mice lacking the enzyme β-hexosaminidase, responsible for the generation of iGb3, had significantly fewer iNKT cells compared to wild type mice [310]. iGb3 has also been shown to be produced during *Salmonella typhimurium* infection, where it is thought to act as a danger signal following TLR4 activation [300]. However, the role of iGb3 has since been questioned by several studies showing that the decrease in iNKT cell function in these mice was attributable to altered lysosome function, having consequential implications on CD1d trafficking [311, 312]. Nevertheless, iGb3 itself is unlikely to be an important antigen in humans due to a single amino acid variation between human and mouse CD1d, which renders its binding ineffective in humans [313].

To identify other self-lipid antigens, Cox et al. eluted natural (endogenous) lipids from the CD1d molecule and observed lyso-phosphatidylcholine (LPC) to be antigenic for human cell lines [314, 315]. Importantly, LPC is produced by phospholipase A2 (PLA2) during

inflammatory conditions such as atherosclerosis, where it may play a role in altered iNKT cell responses [316]. Structural analysis of LPC-CD1d complexes provided insight into the particular amino acids required for antigen binding within the variable or CDR3β region, indicating that LPC may only serve as an antigen for a subset of iNKT cells [317].

A more recently identified self-antigen is β-GlcCer, which is abundant in the thymus and peripheral lymphoid tissues and accumulates in response to danger signals [318]. There have been several recent breakthroughs involving other physiologically relevant iNKT cell agonists, but mainly from a therapeutic perspective. For example, β-GlcCer has been shown to induce anti-tumour immunity as well as enhancing the immune response to Hepatitis B, in an iNKT cell-dependent manner [319, 320]. However, CD1d-bound β-GlcCer has been reported not to stimulate iNKT cells directly, suggesting that this is due to presentation of its derivatives instead [321], which was since confirmed by Brennan et al [322]. Additionally, certain lipids such as the ganglioside GD3 have dose-dependent inhibitory effects on iNKT cells, through a CD1d-specific mechanism [323].

Identification of such self-antigens has been extremely challenging due to differences in in lipid antigen uptake, intracellular transport, loading efficiency onto CD1d, stability of the CD1d-binding cleft, clustering of CD1d (e.g. in lipid rafts) and antigen half life [324]. Even once at the cell surface, individual CD1d-lipid complexes vary in the affinity for the iTCR; some may persist at the cell surface, whilst others rapidly decay, which can alter the dynamics of the iNKT cell response and lead to cytokine skewing [325]. However, since no physiologically relevant agonists as strong as α-GalCer have been identified in humans, further characterisation of iNKT cell antigens is necessary, particularly in disease states where altered self-antigens may be present.

The innate ability of such endogenous antigens to activate iNKT cells also poses the question of how autoreactivity is prevented under normal physiological circumstances. As extremely abundant and heterogeneous components of cell membranes, certain phospholipids have also been shown to bind CD1d and activate iNKT cells. Those identified include phosphotidylethanolamine, phosphatidylinositol and phospholipase E (PLE) [326-328]. However, it appears that in healthy individuals the signalling induced by endogenous antigens is far too weak to trigger iNKT activation [294]. In the disease state however, alterations in the biosynthesis or degradation of intracellular lipids may augment iNKT cell

signalling resulting in activation, cytokine secretion and proliferation [326]. In addition to providing early danger signals during infection, disrupted lipid metabolism may also contribute to autoimmune diseases such as SLE through enhanced iNKT cell signalling [318].

1.3.7 Molecular basis for CD1d-iTCR interactions

It is unsurprising that conventional T cells have a diverse TCR repertoire given the high rate of polymorphism in classical MHC molecules and the large diversity of possible peptide antigens capable of recognition. For this reason, iNKT cells were originally thought to have a single iTCR which lacked variability, enabling its interaction with non-polymorphic MHC-like molecules such as CD1d. This clearly is not the case from the finding that several different iTCR clones exist in the peripheral blood of both mice and humans [329]. However, it has been extremely challenging to assess the roles of different iTCR clones due to their relatively small size [330].

Compared to humans, the iTCR β chain in mice appears more diverse since it includes include Vβ2, Vβ7 and Vβ8 compared to only Vβ11 in humans [329]. However, sequencing of Vβ11 chains revealed extensive N-region diversity in multiple clones from a single donor demonstrating that these clones were derived from V-(D)-J recombination and subsequent positive selection, as with T cells [331]. Moreover, Van Schaik et al. found 16 clones to be expanded in healthy donors, indicating that these iTCR clones play an important role in normal physiological conditions [332].

In vivo expansion of iNKT cell clones is thought to occur through two different mechanisms: homeostatic iNKT cell expansion and antigen-specific iNKT cell expansion [330]. The former occurs in response to cytokines or other inflammatory signals which drive general expansion of all T cells expressing stimulatory receptors other than TCRs (e.g. cytokine receptor) [332]. The latter occurs in response to a specific antigen, and results in clonal expansion of iNKT cells containing an iTCR specific for that particular antigen [332]. This is achieved through differences in the interactions between the Vβ chain, CD1d and the lipid in question [324]. Crystal structures have revealed that the iTCR-α chain is predominantly responsible for the interaction with CD1d and lipid antigen (up to 65% of the buried surface area)[271], through

interactions mediated by the CDR1α and CDR3α loops. The iTCRVβ loop forms most of its contacts through the CDR2β loop, which solely binds the α1 helix of CD1d, and is dependent on the presence of two tyrosines and a glutamic acid [333]. Importantly, specific regions of the iTCR are required for binding of different CD1d-antigen complexes. For example, whilst the galactose moiety of αGalCer binds CDR1/3α, the inositol ring of phosphatidylinositol instead binds CDR1/2α but not CDR3α, and the trisaccharide head group of iGb3 interacts with all three CDRα loops [334].

Previous binding affinity measurements have shown that different TCRVβ compositions can drastically affect the kinetics and affinity of CD1d-α-GalCer complexes [334]. This has further been explored in mutational studies which found that the CDR2β loop in mice and the CDR3β loop in humans can influence the binding avidity of CD1d tetramers and various lipid antigens [335-337]. Though this mechanism, gradual alterations to the iTCR during clonal expansion enable iNKT cells to alter their range of antigen recognition with increased iTCR affinity [324]. In support of this, iNKT cells with both naturally occurring or genetically modified high affinity iTCRs are able to recognise less potent self-lipids (e.g. iGb3 and phospholipids), in addition to more potent lipids such as α-GalCer [335].

Other molecular interactions influence the stability of the CD1d-lipid complex, which for Gb3 occurs between the most distal galactose and the α2 helix of CD1d [338]. However, this is not the case for iGb3 and β-lactosylceramide (β-LacCer) which lack this residue rendering such interactions impossible [338]. As a result these lipids are much less potent, which supports previous research where substitution of the terminal sugar on iGb3 was able to influence antigenicity [339]. Furthermore, lipids with bulky head groups such as gangliosides are even able to block interactions between CD1d and the iTCR, possibly acting as a homeostatic mechanism, as demonstrated by Mallevaey et al. who observed that engineered high affinity iTCRs were only able to weakly bind CD1d tetramers loaded with gangliosides [335].

Studies have shown that β-linked antigens adopt a perpendicular orientation with protruded head groups [340-342], yet it is unclear how iNKT cells cope with such drastic structural changes. One theory puts it down to remodelling of the CD1d-antigen landscape [338], in a similar way to the “induced fit” hypothesis in conventional T cells. For example, published structures suggest that the iGb3 trisaccharide head group is pushed away from the iTCR

and instead lies flat against the α2 helix of CD1d to enable tighter interactions between CD1d and the iTCR [343]; further maintained through polar forces and van der Waals interactions. Similar remodelling of the iTCR has also been shown to occur in response to other lipids including β-LacCer and β-GalCer [338].

Whilst it is possible to quantify the strength of interactions between the MHC and the conventional TCR, it is more of a challenge for interactions between CD1d and the iTCR. In the case of T cells, the rate of association and dissociation between the TCR and the MHC antigen complex can be measured through surface plasmon resonance [324]. Evidence suggests that the outcome of MHC-peptide interactions with T cells can be clearly defined [344]; with low-strength interactions generally promoting Th2 differentiation and IL-4 secretion, and high strength interactions inducing a Th1 phenotype with IFN-γ. Similarly for iNKT cells, the study of Th2-inducing lipid antigens such as C:20 and OCH9 analogues have indicated that such antigens bind with lower affinity compared to α-GalCer, and have a higher rate of dissociation [334]. α-GalCer on the other hand, which predominantly induces IFN-γ secretion, binds with stronger affinity and leads to increased stability between CD1d and the iTCR [334, 345]. The ability to induce both IFN-γ and IL-4 secretion simultaneously could therefore be due to interactions of intermediate affinity, which are dictated by antigen structure.

Whilst antigen affinity and dissociation rate may prove a valuable measure to characterise iNKT cell responses, in reality it becomes extremely difficult to measure long term interactions (over 2h), since iNKT cells downregulate their receptors following prolonged stimulation, meaning they can no longer be tracked [324]. Furthermore, iNKT cells are known to contain preformed cytokine messenger RNA (mRNA) [346], which raises the question on whether iNKT responses depend more on the nature of their previously transcribed DNA, rather than the effects following antigen recognition in the periphery.

1.3.8 iNKT cell activation and responsiveness

When considering iNKT cell activation in response to lipid antigens, one should not only consider cytokine production by the individual cell, but the effect on the whole iNKT cell population and transactivation of other immune cell types. Following activation via the iTCR, iNKT cells rapidly proliferate and secrete a vast range of cytokines and chemokines, mediated by the presence of preformed mRNA (Figure 1.6) [234]. The response which ensues, results in immune cell transactivation either through cytokine and chemokine production, or through contact-dependent mechanisms. Reciprocal signals provided by the iNKT cell can also increase CD40, CD80 and CD86 expression on the APC, and in the case of B cells, can provide stimuli for class switching, expansion and memory cell formation.

The potential for iNKT cells in immunity is often underemphasized. Despite displaying phenotypic markers from the adaptive immune system, iNKT cells behave more like innate immune cells in terms of their proliferation and cytokine release upon contact with lipid antigens. iNKT cells exhibit poor memory and secondary responsiveness due to the fact that they become anergic following activation, through downregulation of the iTCR [234]. The reason for this is unknown but in healthy individuals may represent a negative feedback mechanism to limit the extent of the inflammatory response.

In humans, iNKT cells are either CD4 or CD8 positive whereas in mice most cells are CD4 single positive, with a small population expressing CD8α alone. Other surface markers expressed by iNKT cells include NK surface receptors such as CD161 in humans (NK1.1 in mice), as well as activated or memory T cell markers including CD44, CD69 and CD122 [234]. However, not all iNKT cells express NK cell markers, so in addition to the iTCR, the characterization of these cells must also rely on their functional characteristics [231].

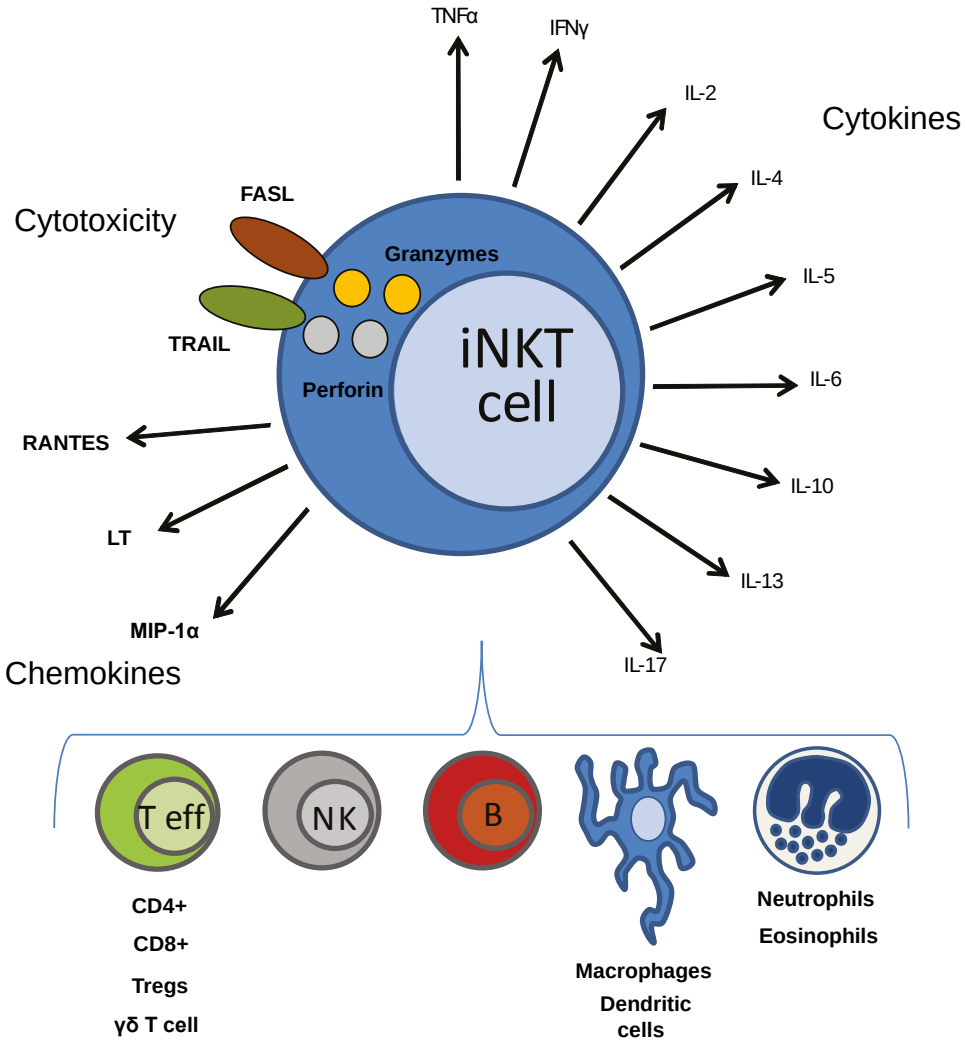


Figure 1.12 iNKT cell effector functions and transactivation of other immune cells

iNKT cells can produce a range of pro- and anti-inflammatory cytokines and chemokines, as well as carrying out cytotoxic functions mediated by FASL and TRAIL expression or Granzyme and Perforin release. iNKT cell cytokine release results in transactivation of many different cell types.

Studies have also characterized iNKT cells based on their CD4 and CD8 positivity. However, there seems to be little consensus on the roles of these subsets in human health and disease; perhaps due to low iNKT cell frequencies in certain individuals. A study by Lee et al found $CD4^+$ iNKT cells to have a Th2 cytokine response characterized by IL-4 and IL-13, whilst double negative and $CD8^+$ iNKT cells were found to have a Th1 pro-inflammatory cytokine response following stimulation with α-GalCer in mice [347]. This is supported by Crowe and colleagues who discovered $CD4^+$ iNKT cells to be tolerogenic, and $CD4^-$ iNKT cells to have better inducers of anti-tumor responses in animal models of cancer [348]. A different study showed that CD4+ NKT cells are producers of both Th1 and Th2 cytokines and can induce FasL following ionomycin stimulation.

In humans, Osada et al. found CD4+ iNKT cells in healthy individuals to be Th2-biased and characterized by IL-4, IL-10 and IL-13 secretion [349], whereas Takahashi et al. observed that CD8+ iNKT cells predominantly produced IFN-γ [350]. However, iNKT cells had been expanded using α-GalCer, meaning that data may not represent the *ex vivo* characteristics of these subsets in humans.

1.3.9 iNKT cell subsets

Functional iNKT cell subsets have been described including NKT1, NKT2, and NKT17, which mostly resemble CD4+ conventional Th1, Th2 and Th17 cells in terms of their transcription factor expression and cytokine production [351-354] (**Figure 1.13**). However, several differences have been observed. For example, whilst NKT1 cells express T-bet and produce IFN-γ, they are also capable of producing Th2 cytokines such as IL-4. NKT17 cells on the other hand express RORγt; a transcription factor which is not found in any of the other subsets. Alternatively, NKT1, NKT2 and NKT17 cells can be distinguished based on the level of PLZF expression, whereby NKT2 cells express the most and NKT1 cells express the least. The high expression of PLZF on NKT2 cells is essential for Th2 function, since PLZF has been shown to induce c-Maf, whose expression is necessary for both IL-4 and IL-10 production [355].

Similar to conventional T cells, iNKT cell subsets can also be characterized based on their surface marker expression. In mice NKT1 cells express NK1, yet NKT17 cells lack both NK1.1 and CD4 expression, and instead express CCR6 [351, 354]. iNKT cell subsets also vary in terms of their cytokine receptor expression whereby NKT1 cells high levels of IL-12R, whilst NKT2 cells express high IL-17Rβ, and NKT17 cells express high IL-23R. Most research has focused on NKT1 cells as they are the predominant subset in C57BL/6 mice [352], although other inbred strains have been described with a predominance for NKT2 or NKT17 cells.

Other iNKT cell subsets include NKT10 cells which exert immunoregulatory functions and are present at a very low frequency [356]. This particular subset is characterized by expression of several T cell markers and IL-10 production following activation by α-GalCer, as well as expression of transcription factor *Foxp3* [357]. Surprisingly, NKT10 cells were found not to downregulate their receptor following activation [357]. Interestingly, regulatory iNKT cells lack *PLZF* expression and instead express the transcription factor E4BP4, which is responsible for IL-10 production. NKT_{FH} cells have also been described, which are similar to T follicular helper cells in that they provide help for B cells to undergo immunoglobulin class switching and clonal expansion [358].

Interestingly, different NKT cell subsets have been shown to localize to different tissues in the body. NKT1 cells preferentially localizing to the liver [352]. NKT2 have been shown to reside in the thymic medulla and the T cell zone of lymph nodes, where their high STAT6 expression promotes IL-4 secretion, enabling them to condition neighbouring cells [359]. NKT17 cells reside in the lymph nodes, skin and lung [351].

Different routes of antigen administration can also influence the iNKT cell pool in various locations of the body. For example intravenous injection of α-GalCer stimulates systemic IFN-γ and IL-4 secretion by NKT1 cells in the liver and spleen, whereas oral administration of α-GalCer induced only local secretion of IL-4 by NKT2 cells in the mesenteric lymph nodes [359].

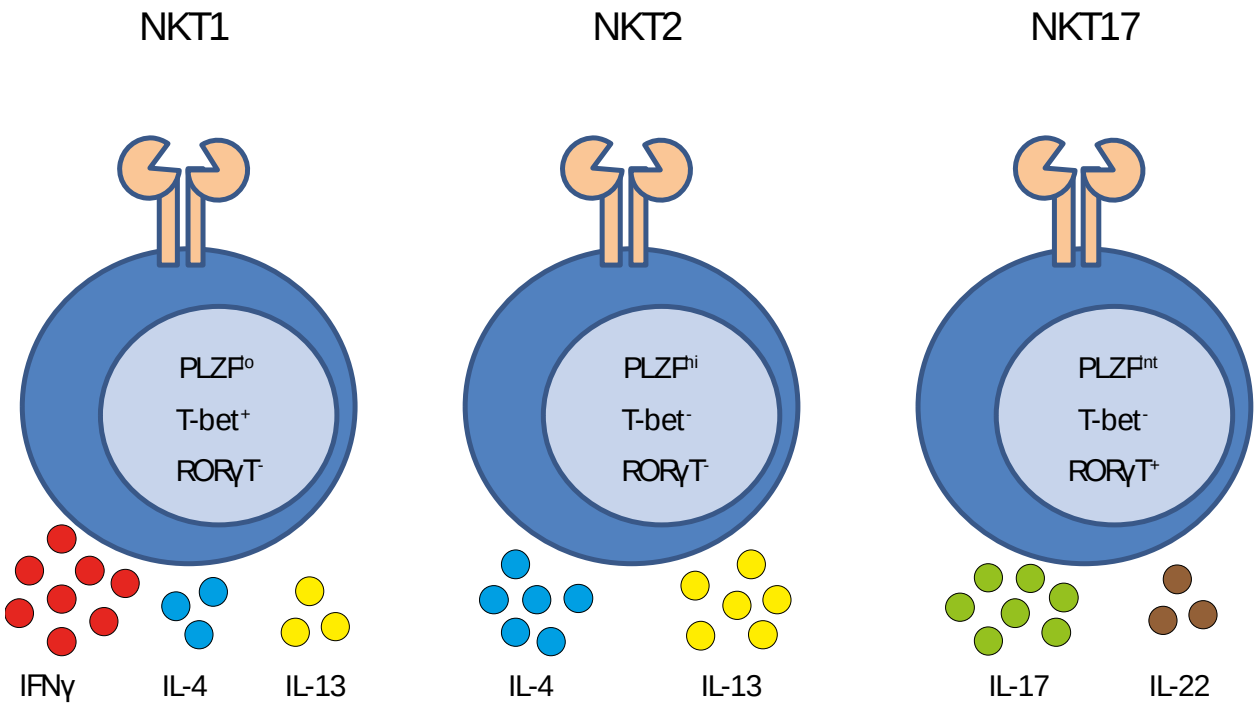


Figure 1.13 Main functional subsets of iNKT cells

The NKT1, NKT2 and NKT17 iNKT cell subsets are similar to Th1, Th2 and Th17 cells in terms of the cytokines that they produce. They can be defined based on their expression of transcription factors PLZF, T-bet and RORγt as well as differences in surface marker expression.

1.3.10 Determinants of iNKT cell responses

The innate ability of iNKT cells to respond in a proinflammatory versus a tolerogenic manner is governed by a number of factors including the lipid antigen being presented, the APC involved, and the association between CD1d and lipid rafts. The importance of antigen structure in determining iNKT cell responses was demonstrated by Aspeslagh et al. using a synthetic α-GalCer analogue naphthylurea-α-GalCer (NU-α-GalCer) [298]. Whilst α-GalCer and NU-αGalCer were both shown to induce IFN-γ and IL-4 secretion, α-GalCer was found to stimulate greater amounts of IL-4, whereas NU-α-GalCer predominantly stimulated IFN-γ secretion [360]. Such studies demonstrate that iNKT cell responses can be drastically altered by even minor variations in the structure of the lipid (such as the addition of a napthyl or urea group).

It is thought that different cell types may determine the extent of iNKT cell cytokine production and transaction of other immune cells in vivo through [361]. These include differences in expression of CD1d between B cells, monocytes and dendritic cell subsets, which may each have different influences on the iNKT cell response which follows. This is particularly evident in the case of α-GalCer, where use of a microparticle-formulated α-GalCer-based cancer vaccine enabled the targeting to dendritic cells instead of B cells, preventing the anergy normally associated with iNKT cell responses [362]. Arora et al. have suggested that different dendritic cell subsets contribute to altered iNKT cell function, with a predominant role for the $CD8\alpha^+$ $DEC205^+$ DC subset, which are capable of presenting multiple forms of α-GalCer to induce either a Th1 or Th2 response [361].

Furthermore, different B cell subsets have been shown to have an influence on iNKT cell function. CD1d is expressed on immature, naïve and mature B cells, as well as plasma cells. Blair et al. demonstrated that immature B cells defined by $CD19^+CD24^{hi}CD38^{hi}$ express higher levels of CD1d compared to naïve, mature and memory B cells [363]. In addition, immature B cell population were found to be more important than mature or memory B cells for both iNKT cell proliferation and cytokine secretion [233].

1.3.11 Lipid rafts and CD1d-mediated antigen presentation

Lipid rafts are highly ordered areas of the plasma membrane, which play a role in regulating immune cell activation and function. Cholesterol constitutes approximately 25% of the plasma membrane of human cells, where it aggregates due to its rigid hydrophobic properties to form lipid raft microdomains [364]. Differences in the cholesterol composition of cell membranes determine the fluidity of cell membranes, with cholesterol acting to stabilise cell membranes and to reduce permeability of polar molecules [364]. Several methods have been widely used to characterise both the composition and properties of lipid rafts. Visualisation of lipid rafts relies on the use of molecular probes; commonly cholera toxin B subunit (CTB) binding to the glycosphingolipid GM1, which is particularly abundant in cell membranes [365]. Filipin, which is a fluorescent mixture of antibiotics, recognises membrane cholesterol [366].

Previous work by Jury and colleagues has shown that lymphocytes from SLE patients have differences in lipid raft composition; namely increased levels of glycosphingolipids and cholesterol in the membrane of lymphocytes (Figure 1.14) [367]. These differences influence the position of key signaling molecules, such as lymphocyte-specific protein tyrosine kinase (Lck), linker for activation of T cells (LAT), Zeta-chain-associated protein kinase 70 (ZAP-70) and CD45 in T cells, or Lck/Yes novel tyrosine kinase (Lyn) expression on B cells [368-370]. Such signaling molecules are critical for regulating immune cell function, meaning their dysregulation in SLE increases cell susceptibility to prolonged activation and hyper-responsiveness [365, 371, 372]. Evidence for a defect in lipid metabolism and cell signalling in SLE is strengthened by the finding that atorvastatin has the capacity to normalize Lck and CD45 distribution, as well as lipid raft-associated signaling in T cells from SLE patients [373].

Lipid raft expression can be influenced by alterations in lipid biosynthesis, for example through LXR activation which acts to regulate the cholesterol content, and therefore stability of plasma membranes [374]. This can have functional consequences regarding immune cell activation, potentially influencing iNKT cell activation. This will be discussed in section 1.3.12.

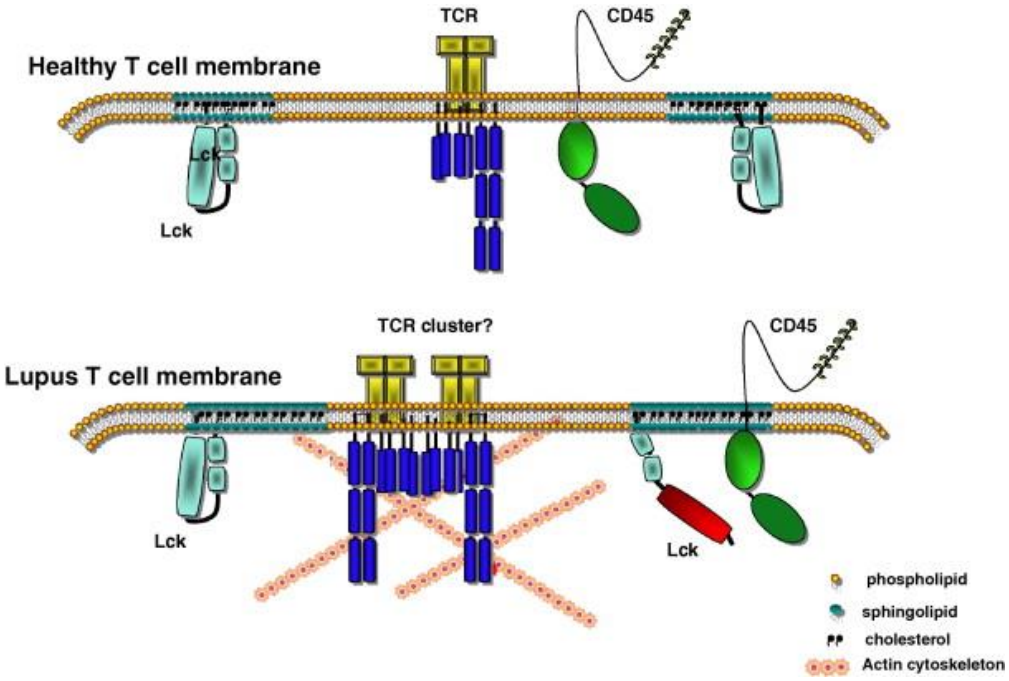


Figure 1.14 Role of lipid rafts in T cell activation in healthy donors and Lupus patients

Changes in the membrane of lupus T-cells may associate with their autoimmune phenotype. The higher cholesterol and GM1 content of lupus T-cell membrane may result in larger and/or less mobile lipid rafts. This in combination with the increased association of CD45 with lipid rafts, higher Lck activity, and changes in the actin cytoskeleton seen in these cells could reduce the threshold for activation. Figure from Karbourdis et al, 2008 [375].

CD1d association with lipid rafts was first described by Lang et al. in 2004 [376], suggesting that lipid rafts may play an important role in the presentation of glycolipid antigens to iNKT cells. Moreover, disruption of lipid rafts ex vivo using the short chain ceramide C2-Cer was found to inhibit co-localisation between murine CD1d and the lipid raft surrogate marker GM-1 [376]. This resulted in subsequent inhibition of IL-2 secretion following iNKT cell presentation, which was especially evident at low concentrations of α-GalCer (1000ng/ml) [376], thus suggesting an ability for lipid rafts on APCs to regulate iNKT cell responses.

The importance of lipid rafts is epitomized by the finding that different analogues of the iNKT cell agonist α-GalCer can induce different levels of colocalisation between CD1d and lipid rafts. Using the monoclonal antibody L363, which recognizes CD1d-α-GalCer complexes, Im and colleagues demonstrated that α-GalCer C:20 induces strong colocalisation between CD1d-α-GalCer and lipid rafts, in contrast to α-GalCer C:10 where CD1d-α-GalCer was excluded from lipid rafts [377]. Moreover, treatment of DCs with MβCD, in addition to different α-GalCer agonists showed that cholesterol rich plasma membrane lipid rafts were required for the Th1 and Th2 iNKT cell cytokine response to αGalCer C:26, but were dispensable for the Th2-biased response to α-GalCer C:10 and α-GalCer C:20 [377]. This is supported by earlier work in mice showing that lipid raft colocalisation promoted Th1 cytokine release, whilst lipid raft exclusion induced Th2 anti-inflammatory responses (Figure 1.15) [296].

Since iNKT cell responses are determined by downstream signaling, it seems logical that altered CD1d and lipid raft distribution results in differential downstream effects. Indeed, disruption of raft microdomains by β-Glycosphingolipids was associated with decreased STAT1 and STAT3 levels in iNKT cells, an effect which was shown to be CD1d-dependent [378]. However, it remains to be seen whether this is also the case in humans.

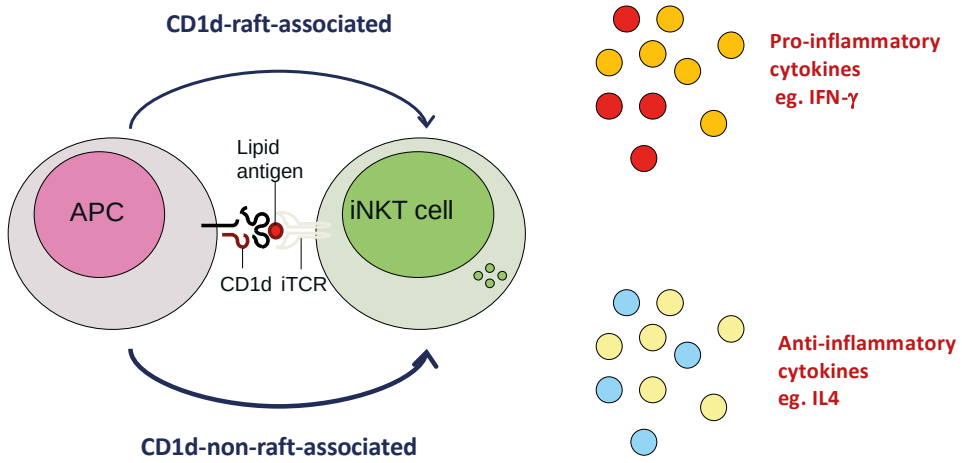


Figure 1.15 The role of lipid rafts in CD1d-mediated lipid presentation to iNKT cells

Lipid rafts on APCs play a role in regulating iNKT cell activation and responsiveness. The incorporation of murine CD1d into lipid rafts has been shown to induce proinflammatory cytokine secretion, whilst lipid raft exclusion induces the secretion of anti-inflammatory cytokines.

1.3.12 Lipid biosynthesis and CD1d-mediated antigen presentation

Little is known about the consequence of altered lipid metabolism on iNKT cell number and function. Through studying the lysosomal storage disorder Niemann-Pick type C2 disease, where patients are deficient in enzymes controlling GSL degradation, we are beginning to understand how lipid metabolism can influence presentation to lipid-reactive T cells. Schumann et al. demonstrated that iNKT cell development is impaired in mice deficient in the lysosomal lipid transfer protein NPC2 or the enzyme β-galactosidase [312]. Moreover, these NPC2 knockout mice displayed an altered TCR repertoire and CD4 distribution, which may be a consequence of differential CD1d-lipid antigen activation complexes [312]. However, a more recent study found no numerical or functional deficiencies in iNKT cells from Niemann-Pick disease patients, which they attributed to differences in iNKT cell development between mice and humans [379].

LXR stimulation has been shown to influence the position of lipid rafts on T cells; a factor contributing to T cell dysfunction in SLE [380]. As previously described, lipid rafts are required for signalling in a range of immune cell types. Altered lipid raft composition is characteristic of SLE where increased levels of Gb3 and GM1 lead to hyper-responsiveness. Use of an LXR antagonist was found to restore GSL metabolism and T cell function in SLE patients, indicating a dysfunction in LXR signalling [380]. It is unknown however, whether altered LXR signalling occurs in other cell types in SLE, as well as the effect on CD1d-mediated presentation to iNKT cells.

PPARγ is another regulator of lipid biosynthesis, which could influence CD1d-mediated antigen presentation through multiple mechanisms and thus contribute to atherosclerosis in SLE patients. For example, PPARγ has been shown to regulate expression of the class B scavenger receptor CD36, which consequentially results in enhanced oxLDL uptake and foam cell formation [381]. In line with this, PPARγ stimulation is thought to increase intracellular recycling necessary for lipid presentation. This is evident in DCs where PPARγ regulates the expression of the lysosomal protease Cathepsin D, which is responsible for the production and maturation of saposins, and is also required for lipid antigen processing [382]. Furthermore, global gene analysis has indicated that PPARγ activation of DCs is associated with increased expression of the CD1 proteins, including CD1d, required for presentation to iNKT cells [383]. However, the effect appears to be indirect through

activation of the retinoic acid receptor (RAR), and it is unknown whether this mechanism also exists in B cells and monocytes.

This association can also between PPARγ and CD1d can also be seen in patients with severe obesity, which have been shown to have defective expression of both PPARγ and CD1d expression on adipocytes [384]. Since adipocytes can present to iNKT cells, this was also associated with reduced iNKT cell numbers in adipose tissue [384]. It is unknown whether such mechanisms could influence iNKT cell frequency in atherosclerosis.

Alternatively, PPARγ can regulate LXRα expression on macrophages, which upon activation induces ATP Binding Cassette A1 (ABCA1) expression to increase lipid export from the cell [385]. In a review by Mosser and Edwards, it is hypothesised that such differences may be attributable to macrophage plasticity, depending upon whether they are closer in phenotype to the classically activated M1 macrophage or the alternatively activated M2 macrophage [386], which play immunogenic and regulatory roles respectively.

Other factors which play a role in the control of CD1d-mediated lipid presentation include Vitamin D, which has been implicated in iNKT cell development in mice due to the requirement of the vitamin D receptor (VDR) whereby knockout of VDR was found to significantly reduce iNKT cell numbers in mice [387]. Importantly, such iNKT cells were further found to be hypo-responsive suggesting that vitamin D is required for iNKT cell functional activity. This is supported by Waddell et al. who showed that Jα18-/- mice lacking iNKT cells were not protected against experimental autoimmune encephalomyelitis (EAE) upon treatment with vitamin D, to the same extent as their wild type counterparts, thus indicating that iNKT cells mediate the protective effects of vitamin D in EAE [304].

1.3.13 Co-stimulatory molecules and iNKT cells

In addition to the signal provided by CD1d, costimulatory molecules are important for maintenance of the immune synapse for lipid presentation and iNKT cell activation to occur. Unlike conventional T cells, costimulatory molecules have been proposed to play less of a role in iNKT cells due to their lower threshold for activation [388]. In mice, interactions between CD28 and B7.1/B7.2 are important for intrathymic maturation as it induces the expression of activation markers CD44, CD69 and CD122 as well as transcription factor T-bet to regulate IFN-γ production [389, 390]. However, it appears that unlike conventional T cells, CD28 is not critical to iNKT cell function since CD28 and B7 disruption in α-GalCer-treated mice had no impact on intracellular cytokines produced by iNKT cells despite a reduction in serum cytokines overall [391]. Similarly, the coinhibitor (CTLA-4) is not thought to play a role in the iNKT cell proliferative response to α-GalCer [392], suggesting that iNKT cells become anergic and self-regulate themselves through an alternative mechanism.

iNKT cells have been shown to constitutively express inducible costimulator (ICOS)[393], expression of which is further upregulated upon α-GalCer treatment, and unlike conventional T cells is independent of CD28 engagement [394]. ICOS is essential for iNKT cell survival in the periphery with a lack of ICOS signaling resulting in reduced iNKT cell numbers in the mouse spleen and liver [390, 393]. Inhibition of the ICOS-ICOSL signaling pathway results in decreased IFN-γ, IL-4, IL-5, IL-10 and IL-13 production, suggesting an important role for ICOS in the rapid cytokine production characteristic of iNKT cells [393, 394].

Expression of CD40L on iNKT cells is induced mainly in CD4+ populations following presentation of glycolipid antigen by APCs [395]. CD40L is necessary for providing help to B cells to enable maturation and class switching of antibodies [396], as well as enabling crosstalk with DCs to stimulate IL-12 production [397]. IL-12 subsequently acts in a positive feedback manner to skew iNKT cells towards an NKT1 type response, characterized by IFN-γ production. This association between CD40L and the NKT1 response has been characterized using mouse models of autoimmunity, where treatment with a CD40 agonistic antibody induced a Th1 response *in vivo* and exacerbated EAE [398].

1.3.14 iNKT cell signalling

There is evidence to suggest that the signaling mechanisms used by iNKT cells are different to conventional T cells. For example IL-17A and IL-22 production in αβ T cells depends upon transcription factor interferon regulatory factor (IRF)-4, yet IL-17A and IL-22 secretion by iNKT cells is normal in *IRF4* deficient mice, indicating the presence of an alternative pathway, which is thought to rely on STAT3 [399].

iNKT cell responsiveness is mostly associated with signalling through mTOR, which is an important serine/threonine kinase pathway controlling cell growth proliferation and survival [400]. Deletion of the mTOR adaptor molecule Raptor in mature iNKT cells using *Raptorf/f-CreER* mice treated with tamoxifen resulted in impaired iNKT cell expansion and decreased IFN-γ and IL-4 production following α-GalCer stimulation [264]. Interestingly, Raptor deficient mice have altered ratios of iNKT cell subsets where NKT1 cells are decreased, NKT2 cells are increased, and NKT17 iNKT cells remain the same [400]. On the other hand, mice deficient in the mTOR adaptor molecule Rictor show normal NKT1 cell frequencies, but decreased NKT2 cell frequencies [401], together indicating that mTOR signalling is important in the differential expression of iNKT cell transcription factors [400].

Other signalling pathways found to be involved in iNKT cell responses following stimulation include ERK, JNK and p38, which play differential roles following α-GalCer stimulation [402]. In particular, ERK was shown to play a positive role in iNKT cell signalling, whilst p38 was found to be a negative regulator. More recently, work on primary iNKT cells has confirmed this finding that mTOR, ERK and JNK play crucial roles in regulating cytokine production following activation [403], yet whether any of these pathways are more important remains to be seen.

iNKT cell signalling can also be influenced by the expression of suppressor of cytokine signaling (SOCS) genes, which inhibit the activation of inflammatory genes. In addition to inducing IL-10 production by APCs, *SOCS3* overexpression was found to reduce iNKT cell activation, evident through a reduction in CD69 expression and decreased IFN-γ and IL-4 secretion [404].

1.3.15 iNKT cells and B cell-mediated responses

iNKT cells participate in the regulation and maintenance of humoral (or antibody mediated) immune responses by B cells in a similar way to conventional T cells. This is evident from the finding that α-GalCer injection in mice was able to activate B cells in an IL-4-dependent manner and induce IgE secretion [405]. This was associated with acquisition of a Th2 phenotype in conventional T cells. Furthermore, iNKT cells have been shown to participate in models of allergy in mice such as ovalbumin (OVA)-induced asthma [406]. Here, iNKT cell deficient TCRJα18$^{-/-}$ mice showed impaired IL-4, IL-5 and IgE production in response to OVA immunization, whilst iNKT cells from wild type mice were found to be more activated due to increased CD69 expression [406].

The mechanisms by which iNKT cells provide help to B cells (e.g. to enable class switching or memory formation) occur through cognate and non-cognate interactions. It was initially thought that iNKT cells promoted B cell proliferation and immunoglobulin production in a CD1d-dependent manner, as demonstrated in vitro using anti-CD1d [407]. Furthermore, this effect on B cells was present even in the absence of α-GalCer, indicating that self-lipids could also be responsible for this phenomenon [407]. More recently, through studying B cell chimeras lacking CD1d *in vitro*, it is now clear that iNKT cells can provide help to B cells through in a non-cognate manner [396]. This is thought to occur through presentation by other APCs, and subsequent activation of T follicular helper cells by iNKT cells. The long term consequences are stronger interactions between B cells and conventional T cells to induce formation of B cell memory [408].

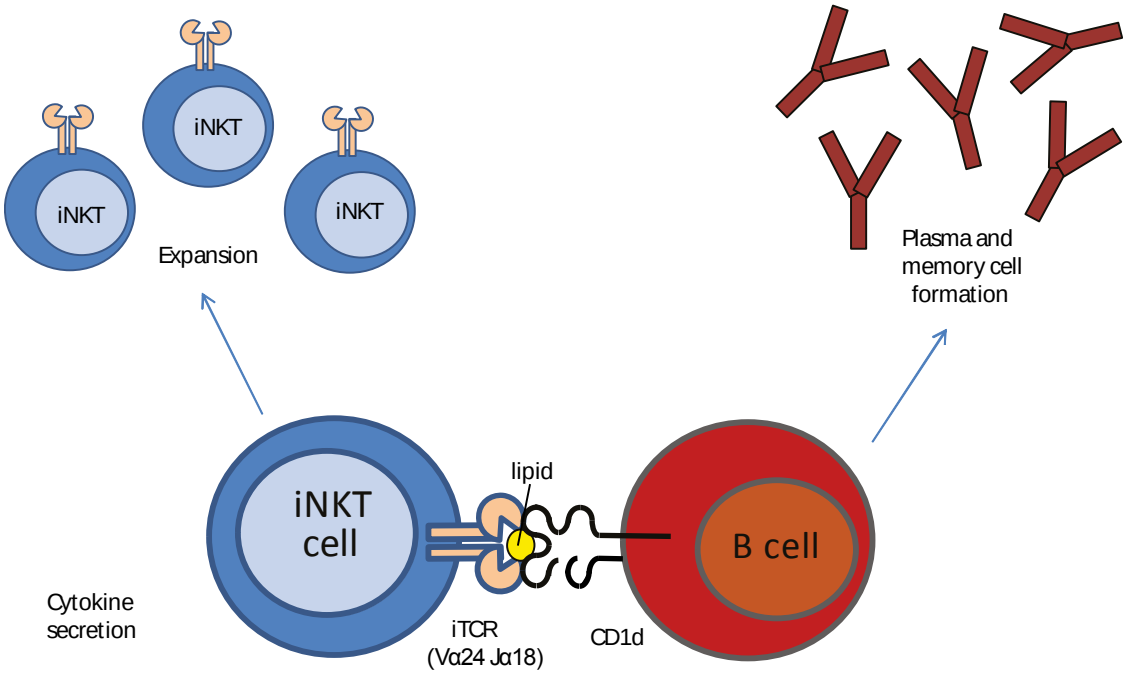


Figure 1.16 The role of B cells in iNKT cell expansion

Upon lipid antigen presentation to iNKT cells, there is a reciprocal signal inducing various effector functions in the antigen presenting cell. In the case of B cells (shown), this includes differentiation into memory and plasma B cells and the release of antibodies. Based on figure by previous PhD student, Anneleen Bosma.

1.3.16 iNKT cells and immune regulation

The fact that iNKT cells are more numerous in healthy individuals compared to in autoimmunity suggests that they play a role in immune regulation under normal homeostatic conditions [409]. This regulatory function of iNKT cells is evident in the autoimmune disease type I diabetes. Non-obese diabetic (NOD) mice, which spontaneously develop the disease, can be protected from type I diabetes by adoptive transfer of iNKT cells [410]. Furthermore, iNKT cells have been shown to impair differentiation of autoreactive anti-islet $CD4^+$ and $CD8^+$ T cells in mice, possibly through the recruitment of tolerogenic DCs [411, 412]. Instead, these T cells are rendered anergic, unable to destroy pancreatic cells.

A recent study suggests that under normal circumstances iNKT cells play a regulatory role which prevents obesity and the development of the metabolic syndrome. Martin-Murphy et al. demonstrated that $CD1d^{-/-}$ mice (which lack NKT cells) are more susceptible to weight gain and fatty liver following consumption of a high fat diet [413]. This was further associated with the induction of inflammatory genes in the liver as well as a decreased metabolic rate [413].

Several studies have shown that iNKT cells from peripheral blood can acquire *Foxp3* expression and an immunosuppressive phenotype upon exposure to TGFβ, IL-10 or the mTOR inhibitor rapomycin [414, 415]. However, it appears that Foxp3 expression alone is not required for the suppressive function induced by IL-10, as measured according to the proliferation of responder T cells [416]. Instead, rapamycin is responsible for the suppressive capacity of iNKT cells due to its involvement in the nuclear localization of *Foxp3*. The induction of *Foxp3* expression upon culture with IL-10 and rapamycin is also associated with the acquisition of Treg markers CD25 and CTLA-4 [221, 416]. However, Helios expression was only observed only in iNKT cells cultured in the presence of rapamycin, indicating a role for mTOR inhibition in the acquisition of natural Treg properties.

This could be particularly detrimental in cancer immunotherapy where mTOR inhibitors have been used [417, 418], since conversion of iNKT cells to suppressive nTreg-like cells could make them less effective at carrying out anti-tumour responses [419]. On the other hand, mTOR inhibition could be useful as a potential therapeutic for autoimmune diseases due to

the creation of a more suppressive environment characterized by decreased iNKT cell activation.

Another regulatory function of iNKT cells involves the crosstalk to pDCs through secretion of IL-10 in the pancreatic lymph nodes [420] (Figure 1.17). This promotes TGFβ secretion by pDCs, which promotes the passage of naïve $CD4^+$ T cells into $Foxp3^+$ Tregs (Figure 1.9). This tolerogenic influence on dendritic cells is supported by the finding that treatment of NOD mice with α-GalCer can increase tolerogenic mDC accumulation in pancreatic lymph nodes, which is associated with an increase in iNKT cell numbers [185].

iNKT cell crosstalk with Tregs has also been shown in murine models allogenic hematopoietic stem cell transplantation, where purified $CD4^+$ iNKT can prevent graft versus host disease (GVHD) through expansion of donor $CD25^+Foxp3^+$ Tregs [421]. This is supported by Pillai et al. who found iNKT cell-mediated Treg expansion to be IL-4-dependent [422]. Moreover GVHD protection was lost in donors lacking Tregs or in $Jα18^{-/-}$ recipient mice which are deficient in iNKT cells, suggesting a requirement for both iNKT cells and Tregs in protection from GVHD [422].

However, following iNKT cell activation, IFN-γ has been shown to suppress iTreg development in vivo [423]. iTregs are generated in the periphery from naïve $CD4^+$ precursors, which gain CD25 and Foxp3 expression upon activation through CD3 and CD28, and stimulation with TGF-β and IL-2 [424]. They are characterized by secretion of the immunosuppressive cytokines TGFβ and IL-10, as well as IFN-γ [425].

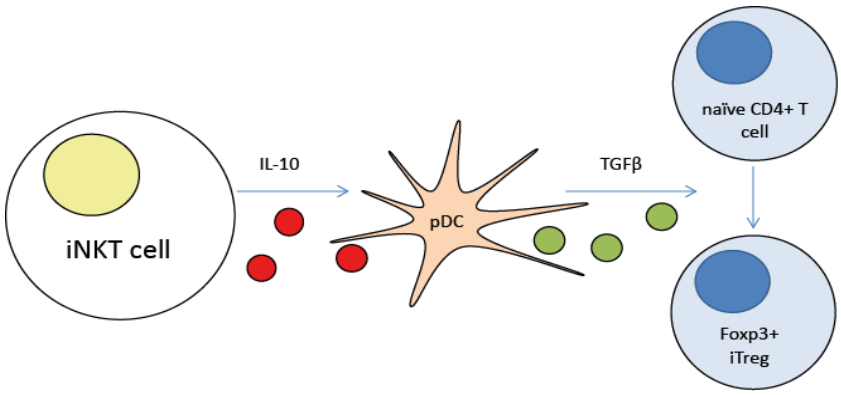


Figure 1.17 Indirect iNKT cell mediated iTreg induction via plasmacytoid dendritic cells

IL-10 production by iNKT cells can induce TGFβ secretion by plasmacytoid DCs, which in turn induces differentiation of CD4+ T cells into inducible Tregs (iTregs), thus promoting a regulatory environment.

Furthermore, iNKT cells have been shown to interact with Tregs through the secretion of IL-2 and IL-4, which induces Treg expansion, as well as regulating Treg homing to the liver [426]. Tregs can also act in a reciprocal manner to inhibit iNKT cell proliferation and cytokine production in vitro. This iNKT cell priming of Tregs was found to be dependent upon cell-cell contact and subsequent IL-10 secretion from Tregs, and did not require the presence of APCs [360, 427]. This was recently confirmed in a study by Huijts et al. where iNKT cells couldn't exert their suppressive function when cultured in a transwell system with responder T cells [416]. It is unknown however which iNKT cell receptors are responsible for contact-dependent suppression of responder T cells, although this is not thought to occur via CTLA-4 or latency associated peptide (LAP) due to comparable expression on both suppressive and non-suppressive iNKT cells [416].

Activation by strong agonists such as α-GalCer and NU-α-GalCer appeared to limit the suppressive ability of Tregs, in comparison to weaker agonists such as bacterial DAGs and activation by IL-12 and IL-18 secretion [360]. There was no preference between $CD4^+$ and $CD4^-$ subsets regarding inhibition of iNKT cell cytokine production, although IL-4 was more prone to inhibition compared to IFN-γ [360]. Tregs can also able to exert negative feedback indirectly towards iNKT cells through the production of TGFβ, which activates SMAD-2, -3, and -4 in APCs and has been shown to inhibit CD1d-mediated lipid antigen presentation [428].

In type I diabetes for example, both *in vivo* and *ex vivo* data from diabetes susceptible NOD mice has shown that repetitive stimulation of iNKT cells by α-GalCer is protective against disease onset [304]. This effect is due to the induction of tolerance following repetitive (but not single) α-GalCer stimulation, whereby iNKT cells induce tolerogenic dendritic cells capable of converting naïve T cells into Tregs [304]. This occurs through the upregulation of CXCR3 expression on Tregs, which enables their recruitment in the pancreas thus preventing type 1 diabetes onset.

iNKT cells can also induce immune regulation through crosstalk with myeloid derived suppressor cells (MDSCs). This is demonstrated by Parekh et al. in the context of autoimmunity in the central nervous system, where the mouse model EAE was used. iNKT cell activation in EAE mice was associated with MDSC expansion and enrichment of

immunosuppressive markers including iNOS and Arg1 [429]. In contrast, other studies have shown that iNKT cells can suppress MDSC development during influenza [430], and that activated iNKT cells can reduce the immunosuppressive capacity of MDSCs in the tumor environment [431]. Such studies demonstrate that other factors in the local environment may be driving altered iNKT cells.

Immunoregulation can also be induced by iNKT cells through communication with macrophages, which can have an influence of the balance between the pro-inflammatory M1 or classical macrophage and the anti-inflammatory M2 or alternatively activated macrophage. This has been demonstrated during Coxsackievirus B3 infection in mice where the iNKT cell response was associated with increased numbers of M2 macrophages [432]. However, the balance was shifted in favour of the M1 macrophage in NKT cell knockout mice, thus demonstrating a requirement for iNKT cells in M2 macrophage polarisation [432].

Likewise, in the mouse model EAE iNKT cell deficient mice were found to have fewer M2 macrophages and worse prognosis during early stages of the disease [433]. Similar results have also been observed in obesity, where M2 macrophage polarisation was shown to be IL-4 and STAT6 dependent [229].

1.4 iNKT cell number and function in SLE

1.4.1 iNKT cells in autoimmunity

Due to their dual role in immune activation and regulation, it is unsurprising that iNKT cells are dysfunctional in many autoimmune disorders. Reduced iNKT cell frequency has been reported in SLE, psoriasis, RA and myasthenia gravis [434]. This is particularly striking in SLE, where various methods have been used to confirm this association. Quantification of Vα24Jα18 mRNA expression indicated reduced numbers of iNKT cells in both the peripheral blood and synovium of SLE patients compared to healthy controls [435]. Similarly, studies using flow cytometry found reduction in both the proportions and absolute numbers of type I and type II NKT cells, with $CD161^+$ cells within the $Vα24^+CD4^-CD8^+$ and $Vα24^+CD4^-CD8^-$ iNKT cell subpopulations being reduced in SLE patients compared to healthy controls [436-438]. Importantly, this reduction in iNKT cells was not observed in relatives of SLE patients, highlighting that the iNKT cell deficiency observed was disease-associated and not genetic [439]. This relationship has further been shown to occur independently of treatment with immunosuppressive drugs in SLE patients [436, 440].

In addition to the numerical deficiencies observed in SLE patients, iNKT cells are also functionally impaired, as demonstrated by poor iNKT cell responsiveness to α-GalCer [233, 436]. The median proliferative index following α-GalCer stimulation was significantly lower in SLE patients compared to healthy controls, whilst impaired IL-4 and IFN-γ mRNA expression and cytokine secretion were also observed [436]. Furthermore, studies have shown that this poor functionality is not a consequence of a defect in CD1d-mediated antigen presentation, as similar levels of CD1d expression were observed in PBMCs from both SLE patients and healthy controls [436]. This result is supported by the lack of iNKT cell proliferation seen when APCs from healthy controls were cocultured with iNKT cells from SLE patients [436]. This functional impairment of iNKT cells is also seen in other autoimmune diseases [441].

One original hypothesis for poor iNKT cell function in SLE patients comes from the finding that iNKT cells from SLE patients are more susceptible to apoptosis compared to those from healthy controls following 7-day incubation with α-GalCer [436]. This suggests that genetic defects regulating activation induced cell death in iNKT cells may contribute to SLE

pathogenesis. Interestingly, this lack of iNKT cell responsiveness can be reversed as shown in mouse models. Morshed et al. found that β-GalCer with a 12 carbon acyl chain can reduce iNKT cell cytokine secretion in C57BL/6 mice following iNKT cell stimulation with α-GalCer, in addition to diminishing iNKT cell numbers in the liver and spleen [442, 443]. Furthermore, oral or intraperitoneal β-GalCer treatment in mice was found to improve SLE disease activity as measured by proteinuria, IgG-anti-dsDNA and rate of survival [442]. Such findings suggest that this reduction in iNKT cells seen in SLE is more than just a defect in cell death, but one concerned with the recognition of lipid antigens, which play a role in the pathogenesis of the disease.

1.4.2 iNKT cells and the pathogenesis of SLE

Mouse models have been used to clarify the role of iNKT cells in SLE; typically through elimination of iNKT cells by knocking out genes for CD1d or the iTCR. Mice deficient in iNKT cells are generally healthy, but have a predisposition to developing autoimmunity and cancer as well as certain infections [444]. Moreover, mouse strains with a genetic predisposition towards SLE (MRL/lpr and NZB/W F1) show similar defects in iNKT cell frequency and function compared to humans [445]. However, there is evidence that the disease and mechanisms involved may differ considerably according to the model of SLE and strain used. This is highlighted in the pristine-induced nephritis model, where α-GalCer injection induces a protective Th2 response in BALB/c mice, but a harmful Th1 response in SJL/J mice [446]. Similar differences can be found in $CD1d^{-/-}$ mice, where CD1d deletion results in more advanced skin lesions in MRL/lpr mice, which develop SLE-like disease due to a point mutation in Fas [447], whilst in NZB/WF1 mice CD1d deficiency was shown to accelerate nephritis [448]. Despite this, some useful advances to the field have come from the study of iNKT cells in mouse models of SLE.

It was previously unknown whether the iNKT cell deficiency seen in SLE patients preceded the onset of the disease or whether it occurred as a consequence. However, data from lupus-susceptible NZB/WF1 mice indicates that iNKT hypo-responsiveness to α-GalCer is present at a young age, prior to any physical signs of autoimmunity [449]. The finding that mice also had lower transactivation of T and B cells and reduced cytokine secretion suggests that in SLE patients, defective iNKT cell responses may have systemic consequences, even

before any visible signs of disease [449]. This finding is supported by Zeng et al. who identified age as a factor in iNKT cell responsiveness using NZB/WF1 mice with a form of hereditary Lupus closely resembling human disease [450]. Moreover, treatment with α-GalCer exacerbated disease symptoms and induced IFN-γ secretion in mice aged 8-12 weeks but improved disease in mice aged 4 weeks [448, 450]. Interestingly, transfer of $NK1.1^{+}$ T cells from the older to younger group worsened disease, implicating that iNKT cells play different roles in the immune response through age.

1.4.3 Autoantibody production and iNKT cells

Evidence also suggests iNKT cells may promote autoimmune-like disease in mice by inducing IgG autoantibody production by B cells [448], through a mechanism subsequently shown to be dependent upon CD1d and CD40 expression in vivo [451]. Wermeling and colleagues subsequently observed that specific knockout of iNKT cells (using $J\alpha18^{-/-}$ mice) resulted in autoantibody production by B cells following injection with apoptotic cells [452]. Similarly, Yang et al. demonstrated that the iNKT cell ligand α-GalCer could suppress autoantibody production both in vivo and in vitro in mice [453], suggesting that glycolipid presentation to iNKT cells may contribute to SLE by promoting autoantibody production. Moreover, this is in line with observations in SLE patients where a reduction in iNKT cell number has been shown to be associated with increases in plasma IgG and anti-dsDNA [439]. Overall, these studies highlight the regulatory role of iNKT cells, which enables them to protect against the inflammatory effects in SLE, when present in adequate numbers. Quantification of iNKT cell frequencies, as well as phenotyping to determine iNKT cell activity, could therefore provide an indication of SLE prognosis in terms of immune function.

1.4.4 Altered self-lipids in SLE patients

It has previously been hypothesized that the lack of iNKT cells in autoimmune disease settings may be due to altered presentation of self-lipids in these patients. This theory has been proposed by Tan et al. and others; supported by the finding of an altered glycosphingolipid profile in immune cells from SLE patients [367]. Using liquid chromatography mass spectrometry, Tan et al. showed that $Fas^{f/f}$ $Cd19^{Cre/+}$ mice, which have a B cell-specific Fas mutation, develop an altered B cell lipidome from birth [454]. They observed that Fas deficient B cells had increased expression of certain globotrihexosylceramides (Gb3 [d34:1], Gb3 [d42:2] and Gb3 [d42:1]) and phosphatidylglycerol PG [32:0], with a reduction in cholesterol sulfate compared to Fas sufficient B cells [454].

Moreover, B cells from SLE patients may have altered expression of enzymes, which regulate the generation of self-lipids. α-galactosidase A is involved in the generation of self-lipids in mice; expression of which has been shown to be reduced in B cells from $Fas^{f/f}$ $Cd19^{Cre/+}$ mice, which are deficient in Fas [454]. Since both $Fas^{f/f}$ $Cd19^{Cre/+}$ and α-galactosidase A knockout mice have reduced numbers of iNKT cells [454, 455], it stands to reason that reduced α-galactosidase A expression may influence the self-lipids presented via CD1d to iNKT cells, resulting in strong iNKT cell activation and activation induced cell death, which is thought to occur in SLE patients. This is in support of work by Bosma et al. showing that defective CD1d-mediated lipid antigen presentation by B cells contributes to SLE pathogenesis [233]. This was attributed to increased CD1d recycling, which could have resulted in altered self-lipid presentation to iNKT cells [233].

1.5 iNKT cells in atherosclerosis

1.5.1 iNKT cells and atherosclerosis in humans

Unlike SLE where iNKT cells protect against the inflammatory response, in atherosclerosis iNKT cells are seen as pro-atherogenic. Whilst few detailed studies have been carried out on atherosclerosis in humans, the presence of CD1d expression within human atherosclerotic lesions indicates a role for iNKT cells in plaque progression [456]. Moreover, the finding that CD1d expression was restricted to foam cells and areas containing T lymphocytes and DCs suggested a role for CD1d-mediated lipid antigen presentation to iNKT cells in atherosclerosis [456, 457].

Previous studies investigating atherosclerosis in humans have observed that iNKT cells within the plaque are abundant and comprise up to 3% of total infiltrating T cells, yet are scarce in the peripheral blood of patients who have had a cardiovascular event [219, 458]. It has been hypothesized that the reduction in iNKT cells within the peripheral blood is due to accumulation within the plaque sites, although it may also be as a consequence increased iNKT cell activation and downregulation of the iTCR following stimulation [458].

1.5.2 iNKT cells and animal models of atherosclerosis

On the contrary, iNKT cell numbers are generally increased in mouse models of atherosclerosis. When apoE$^{-/-}$ mice, which are extremely susceptible to atherosclerosis, are crossed with CD1d$^{-/-}$ mice which lack iNKT cells, there is a significant reduction in the extent of atherosclerosis compared to apoE-/- controls [459-461]. Similarly, CD1d-/- mice fed an atherogenic diet developed smaller lesions compared to wild type mice fed the same diet [459]. Interestingly however, adoptive transfer of wild type iNKT cells to RAG1$^{-/-}$LDLR$^{-/-}$ mice, where cells are unable to phagocytose oxLDL, resulted in the development of plaque, inferring that iNKT cells themselves are pro-atherogenic in nature – even in the absence of exogenous lipids [462]. This suggests that cytokines or endogenous lipid antigens may play a larger role in plaque progression than previously thought.

Jα18$^{-/-}$ mice, which specifically lack type I (and not type II) NKT cells, have been used to clarify the importance of iNKT cells in atherosclerosis. Targeted deletion of the Jα18 gene in LDLR$^{-/-}$ mice was found to decrease both the extent of atherosclerosis in the aortic root and arch, as well as IFN-γ levels in both male and female mice [463]. In Vα14 LDLR$^{-/-}$ transgenic mice, where iNKT cells are expanded, macrophage accumulation was found to occur in adipose tissue, which may promote iNKT cell activation through presentation of lipid antigens [464]. Vα14 transgenic mice also have increased hyperlipidemia, insulin resistance and aortic atherosclerosis indicating a positive correlation between iNKT cell number and cardiovascular disease risk. In addition, these studies also highlight the importance of targeting the type I or invariant (and not type II) NKT cells to prevent atherosclerosis.

Interestingly, the characterization of murine iNKT cells by CD4$^+$ and CD8$^+$ subsets as previously described found the CD4$^+$ iNKT cell subset as the most atherogenic [465]. This may be linked to the observation that the CD4$^+$ subset expressed lower levels of the inhibitory NK cell receptor Ly49 and were stronger producers of IL-2, TNF-α and IFN-γ in response to α-GalCer stimulation than the other subsets [465]. Moreover, evidence suggests that these CD4$^+$ iNKT cells contribute to atherosclerosis through production of perforin and granzyme B-mediated cell cytotoxicity [225]. This is in contrast to a study in humans where no differences were observed in the proportion of CD4$^+$ and CD4$^-$ iNKT cells within the peripheral blood of healthy individuals versus patients who had suffered an acute MI [466].

In addition, there is evidence to suggest that iNKT cells are capable of altering the content of atherosclerotic lesions. Nakai et al. demonstrated using apoE$^{-/-}$ mice that chronic α-GalCer stimulation of iNKT cells could result in collagen depletion from atherosclerotic lesions and an increase in cellular content [459]. This suggests that iNKT cells display other effector functions in humans, which promote inflammation and liability of plaque to rupture.

Importantly, iNKT cells may play a protective role in certain experimental conditions of atherosclerosis. Aslanian et al. demonstrated the effects of iNKT cells in atherogenesis to be more pronounced during early stages of disease, suggesting that iNKT cell involvement in atherosclerosis is transient [467]. This may explain why one study observed a reduction in plaque size in LDLR$^{-/-}$ mice following treatment with α-GalCer indicating that iNKT cells may play an atheroprotective role in subclinical atherosclerosis [468]. Alternatively, Braun

et al. reasoned that this discrepancy could have been due to the measurement of different regions of the vasculature [469].

Data from animal models of atherosclerosis generally supports the theory that increased iNKT cell accumulation within plaque is a direct result of increased CD1d-mediated lipid presentation by antigen presenting cells. This is emphasized in a study by Strom et al. where mice lacking CD1d were found to have a 60% reduction in carotid plaque formation compared to wild type mice following carotid collar injury [470]. Furthermore, iNKT cell expansion occurred in the spleen and periadventitial space of the arteries in wild type mice, and was associated with increased carotid injury. On the whole, this suggests a pathogenic role for iNKT cells in atherosclerosis, although this could vary at different stages of disease and may not be representative of data from humans.

1.6 iNKT cells in SLE patients with atherosclerosis

Whilst animal models have proved useful in clarifying the roles of iNKT cells in SLE and atherosclerosis, the two diseases have rarely been studied in combination, with very little data from human studies. This is particularly important in light of the differences in CD1d and lipid presentation between mice and humans, as has been described. Taking previous research into account, the role of iNKT cells in SLE patients with atherosclerosis appears extremely confusing due to their conflicting roles in both diseases. In SLE, iNKT cells have a defect in cell number and function, resulting in a defective iNKT cell pool, and loss of immunoregulation. This is in stark contrast to atherosclerosis, which appears to be mediated through increased presentation of glycolipids and iNKT cell activation, resulting in a proinflammatory response. As a consequence, this dichotomy exists whereby iNKT cells are viewed as protective against autoimmunity, but pathogenic in the context of atherosclerosis (Figure 1.18).

It is possible however, that the concept of iNKT cells playing dual roles in both diseases may not be too farfetched. Evidence from a study on $CD4^+$ T cells by Major et al suggests that T cells are able to convert phenotype upon transfer from a mouse model of SLE to atherosclerosis [198]. Despite this, it is unknown whether this would also true for iNKT cells, and whether this plasticity would be true *in vivo* in humans for SLE patients who later develop atherosclerosis. What is clear is that iNKT cell phenotype in SLE patients with atherosclerosis likely depends on numerous factors including disease activity, stage of atherosclerosis, smoking, age, medication and lipid profile. This conundrum therefore presents a challenge to understanding the mechanistics of plaque development in SLE patients, as well as the possibility of indirectly targeting iNKT cells in order to protect against both atherosclerosis and SLE.

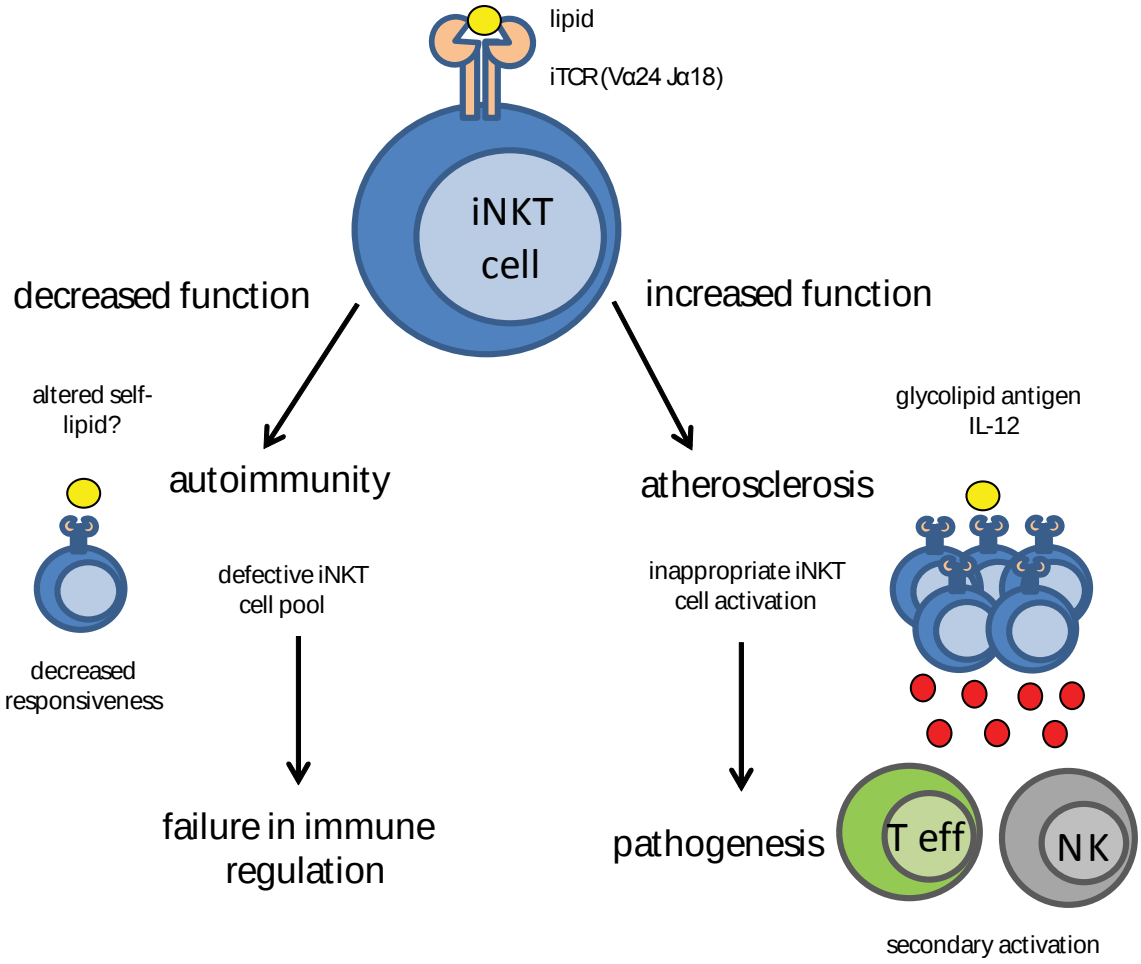


Figure 1.18 The role of iNKT cells in autoimmunity and atherosclerosis
iNKT cells mediate a balance between activation and tolerance under normal homeostatic conditions and a disturbance in this contributes to a number of immunopathologies associated with either increased or decreased iNKT cell function.

1.7 Hypothesis and aims

Hypothesis: iNKT cell phenotype and function are defective in SLE patients due to altered lipid metabolism, and this contributes to the increased cardiovascular risk observed in these patients.

Outline of PhD project:

Using a cohort of 100 SLE patients who were scanned to determine the presence of preclinical atherosclerosis my aims were to:

- Assess iNKT cell frequency, phenotype (surface markers) and function (cytokine profile) in patients with and without plaque.
- Investigate whether serum lipids and metabolites were different in SLE patients and whether they affected iNKT cell function.
- Explore the role of lipid biosynthesis and CD1d-mediated lipid antigen presentation in SLE patients and its influence on iNKT cell function.
- Understand how altered iNKT cell function in SLE patients may contribute to atherosclerosis through transactivation of other immune cell types.

CHAPTER II: Materials and Methods

Materials and Methods

2.1 Patients and controls

650 SLE patients who met the 2004 revised criteria for SLE set out the America College of Rheumatology were assessed for disease activity and a personal history of cardiovascular as part of their routine visits to University College London Hospital (UCLH) [10, 38]. Of these, 100 SLE patients who had not previously suffered a cardiovascular event were randomly selected for ultrasound scan by collaborators Dr. Sara Croca and Prof. Anisur Rahman at UCLH. Ultrasound scans were performed by Prof Andrew Nicolaides, Dr Maura Griffin and Dr Sara Croca, as follows:

Vascular ultrasound of the common carotid artery (CCA), carotid bulb, carotid bifurcation, common femoral artery (CFA) and femoral bifurcation were performed bilaterally using the Philips IU22 ultrasound computer and the L9-3 MHz probe. IMT was measured using QLAB Advanced Quantification Software® version 7.1 (Philips Ultrasound, Bothell, USA), in order to provide an indication on the stage of atherosclerosis. A longitudinal section of the mid-CCA was obtained and a 2 cm segment of the posterior wall was measured with at least 97% accuracy. IMT was also measured in a transverse view of the mid-CCA using manual callipers with variability <1% compared with the longitudinal view. The mean IMT value was obtained from combining the bilateral measurements. Measurements were taken from the carotid and femoral arteries to quantify intima-media thickness and plaque presence, as well as calcium content, which is a key determinant of plaque stability [471].

Regardless of their location, all the plaques which were identified were included in the analysis. When plaque was present, colour and Power Doppler images were obtained to ensure that the outline of the plaque was accurately determined. Plaque thickness was measured at the point of maximum thickness using manual callipers on longitudinal and transverse views and the mean value was obtained (variability <1%). Plaque echogenicity was characterized qualitatively by two trained observers independently (variability<10%) using a visual scale [472], and analysed for ultrasonic arterial wall changes and atherosclerotic plaques using “The Plaque Texture Analysis” software version 4.5 (LifeQ Ltd).

Plaque echogenicity measured by the later method was expressed numerically by Grey Scale Median (GSM) [202, 216, 473-475], which has been shown to reflect visual assessment of plaque using the Grey Weale classification [476]. Plaque area was measured using the same automated plaque texture analysis software using a method previously described [217, 218, 473]. Lipid rich, soft and unstable plaques were characterised by an area >95mm^2 and GSM of <25 [477]; high GSM values were indicative of hard plaque, which is characterised by calcification [478]. Very few patients had lipid rich or unstable plaques or lipid cores of a quantifiable size.

Plaque presence and size was characterised using a previously described arterial ultrasound score (AUS), which ranges from 0-8 for each artery [479, 480]. AUS was allocated to each arterial site according to both IMT measurements and assessment of plaque morphology (Table 2.1), using a classification system based on the one described by the American Heart Association [481]. Examples of class I, II and III lesions can be seen in Figure 2.1. This scoring system has previously been shown to predict cardiovascular event rate in a 6 year follow-up study where cardiovascular events were observed only in subjects with class II, III and IV lesions, with patient deaths occurring only in subjects with class III or IV lesions [480]. Participants' total AUS was quantified by adding scores from the four individual sites to give a combined score ranging from 0-32. Class III and IV lesions were indicative of plaque, which enabled patients to be grouped according to whether or not they had plaque (SLE-P or SLE-NP) as well as the number of sites with plaque (Table 2.2).

Supine blood pressure (BP) was measured in the left upper arm using an automated BP reader (Omron M6 HEM-700-E). Three measurements were taken and the first discarded. The average systolic and diastolic BP value was calculated using the second and third readings. Pulse pressure was calculated as the difference between systolic and diastolic BP, Mean BP value was calculated for each patient using:

Mean BP= Diastolic BP + 1/3(systolic BP – diastolic BP).

Blood samples were collected at the time of the scan and at regular time points following the scan, according to patients' routine appointments, which on average were scheduled every 3 months or sooner depending on clinical need. Routine blood was taken from patients at the time of each appointment to measure serum lipids, homocysteine, CRP and vitamin D. Research blood was also taken from SLE patients identified as having suffered a

cardiovascular event (stroke or MI), and healthy donors; both of which did not receive ultrasound scans. All participants completed a cardiovascular risk factor questionnaire at the time of blood donation to evaluate individual risk factors including medication, smoking and family history of cardiovascular disease. Basic characteristics of all participants can be seen in Figure 2.2, with more detailed analysis throughout Results sections 1&2.

For all SLE patients, disease activity calculated using the British Isles Lupus Assessment Group (BILAG) index on every visit [38]. Those who had recently underwent rituximab therapy were excluded from the main analysis due to the known influence of B cell depletion on iNKT cell frequency [233, 482].

The study had research ethics committee (06/Q0505/79) and National Health Service approval. Ultrasound scans and blood donation were carried out subject to informed consent, which was obtained from all participants, under the guidelines of UCLH.

Class	Ultrasound morphology	Arterial Ultrasound Score
0	Normal: Intima-media thickness (<1mm) with no gaps or acoustic holes, which correspond to fatty streaks.	0
I	Fatty streaks: Three ultrasonic layers (intima-media, adventitia, and periadventitia) clearly separated. No disruption of lumen-intima interface for at least 3.0 cm, and/or initial alterations (lumen-intima interface disruption at intervals of <0.5 cm).	2
II	Intima-media granulation or thickened intima-media: Granular echogenicity of deep, normally unechoic intimal-medial layer and/or increased intima-media thickness (>1 mm).	4
III	Small plaque without hemodynamic disturbance†: Localized wall thickening and increased density involving all ultrasonic layers. Intima-media thickness >2 mm.	6
IV	Large or stenotic plaque: As in III, but with hemodynamic stenosis on duplex scanning (sample volume in the center of the lumen), indicating stenosis >50%.‡	8

Table 2.1: Ultrasound Arterial Morphology Classification

Each artery was scored based on the above classification criteria, as previously described by Belcaro et al [480]. Class V (AUS of 10) refers to symptomatic plaques, regardless of size and is not included since none of the patients had suffered a cardiovascular event at the time of the scan. Scores for all four arteries were combined to give a total score for each patient.

†Hemodynamic disturbance is defined as moderate spectral broadening (downstroke of systole); systolic window present; diastolic window reduced and/or absent. Ratio $a-b/a<0.5$, where a is the peak systolic velocity and b, the first peak end-systolic velocity.

‡Peak frequency >4 kHz and spectral broadening throughout systole; no systolic window. Ratio $a-b/a<5$.

Table obtained from Belcaro et al, 1996 [480].

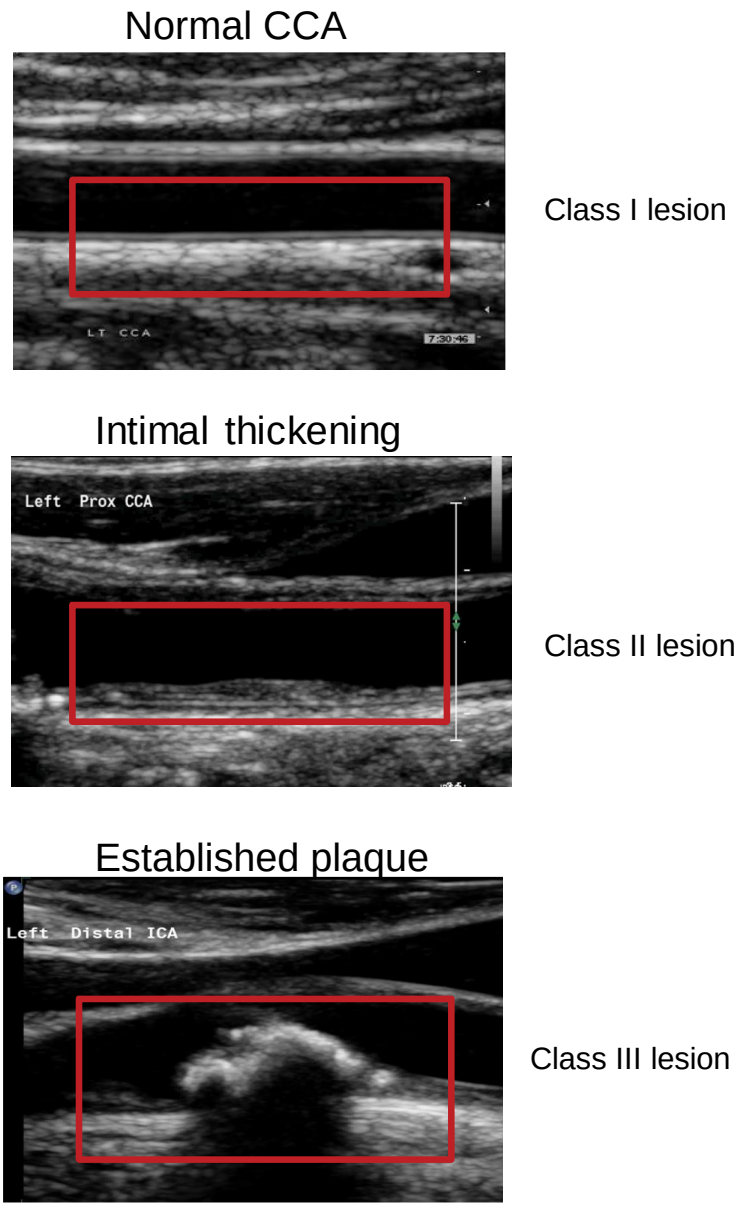


Figure 2.1: Representative Images of Ultrasound scans from patients in this study

Examples of ultrasound images from scanned SLE patients, and their classification according to AUS score. Very few patients had lipid cores which could be quantified for size.

	Healthy donors (n=50)	SLE-NP (n=64): 0 sites with plaque	SLE-P (n=36): 1-4 sites with plaque	SLE-CV (n=11)	ANOVA or T test (as applicable)
Mean Age (range)	39 (22-67)	40 (20-66)	54 (27-69)	50 (20-78)	[a]HC vs SLEP p<0.001[c] [a]SLENP vs SLEP p<0.001 [c]
Years since diagnosis (range)	NA	13 (2-32)	21 (2-46)	18 (1-34)	[a]P=0.0008 [c]
Sex: female: male	47:3	62:2	33:3	9:2	[a]NS
Ethnicity: C/A/AC/O (%)	40/4/6/0 (80: 7: 13: 0)	33/9/17/5 (51.6/14.1/26.6/ 7.8)	24/2/7/3 (66.6/5.5/19.4/ 4.7)	8/1/1/1 (73/9/9/9)	[a]H vs SLE-NP p<0.05 [c] [a]H vs SLE-P p<0.05 [c]
Personal history of CVD (%)	0	0	0	11	N/A
Mean RT CCA IMT (range)	ND	0.051 (0.04-0.08)	0.057 (0.04-0.08)	ND	[b]0.002**
Mean RT BULB MIT (range)	ND	0.080 (0.05-0.12)	0.178 (0.07-0.42)	ND	[b]<0.0001***
Mean LT CCA (range)	ND	0.061 (0.04-0.06)	0.061 (0.05-0.10)	ND	[b]<0.0001***
Mean LT BULB (range)	ND	0.078 (0.05-0.13)	0.196 (0.07-0.35)	ND	[b]<0.0001***
Mean total arterial score (range)	ND	3.59 (2-8)	16.97 (8-26)	ND	[b]<0.0001***

Table 2.2: Basic characteristics of all study participants

2.2 Peripheral blood mononuclear cell (PBMC) isolation, storage and thawing

Density gradient centrifugation was used to isolate PBMCs from heparinised blood. Blood was diluted 1:1 in RPMI-1640 medium (Sigma), before gently layering onto 15ml Ficoll-paque (GE Healthcare) in 50ml falcon tubes. Samples were then centrifuged at 800g for 30 minutes at 21°C with minimum acceleration and brake settings to separate blood into layers (Figure 2.2). The white buffy coat layer was carefully removed and diluted 1:1 with RPMI-1640 medium, then centrifuged at 400g for 10 minutes at 4°C. The supernatant was discarded and the pellet resuspended in 10ml RPMI.

Cell concentration was determined by diluting 1:10 in trypan blue and counting cells using a haemocytometer under a light microscope. Lastly, cells were centrifuged using the same settings aliquotted into cryovials, at a concentration of $1x10^7$ PBMCs/ml freezing medium containing fetal calf serum (FCS) and 10% dimethyl sulfoxide (DMSO). Vials were placed into Nalgene™ Mr Frosty freezing containers containing isopropanol in order to achieve a freezing rate of -1°C/minute before transfer to -80°C freezer for storage. For long-term storage samples were transferred to liquid nitrogen storage (-180°C).

For each experiment cells were thawed by pre-warming 20ml complete RPMI (RPMI-1640 medium; 10% FCS, 1% penicillin/streptomycin [Pen/Strep]) per sample to 37°C in a water bath. A sterile Pasteur pipette was used to thaw each vial individually before diluting in pre-warmed complete RPMI, and washed to remove traces of DMSO.

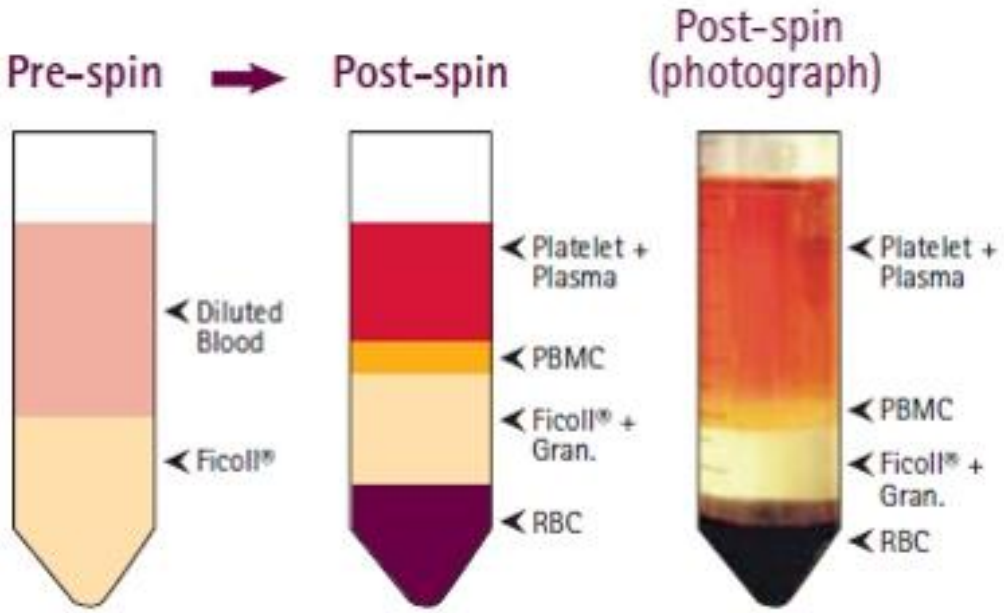


Figure 2.2 Isolation of PBMCs using Ficoll-Paque Plus density gradient centrifugation

Illustration of the layering of blood before and after centrifugation.

2.3 Surface staining for FACS

Multi-parameter flow cytometry was used to carry out immunophenotyping of iNKT cells and APCs. Cell staining was carried out in a 96 well plate at a concentration of 1-2x10^6 PBMCs per well. Cells were stained with Live/dead violet or yellow (50µl/well diluted 1:200 in PBS) for 20 minutes at 4^0C before washing in PBS. Cells were then washed and stained with either a surface marker or intracellular panel using the antibodies indicated in Table 2.3 and Table 2.4. Panels were designed for use on BD LSR Fortessa and all antibodies were titrated for optimal dilution.

For the surface marker panel, staining was carried out in 50µl cold FACS buffer containing 1x phosphate buffered saline (PBS), 1% FCS, 0.01% NaN_3; 0.5% Ethylenediaminetetra-acetic acid (EDTA). A mix was prepared containing the following antibodies, which were diluted accordingly. iTCR-PE (1:10) and CD3-APC/efluor780 (1:50) were used to identify iNKT cells, with subsets CD4-BV605 (1:1000) and CD8-BV705 (1:100). Other markers stained for included activation markers CD25-alexafluor700 (1:25), CD69-APC (1:25) and PD-1-PE/Cy7 (1:25), as well as NK cell marker CD161-BV421 (1:25), and chemokine receptor CCR6-FITC (1:25). Cells were incubated at 4°C in the dark for 20 minutes to enable binding. Cells were then washed in 100ml MACS buffer and centrifuged at 2500rpm for 2 minutes at 4°C, before fixing with 70µl 2% paraformaldehyde (PFA) and incubating in the dark at RT for 15 minutes. Finally, cells were washed and resuspended in 200µl FACS buffer ready for analysis. For each sample, 1-5x10^6 cells were acquired by flow cytometry using BD LSR Fortessa, and analysed using FlowJo version X (TreeStar).

Staining for APCs was carried out with 1x10^6 cells/well in 50µl MACS buffer as previously described. All antibodies were diluted 1:50 in FACS buffer with separate panels for monocytes, B cells and pDCs, which were labelled with CD14, CD19 and CD303 respectively. APCs were stained for CD1d followed by lipid rafts (Section 2.5), or alternatively for scavenger receptors CD36, LOX-1 and LDLR. Monocyte subsets were stained for using CD14, CD16, CD68, CCR2, CD206 and CX3CR1. Cells were washed and fixed as above before acquisition of >3x10^5 cells by flow BD LSR Fortessa. Samples were analysed using FlowJo version X (TreeStar).

Table 2.3 Cell surface antibodies and markers used

Surface marker	Fluorochrome	Clone	Isotype	Company	Concentration in FACS Buffer
Invariant T cell receptor (Vα24Jα18)	PE	6B11	Mouse IgG1	BD Bioscience	3:50
Invariant T cell receptor (Vα24Jα18)	PE/Cy7	6B11	Mouse IgG1	eBioscience	3:50
CD1d	PE	51.1	Mouse IgG1	eBioscience	1:50
CD3	APC/efluor780	SK7	Mouse IgG1	eBioscience	1:50
CD4	Alexa488	OKT4	Mouse IgG2b	BioLegend	1:50
CD4	BV605	OKT4	Mouse IgG1	BioLegend	1:100
CD8	BV711	RPA-T8	Mouse IgG1	BioLegend	1:100
CD14	BV510	M5E2	Mouse IgG2a	BioLegend	1:50
CD14	efluor450	61D3	Mouse IgG1	eBioscience	1:50
CD16	alexafluor700	3G8	Mouse IgG1	BioLegend	1:50
CD19	alexafluor700	HIB19	Mouse IgG1	BioLegend	1:50
CD25	Alexa700	BC96	Mouse IgG1	BioLegend	1:50
CD25	APC	BC96	Mouse IgG1	BioLegend	1:50
CD36	FITC	NL07	Mouse IgM	eBioscience	1:25
CD40L	APC	24-31	Mouse IgG1	BioLegend	1:50
CD68	PE/Cy7	Y1/82A	Mouse IgG2b	BioLegend	1:50
CD69	APC	FN50	Mouse IgG1	eBioscience	1:50
CD161	BV421	HP-3G10	Mouse IgG1	BioLegend	1:50
CCR2 (CD192)	PerCP/Cy5.5	K036C2	Mouse IgG2a	BioLegend	1:50
CCR6 (CD196)	FITC	R6H1	Mouse IgG1	eBioscience	1:50
CD206	PE	15-2	Mouse IgG1	BioLegend	1:50
CD209	FITC	DCN47.5	Mouse IgG1	Miltenyi Biotec	1:50
PD-1 (CD279)	PE/Cy7	EH12.2H7	Mouse IgG1	BioLegend	1:50
CD303	APC	201A	Mouse IgG1	BioLegend	1:50
CX3CR1	FITC	2A9-1	Rat IgG2b	BioLegend	1:50
ICOS	PE	C398.4A	Hamster IgG	BioLegend	1:50
LOX-1	PE	331212	Mouse IgG2b	R&D Systems	1:25
LDLR	APC	472413	Mouse IgG1	R&D Systems	1:25
Cholera Toxin subunit B (CTB)	FITC	N/A	N/A	Sigma	1:100
Filipin	Emission at 470nm	N/A	N/A	Sigma	1:200
ANE	Emission at ~465/635nm	N/A	N/A	Life Technologies	4 mM
Live/dead	Yellow (emission at 405nm)	N/A	N/A	Life Technologies	1:200 (PBS)
Live/dead	Aqua (emission at 405nm)	N/A	N/A	Life Technologies	1:200 (PBS)

2.4 Intracellular FACS staining

For the intracellular panel, PBMCs were cultured for 4 hours in complete RPMI containing 50ng/ml PMA, 250ng/ml ionomycin and 1:1000 Golgiplug (all from Sigma). PBMCs were washed and stained for iTCR, CD3, CD4 and CD8 as before. Cells were then washed and resuspended in 70µl Fix/Perm buffer (1:4) using the diluent provided (eBiosciences) before incubating for 20 minutes at 4°C in the dark and washing twice with PermWash buffer 1:10 dH_2O (eBiosciences). Cells were resuspended, then stained for a maximum of three intracellular cytokines (2µl/well undiluted) and incubated at 4°C in the dark for 20 minutes. Samples were washed using Perm/Wash buffer as previously, then resuspended in MACS buffer for analysis. For each sample, 1-5x10^6 cells were acquired by flow cytometry using BD LSR Fortessa, and analysed using FlowJo version X (TreeStar).

Table 2.4 Antibodies for Intracellular molecules

Intracellular marker	Fluorochrome	Clone	Isotype	Company	Concentration
IFN-γ	efluor450	4S.BS	Mouse IgG1	eBioscience	1:25
IL-4	APC	MP4-25D2	Mouse IgG1	BioLegend	1:10
IL-10	APC	JES3-19F1	Rat IgG	BD Bioscience	3:50
CD1d	PE	51.1	Mouse IgG1	eBiosciences	1:50
EEA1	FITC	14/EEA1 (RUO)	Mouse IgG1	BD Biosciences	1:100
LAMP-1 (CD107a)	PerCP/Cy5.5	H4A3	Mouse IgG1	BioLegend	1:50
Ki67	PE	B56	Mouse IgG1	BD Pharminogen	1:20
Ki67 (isotype control)	PE	MOPC-21	Mouse IgG1	BD Pharminogen	1: 20
TCR zeta (CD247)	Alexa488	K25-407.69	Mouse IgG2A	BD Phosflow	1:50
ERK1/2	Alexa647	20A	Mouse IgG1	BD Phosflow	1:50
T-bet	PerCP/Cy5.5	4B10	Mouse IgG1	BioLegend	1:50
GATA-3	Alexa647	I6E10A23	Mouse IgG2b	BioLegend	1:50

2.5 Lipid detection

Staining for lipid rafts was carried out following staining for B cells, monocytes and pDCs and CD1d using surrogate glycosphingolipid marker CTB [380]. 50µl CTB-FITC (1:100 FACS buffer) was added following re-suspension of PBMCs before incubating at 4°C in the dark for 30 minutes. Cells were then washed and fixed in 2% PFA, then analysed by flow cytometry as described in section 2.3.

Filipin staining was carried out after staining for $CD14^+$ monocytes, $CD19^+$ B cells and lipid rafts. Cells were fixed with 2% PFA for 1 hour at room temperature, before washing in FACS buffer and staining with 50µg/ml filipin for 2 hours at room temperature in the dark. Cells were washed and resuspended in FACS buffer before proceeding to analysis as previously described.

B cells and monocytes from healthy and SLE patients were stained with the membrane order probe di-4-ANEPPDHQ (ANE) [483]. PBMCs were stained for CD14 and CD19 (both 1:50 FACS buffer), and incubated in the dark at 4°C for 20-30 minutes. Cells were washed in 200µl MACS buffer before resuspending in 100µl of 4µM ANE and incubating at 37°C for 30 minutes in the dark. Samples were resuspended in FACS buffer and read immediately on the LSR II using PerCP and PE for ANE.

To calculate membrane order using MFI, the following equation was used:

$$GP = \frac{I_{500\text{-}580} - I_{620\text{-}750}}{I_{500\text{-}580} + I_{620\text{-}750}}$$

Alternatively, samples were analysed according to population by low, intermediate and high order (Figure 2.3).

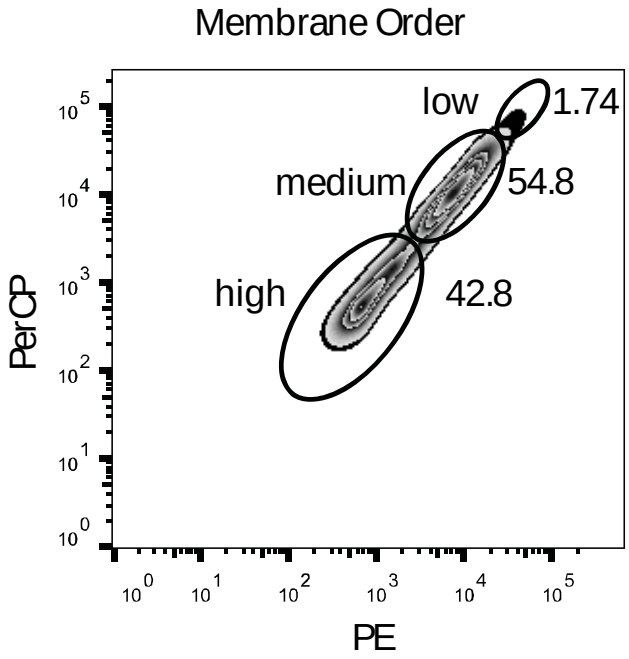


Figure 2.3 FACS plots showing cells populations of high, medium and low membrane order as determined by di-4-ANEPPDHQ (ANE)

2.6 B cell and Monocyte Isolation

$50x10^6$ PBMCs from a healthy individual were stained for CD14-BV510 and CD19-alexafluor 700 (both from BioLegend) in MACS buffer (1x PBS, 1% FCS, 0.5% EDTA). Cells were sorted using BD FACS Aria II according to CD14 and CD19 expression. Monocytes ($CD14^+$), B cells ($CD19^+$) and cells negative for both markers (mainly T cells) were collected for use in co-culture experiments. Alternatively, B cells and monocytes were negatively isolated using magnegic bead isolation kits (EasySep kits, StemCell) according to manufacturer's instructions. B cell and monocyte purification can be seen in Figure 2.4.

Isolated B cells and monocytes were each cultured with RPMI containing 10% human serum from a healthy control, SLE plaque or SLE no plaque patient. Meanwhile, double negative cells were cultured with RPMI containing 10% pooled human serum (1% Pen/Strep). Following 3 hours incubation at 37°C (5% CO_2), B cells and monocytes were washed and co-cultured with the double negative cells. Cells were incubated for 1 and 2 days at 37°C (5% CO_2). Subsequent staining for iNKT cells, CD4 and CD8 expression and intracellular cytokines was carried out as previously described.

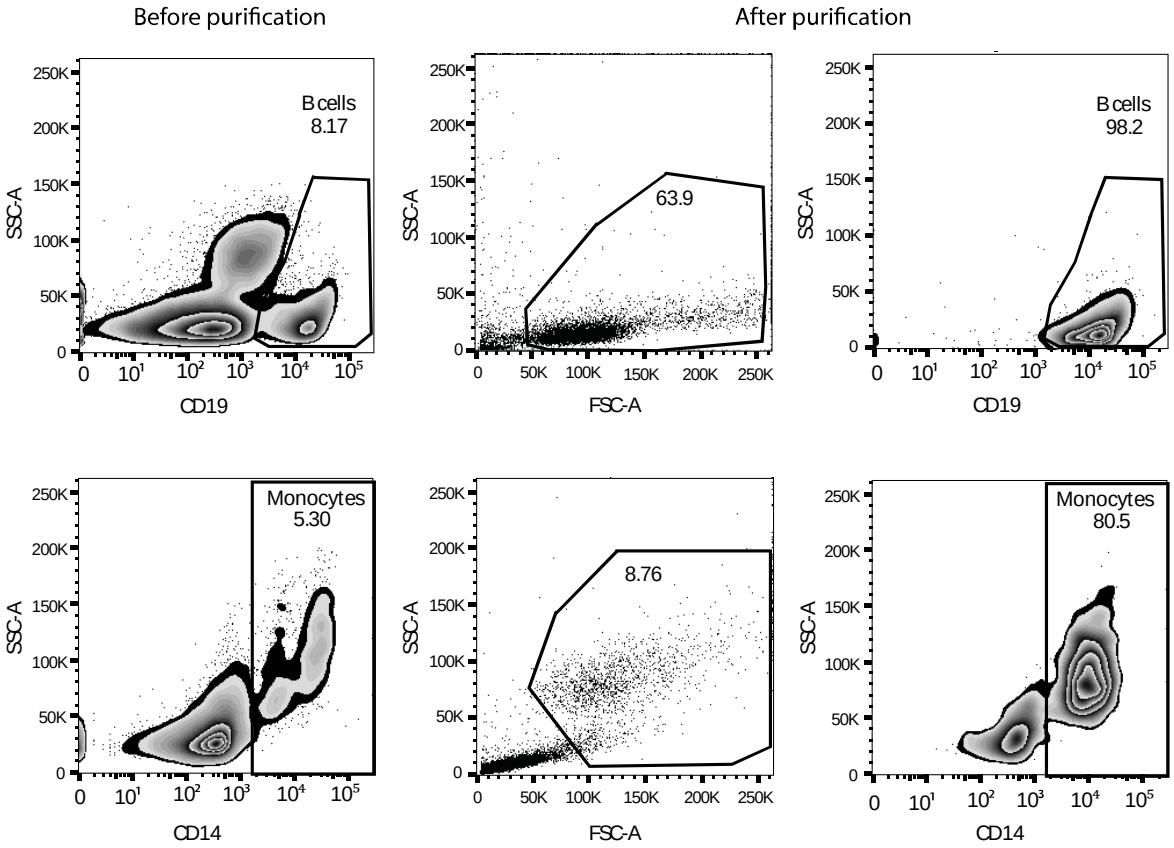


Figure 2.4 B cell and monocyte purification using StemCell EasySep kits

Lymphocytes or monocyte populations were gated according to forward and side scatter, before assessing expression of CD14 (monocytes) or CD19 (B cell expression). Representative plots showing before and after purification using EasySep kits.

2.7 ImageStream analysis of CD1d recycling and lipid raft colocalisation

Cells were stained for B cells (CD19), monocytes (CD14), CD1d and lipid rafts (CTB) as previously described. To induce clustering of CTB, cells were washed in PBS (0.5%BSA) and stained with goat α-human CTB at 1/125 dilution in PBS (0.1% BSA). Cells were incubated in the dark for 20 minutes at 4°C, before transferring to an incubator at 37°C (5% CO_2) for 20 minutes to induce antibody clustering. Finally, cells were washed twice in ice cold PBS, then fixed in 2% PFA.

To measure differences in lipid processing via the lysosomes and endosomes, cells were surface stained for CD14 and CD19, followed by an intracellular stain for CD1d, lysosome marker CD107a and early endosome marker EEA1. Cells were cultured, stained and fixed as previously described, with endosome and lysosome stains carried out according to the manufacturer's instructions. CD45RA and lipid rafts (CTB) were used as a negative colocalisation control.

Acquisition of data was carried out using Amnis® ImageStreamX Mk II, where $1x10^5$ single cells were collected for each sample, based on object diameter and aspect ratio. Data was analysed using Amnis IDEAS software version 6.0. Focused cells were gated according to gradient RMS, and single cells or conjugates were gated according to area and aspect ratio. The inbuilt feature finder wizard was used to determine the appropriate feature and mask combination to quantify each variable. These were as follows:

- Endosome count – Spot Count_Peak (M02, Channel 2, Bright 9.5)
- Lysosome count – Spot Count_Spot (M05, Channel 5, Bright 12.5)
- CD1d-endosome co-localisation – Bright Detail Similarity R3_MC_Ch02, Ch03
- CD1d and lipid raft co-localisation - Bright Detail Similarity R3_Dilate(M01, 6) And Not Erode(M01, 3)_Ch02_Ch03

2.8 Lipid extraction from PBMCs

$10x10^6$ B cells or monocytes from healthy donors, SLE-NP and SLE-P patients were isolated by FACs sorting and washed in PBS to remove traces of serum. Cells were mixed with 250ul PBS and frozen/thawed three times to disrupt the cells before carrying out lipid isolation by chloroform: methanol extraction [484]. Pellets were then and mixed with 1.2ml methanol and chloroform (2:1 concentration) for 30 minutes at room temperature. Samples were centrifuged at 3500g for 5 minutes, before removing the supernatant, which was kept on ice. This was repeated to ensure total lipid extraction. Chloroform and PBS (1:1) were added to the lipid extract before centrifuging at 1000g for 5 minutes. The lower layer (Layer 1) but not the upper layer (Layer 2) was dried under a nitrogen stream. Both layers were stored separately at -80°C.

To isolate lipid subgroups 1ml SupelCo Superclean LC-NH2 columns (Sigma) were pre-treated with 2ml hexane (Sigma). Dried lipid Layer 1 lipid pellets were combined with Layer 2 and dryed under nitrogen followed by reconstitution in 500µl chloroform, before adding to columns. Individual lipid fractions were then eluted using different elution buffers as indicated in Table 2.5. Fractions were dried under nitrogen stream and reconstituted in 50µl DMSO prior to use in cell culture experiments. Samples were stored at -80°C.

Alternatively, total lipids isolated from PBMCs were sent off for HPLC analysis of glycosphingolipid content by Dr Dominic Alonzi in the Glycobiology Department at Oxford University.

Fraction	Lipid group(s)	Elution buffer
1	Cholesterol, di- and tri-glycerides	2ml diethyl-ether
2	Ceramides and monoglycerides	1.6ml chloroform:methanol (23:1)
3	Free fatty acids and free hydroxyl fatty acids	1.8ml di-isopropylether acid: acetic acid (98:4)
4	Neutral glycolipids and sphingoid bases	2ml acetone: methanol (9:1.2)
5	Neural phospholipids, PC, PE and sphingomyelin	2ml chloroform: methanol (2:1)
6	Gangliosides	2ml 0.2M ammonium acetone in methanol

Table 2.5 Isolation of lipid fractions by chloroform: methanol extraction

2.9 PBMC culture

α-GalCer culture:

PBMCs from healthy donors, SLE-NP and SLE-P patients were cultured at a concentration of 1-2x10^6 PBMCs/well for 7 days at 37°C (5% CO_2) in the presence of RPMI containing 10% pooled human serum (Lonza), 1% Pen/Strep, 100ng/ml α-GalCer and 200IU/ml rhIL-2. At 7 days cells were stained with live/dead, CD3 and iTCR (to detect iNKT cells) followed by either Ki67 (to assess proliferation) or costimulatory molecules CD40L and ICOS. Samples were analysed by flow cytometry as previously described.

Healthy and patient serum:

PBMCs from non-autologous healthy individuals were cultured at a concentration of 1-2x10^6 PBMCs/well for 1, 2, 4 and 7 days at 37°C (5% CO_2) in the presence of healthy or SLE serum. Complete RPMI medium was prepared as previously described, but containing 50% human serum from either a healthy control or SLE patient, with the addition of 200IU/ml rhIL-2. A positive control sample was also prepared containing RPMI with pooled human serum (Lonza) and 100ng/ml α-GalCer. In some experiments, 10μg/ml purified CD1d blocking antibody (51.1) or isotype control antibody were also added to the culture medium. At each time point staining for iNKT cell surface markers, intracellular cytokines or CD1d and lipid rafts on APCs was carried out as previously described. Samples were analysed by flow cytometry.

Cellular lipids:

PBMCs from healthy individuals were cultured at a concentration of 1-2x10^6 PBMCs/well for 4 or 7 days at 37°C (5% CO_2) in the presence of RPMI containing 10% pooled human serum (Lonza), 200IU/ml rhIL-2 and 1:100 healthy, SLE-NP and SLEP B cell or monocytic lipid fractions isolated through chloroform: methanol extraction (Section 2.8). An equivalent concentration of vehicle (DMSO) was used a negative control in place of the lipid fractions. At each time point, staining for iNKT cell surface markers and intracellular cytokines was carried out as previously described. Samples were analysed by flow cytometry.

2.10 Generation of an iNKT cell line

An iNKT cell line was produced using monocyte-derived dendritic cells (MDDCs) pulsed with α-GalCer to expand iNKT cells over the course of 7 days. MDDCs were generated as follows, using a method based on a publication by Zhou and Tedder [197], and expanded by culture with iNKT cells using a method optimized by previous PhD student Anneleen Bosma.

For each experiment monocytes were isolated from freshly obtained PBMCs by negative selection using the EasySep CD14 Enrichment Kit, which was followed according to the manufacturer's instructions. Following purification, monocytes were resuspended at $1x10^6$ cells/ml in complete RPMI containing 10% human serum (Lonza), 1% Pen/Strep, 1000IU/ml granulocyte macrophage-colony stimulating factor (GM-CSF; PeproTech), and 500IU/ml recombinant human IL-4 (R&D Systems). Monocytes were incubated 1ml/well in a 12 well tissue culture plate for 4 days, before adding 100ng/ml LPS (Sigma) and 100ng/ml α-GalCer (Alexis Biochemicals) directly to the culture. On day 5, non-adherent MDDC cells were harvested and directly transferred to a separate well. At each step a small sample (200µl) was removed to assess monocyte purity and the presence of dendritic cells by staining for CD14-efluor 450 and CD209 (DC-SIGN)-FITC. Samples were acquired using BD LSR Fortessa II and data were analysed by Flowjo (Treestar). Purity can be seen in Figure 2.5.

At day 5, $2x10^7$ PBMCs from a healthy donor were thawed as previously described. Cells were then resuspended at approximately $3x10^6$ cells/ml of complete RPMI containing 10% human serum (Lonza), 1% Pen/Strep, and 400IU/ml recombinant human IL-2 (R&D Systems). 1ml of PBMCs in complete medium was then added to each of the wells containing the non-adherent MDDCs. Cells were then incubated for 7 days at 37°C (5% CO_2) to enable iNKT cell expansion. iNKT cells were assessed for expansion at day 7 by staining for CD3-APCefluor780 and iTCR-PE. Results can be seen in Figure 2.6.

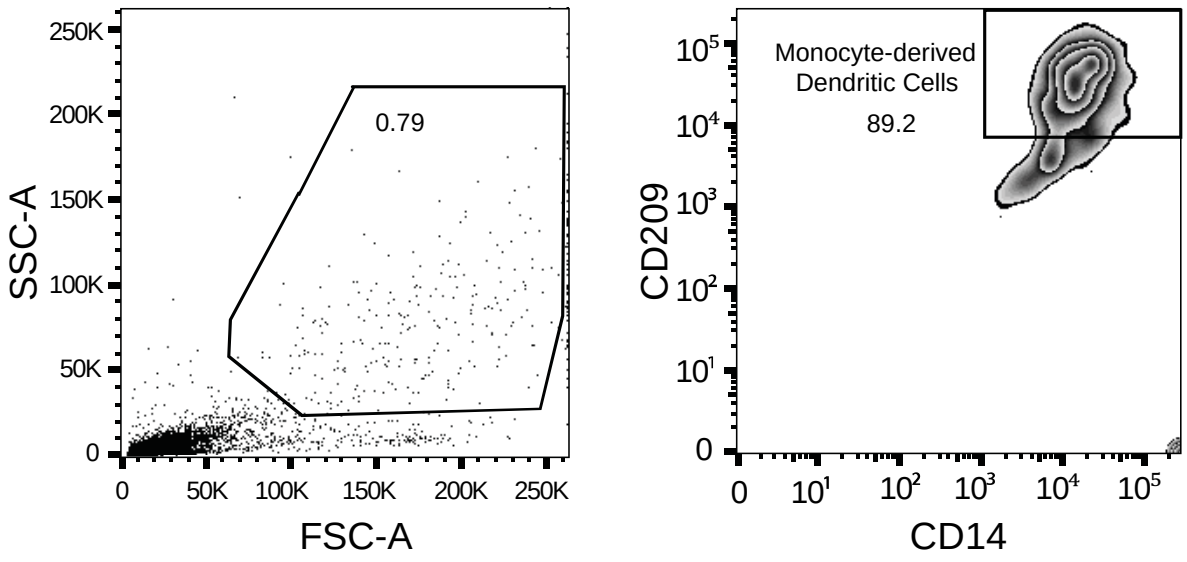


Figure 2.5 Generation of Monocyte derived DCs by monocyte culture

Sample was gated on monocytes according to FSC-A and SSC-A, followed by analysis of CD14 and CD209 (DC-SIGN) expression

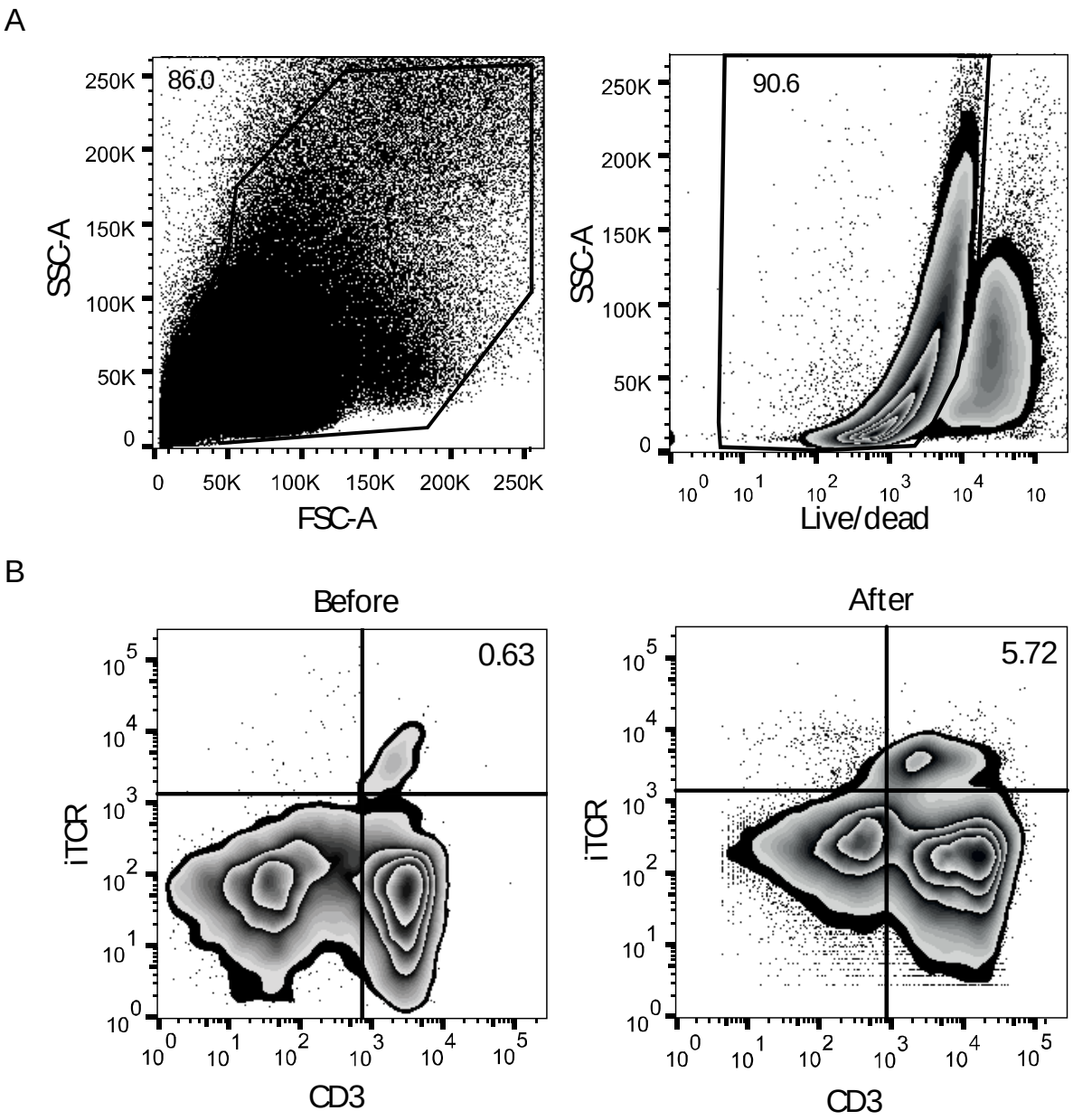


Figure 2.6 Expansion of iNKT cell line

The expanded iNKT cell line was assessed for iNKT cells by staining for Live/dead, CD3 and iTCR. (A) Samples were gated on total PBMCs (according to FSC/SSC) and live cells before (B) quantifying the percentage of $CD3^{+}iTCR^{+}$ iNKT cells both before and after culture with α-GalCer and IL-2.

2.11 Stimulation of APCs with LXR agonist GW3965 and PPARγ agonist Rosiglitazone

$2x10^6$ PBMCs were cultured in the presence of either LXR agonist GW3965 (Sigma, 1µM) or PPARγ agonist Rosiglitazone (2.5 µM, Tocris Biosciences) resuspended in 200µl complete medium (1640 RPMI, 10%pooled human serum, 1% Pen/Strep). An equivalent volume of DMSO was used in place of GW3965 or PPARγ as a vehicle control. At 4h, 24h and 48h cells were washed in FACS buffer and stained for CD14, CD19, CD1d, CTB and filiipin. Flow cytometry was used to analyse expression of CD1d, lipid raft (CTB) and cholesterol (filipin) on B cells and monocytes post stimulation. Alternatively, B cells and monocytes were isolated by negative selection using CD14+ and CD19+ enrichment kits (Stemcell Technologies) then co-cultured with the iNKT cell line shown in figure 2.5. iNKT cell phenotyping was carried out according to method in section 2.3.

2.12 Conjugate formation experiments

PBMCs were thawed as previously described and cultured with RPMI containing 10% pooled human serum, 1% Pen/Strep and α-GalCer. Cells were incubated at a concentration of $1x10^7$ cells/ml for 2 hours at 37°C (5% CO_2) to enable uptake of lipids. Following incubation cells were stained for CD14 and CD19, before being sorted using BD FACS Aria. Purified populations of B cells and monocytes were washed in MACS buffer and resuspended in complete medium containing 10% pooled human serum and 1% Pen/Strep at a concentration of at $1x10^6$/ml and placed on ice ready for use.

Meanwhile, the iNKT cell line culture was harvested at day7 and washed in MACS buffer at 500g for 10 minutes at 4C. Cells were stained using CD3-APCefluor780 and iTCR-PE at a concentration of 1µl/$1x10^6$ cells for 20 minutes at 4°C in the dark before washing. Cells were passed through a filter cap lip into 5ml polystyrene tubes for sorting using BD FACS Aria. iNKT cells (CD3+ and iTCR+) and T cells (CD3+ and iTCR-) were collected.

Sorted iNKT cell and T cell populations were rested for 2 hours at 37°C (5% CO_2) in complete medium containing 10% human serum and 1% Pen/Strep. iNKT cells were then prepared in a suspension of $5x10^5$/ml before being plated 50ul/well. A similar concentration of T cells

was used as a control. Control wells were also prepared containing single cell types; either B cells, monocytes, iNKT cells or T cells alone.

To induce conjugate formation, purified B cells or monocytes were added to the appropriate wells and incubated at 37°C (5%CO2) for 5, 10 or 15 minutes. At each time point, 2 x Fixation/ Permeabilisation Buffer was added to the appropriate wells to stop the reaction. Samples were finally incubated in the dark at 4°C for 20 minutes before staining for cell signalling molecules.

Samples were centrifuged at 800g for 3 minutes before intracellular staining for TCRζ-FITC and ERK1/2-alexa647, as described previously. Cells were resuspended in 200µl FACS buffer prior to analysis using LSR Fortessa II.

2.13 Cytometric bead array

Healthy and SLE patient serum samples, as well as supernatants from culture experiments were assessed for cytokines using BD cytometric bead array Flex kits. Cytokines analysed included IFNα, IFNγ, TNFα, IL-2, IL-4, IL-6, IL-10, IL-12, IL-13 and IL-17. Standards were prepared according to the manufacturer's instructions. To make the top standard, the lyophilised standard spheres for each cytokine were pooled and reconstituted in 4ml of assay diluent, before gently mixing and leaving to stand at room temperature for 15 minutes. 500µl of assay diluent was then added to eight test tubes, and a serial dilution was performed by transferring 500µl of top standard into the first tube, mixing and transferring to the next. This process was repeated for all eight tubes to produce dilutions of 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128, and 1:256.

Serum samples and supernatants were diluted 1:3 in assay diluent, before adding 25µl per well for each test. A solution containing capture beads for each cytokine was prepared by combining 0.5µl of each bead (IFNα, IFNγ, TNFα, IL-2, IL-4, IL-6, IL-10, IL-12, IL-13 and IL-17) and diluting in 25µl capture bead diluent, multiplied by the number of tests. 25µl was then added to each well before incubating in the dark at room temperature for 2 hours. Detection beads were prepared in a similar way to the capture beads, with 25µl added to each well before incubating at room temperature for 1 hour. Finally, wells were washed in

100µl wash buffer and resuspended in 150µl wash buffer for acquisition and analysis using BD FACS Array.

2.14 THP-1 cells

The human monocytic cell line THP-1 was maintained in complete culture medium (RMPI 1640 containing L-glutamine (Sigma), 10% fetal bovine serum and 20ug/mL gentamycin (Sigma)) at 37°C and 5% CO_2. To differentiate into macrophages, cells were plated at 400,000 cells/mL in a 6 well plate in complete medium containing 5ng/mL phorbol-12-myrisate 13-acetate (PMA) (Sigma) for 72 hours, washed twice with phosphate buffered saline (PBS), and allowed to rest for 24 hours in complete medium.

For the co-culture experiment, PBMCs from a healthy donor were incubated for 4 days with serum from either healthy control donors, SLE patients without plaque or SLE patients with plaque (n=3/group). Subsequently $CD3^+$ T cells (that included iNKT cells) were positively selected using the EasySep magnetic isolation following the manufacturer's protocol (Stem Cell) and $1.5x10^6$ T cells were added to each well of THP-1 macrophages. After 40 hours medium and T cells were removed and THP-1 cells were washed once with PBS before being lysed in TRIzol (Invitrogen) for RNA extraction.

2.15 RNA Isolation and qPCR

Following culture of monocytes or THP-1 cells, wells were washed with ice cold MACS buffer, before centrifugation at 1500g, followed by lysis with TRIzol (Invitrogen). After addition of chloroform and phase separation (as per the manufacturer's instruction) the aqueous phase was mixed 1:1 with 70% ethanol (Sigma) and the RNeasy Micro Kit (Qiagen) used for extraction of RNA. RNA was quantified using a Nanodrop Spectrophotometer. All 260/280 and 260/230 ratios were >1.9. 1000ng of RNA were used to make cDNA using the AffinityScript QPCR cDNA Synthesis Kit (Agilent Technologies).

qPCR was performed on the Stratagene Mx3005P qPCR system (Agilent Technologies) using Brilliant III SYBR Green Mastermix (Agilent Technologies). Intron spanning or flanking primer pairs were designed using Primer 3 [485, 486]. Primers were ordered from Sigma Aldrich (Table 2.6), and validated in house to assess the efficiency of amplification and check for primer dimer formation. Primers were used at a concentration of 100nM.

Data analysis was performed using MxPro software. Gene expression was calculated using the relative standard curve method (Bookout AL and Mangelsdorf DJ, 2003), and normalised to the endogenous reference gene cyclophilin A.

Table 2.6 Primers used to amplify RNA for Cyclophilin A, CD206 and CD200R1

Gene name	PubMed ID	Forward Primer	Reverse Primer	Amplicon Length
Cyclophilin A	NM_021130	GCATACGGGTCCTGGCATCTTGTCC	ATGGTGATCTTCTTGCTGGTCTTGC	201
CD206	NM_002438	AATGAACGTGTGTGGATCGC	ATCAGCAGCCCAGTTAGTGT	99
CD200R1	NM_138806	TGAAAGTCAATGGCTGCAGA	TCTGTGTAGCTGGCATAGGG	97

2.16 Metabolomics Analysis

Metabolomics analysis on patient and healthy serum samples was performed by Prof. Mika Ala-Korpela by Brainshake at the University of Eastern Finland using a biomarker analysis platform [196]. The metabolite quantification was performed using hydrogen nuclear magnetic resonance (HNMR) using two spectral windows LIPO and LMWV to give information on lipoprotein subclass distribution and low molecular weight metabolites including systemic amino acids, urea and creatinine. Models were cross-validated against NMR-independent lipoprotein and lipid data, and verified to lie within a 10% limit of the training data set or else were rejected from lipoprotein subclass analysis [487].

2.17 Data Analysis

iNKT cell absolute numbers were calculated for SLE patients by multiplying the percentage of CD3+iTCR+ PBMCs by patient lymphocyte counts obtained at the time of blood donation. For healthy donors iNKT cell frequency was multiplied by $2.5x10^9$ cells/litre, which was used as an average lymphocyte count according to UCLH reference guidelines.

GraphPad Prism software (La Jolla, USA) was used for statistical analysis of data. Data was tested for normal distribution using the Kolmogorov-Smirnov test and parameteric or non-parametric staitiscal tests used accordingly. Data was analysed by one-way ANOVA was used, followed by a Tukey T- test for significance or by students or paired T tests as appropriate. Linear regression was also performed. A 95% confidence interval was used to calculate significance. A bonferroni correction was additionally used for the metabolomics data to determine the threshold for significance.

CHAPTER III: Results 1

Results 1 – iNKT cell phenotype in SLE patients with atherosclerosis

3.1 Introduction and Aims for this chapter

SLE patients have a significantly increased risk of cardiovascular disease compared to the general population; however the mechanisms underpinning the development of atherosclerosis in these patients remain uncertain. Both SLE and atherosclerosis are immune mediated disorders characterised by lipid abnormalities in terms of altered cellular lipids and dyslipidaemia [143, 367, 488]. Furthermore, lipid recognition by iNKT cells has previously been shown to be defective and could therefore play an important role in the pathogenesis of both diseases [234].

Previous studies have shown that iNKT cells protect against the development of autoimmune disease, where they are decreased in number and fail to respond to stimuli such as α-GalCer treatment [233, 436]. This has led many to believe that iNKT cells play an immunoregulatory role under normal homeostatic conditions, which is lost in the context of autoimmune diseases such as SLE [409]. In contrast, iNKT cells are reported to be increased in atherosclerosis, where they play a pathogenic role in atherosclerotic plaque development [459]. However most of the data on atherosclerosis is based on animal models, whilst there is a distinct lack of data on the role of iNKT cells in SLE patients who have subsequently developed plaques. I therefore compared the phenotype and functional characteristics of iNKT cells from SLE patients with preclinical plaque (SLE-P), SLE patients without plaque (SLE-NP) and healthy donors.

3.2 The presence of subclinical plaque in SLE patients was associated with traditional cardiovascular risk factors

To assess the role of iNKT cells in the development of atherosclerosis in SLE patients, I examined a cohort of 100 SLE patients that had not previously suffered a cardiovascular event and had received ultrasound scans to determine the presence of preclinical atherosclerotic plaque in the right and left carotid and femoral arteries (methods section describes patient selection criteria and details of plaque analysis). SLE patients were characterised according to the number of plaque sites which was calculated by arterial ultrasound scores with an AUS score of 6 or more indicative of plaque at each site.

Patients were categorised as having plaque (SLE-P) or no plaque (SLE-NP) based on data from ultrasound scans. Table 3.1 shows clinical, demographic, serological, therapy and disease activity characteristics of patients in the plaque and no plaque groups, which were compared by student's t tests or one way ANOVAs where applicable.

None of the patients or healthy donors were categorised as obese as determined by body mass index over 30, or had previously been diagnosed with diabetes. The only significant differences between groups were that patients with plaque were older, had longer disease duration and higher mean systolic blood pressure than those without plaque. In particular there were no associations between plaque and patient medication or any measure of disease activity including anti-dsDNA, C3 levels and BILAG score at the scan and on subsequent visits.

	Healthy donors (n=50)	SLE-NP (n=64)	SLE-P (n=36)	Statistical Analysis
Mean Age (range)	39 (22-61)	40 (20-66)	54 (27-69)	[a]HC vs SLEP p<0.001** [a]SLENP vs SLEP p<0.001**
Years since diagnosis (range)	NA	13 (2-32)	21 (2-46)	[a]P=0.0008***
Sex: female: male	3:47	62:2	33:3	[a]NS
Ethicity: C/A/AC/O (%)	40/4/6/0 (80: 7: 13: 0)	33/9/17/5 (51:14:27:8)	24/2/7/3 (67: 6: 19: 8)	[a]H vs SLE-NP p<0.05* [a]H vs SLE-P p<0.05*
Smoking (%)	10 (20)	5 (7.8)	6 (16.7)	[a]NS
Personal history of CVD (%)	0	0	0	[a]NS
Systolic blood pressure/mmHg (range)	ND	122.9 (94-166)	132.3 (108-168)	[b]p=0.0045**
Diastolic blood pressure/mmHg (range)	ND	76.1 (57-109)	75.1 (55-96)	[b]p=0.651
Pulse Pressure/mmHg (range)	ND	46.8 (25-72)	57.1 (30-81)	[b]p<0.0001***
Mean blood pressure/mmHg (range)	ND	91.7 (70-125)	94.1 (77-115)	[b]p=0.288
Diabetes	0	0	0	NA
Obesity (BMI>30)	0	0	0	NA
Statins (%)	0 (0)	6 (9.4)	7 (18.9)	[b]p=0.394
ACE Inhibitor (%)	0 (0)	23 (35.9)	12 (33.3)	[b]p=0.830
Aspirin (%)	0 (0)	7 (10.9)	7 (19.4)	[b]p=0.483
Hydroxychloroquine (%)	NA	42 (65.6)	23 (63.8)	[b]p=0.863
Pred ≥10mg/day (%)	NA	9 (14.1)	5 (13.9)	[b]p=0.991
Pred <10mg/day (%)	NA	33 (51.5)	17 (47.2)	[b]p=0.885
HCQ + disease modifying agents (%)	NA	22 (34.2)	6 (16.7)	[b]p=0.060
Pred + disease modifying agents (%)	NA	26 (40.6)	13 (36.1)	[b]p=0.707
Rituximab (%)	NA	18 (28.1)	9 (25.0)	[b]P=0.737

Global BILAG score at time of scan (range)	NA	4.46 (0 – 17)	3.94 (0 – 32)	[b]p=0.655
Persistently active (%)	NA	26 (40.6)	22 (61.1)	[b]P=0.112
Median anti-dsDNA antibodies (range)	NA	89.97 (1-688)	109.0 (1-712)	[b]p=0.192
Median C3 (range) g/L	NA	0.99 (0.49-1.42)	1.026 (0.69-1.46)	[b]p=0.643

Table 3.1: SLE patient and healthy donor characteristics*:*

100 SLE patients fulfilling the revised classification criteria for SLE [36] from the University College Hospital London SLE clinic and who had not previously suffered a cardiovascular event were scanned to determine the presence of plaque. At the time of the scan patients were assessed for smoking, blood pressure, medication and serum concentrations of anti-dsDNA antibodies and complement protein C3. Disease activity was assessed using the BILAG index, which distinguishes activity in 9 organs/systems [38]. Patients with active SLE disease had a BILAG global score >6. Patients with persistently active disease were identified as having a Global BILAG score >5 in at least two consecutive visits from the last four visits. Data expressed as mean except for anti-ds-DNA antibody and C3 levels. 50 healthy donors were also recruited to the study. Healthy donors were not scanned for plaque but demographic information was collected. Data were analysed using either a One-way ANOVA[a] or Mann Whitney test[b] for significance using a 95% confidence interval. *** and ** denote significant results.

BILAG - British Isles Lupus Assessment Group; dsDNA – double stranded DNA; NA – not applicable; ND – not determined; NS – not significant; SLE-NP – SLE patients without plaque; SLE-P – SLE patients with plaque; Pred – prednisolone

3.3 Characteristics of healthy donors

Healthy donors (who were not scanned) were matched to SLE-NP patients according to gender and age. Similar to both SLE-NP and SLE-P patient groups healthy donors had not previously suffered a cardiovascular event. A cardiovascular risk factor questionnaire was used to assess smoking status, the use of anti-hypertensive medication or statins as well as personal and family history of cardiovascular disease (i.e. MI or stroke) (Appendix), calculation criteria described in Figure 3.1. SLE-CV patients who had suffered a cardiovascular event were not included in the ana

Healthy donors included in the study were more similar to SLE-NP patients in terms of the frequency of cardiovascular risk factors reported, suggesting that they were unlikely to have plaque (Table 3.1 and Figure 3.1). For SLE-P patients the most reported risk factors were age>50, hypertension, current or past smoker and a family history of cardiovascular disease.

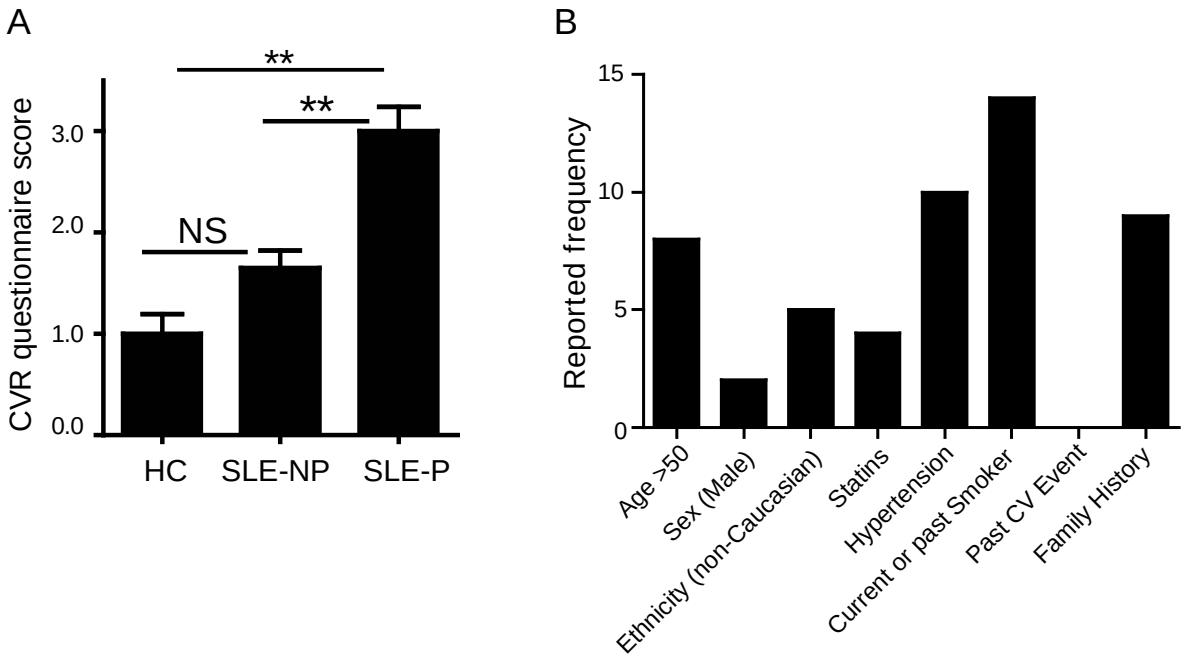


Figure 3.1 Differences in the cardiovascular risk factor profiles of healthy controls and SLE patients with and without plaque.

100 patients from the SLE clinic at University College London Hospital underwent ultrasound scans to determine the presence of carotid and femoral plaque. Patients were given a score, according to the level of thickening at the different sites, with a score >6 being indicative of the presence of plaque at one or more sites. (A) Cardiovascular risk questionnaire scores from healthy controls (n=50) and SLE patients with (n=28) or without plaque (n=34). For each subject, one point was given for each reported risk factor including age (over 55), sex (male), ethnicity (African/Caribbean or Asian), statins, anti-hypertensive medication, smoking (current or past smoker), past cardiovascular event and family history of cardiovascular disease. One way ANOVA **p=<0.01, NS - not significant; HC – healthy controls; SLE-NP – SLE patients without plaque; SLE-P – SLE patients with plaque (B) The most frequently reported risk factors amongst SLE patients with plaque (n=28) as analysed by questionnaire.

3.4 SLE patients with plaque have increased numbers of iNKT cells compared to SLE patients without plaque.

Peripheral blood iNKT cell expression levels were assessed by flow cytometry, according to the gating strategy shown in Figure 3.2A. Previous data showing that SLE patients had reduced iNKT cell numbers compared to healthy donors was confirmed in SLE patients without plaque (SLE-NP) [233], however, this significant reduction was lost in SLE patients with plaque (SLE-P) (Figure 3.2B and C).

In order to determine whether the increase in iNKT cells in SLE-P compared to SLE-NP patients was attributable to factors other than the presence of plaque, iNKT cell absolute numbers were correlated with age, disease duration, systolic blood pressure, patient medication and BILAG (disease activity) score. Despite the observation of a significant negative correlation between age and iNKT cell frequency in healthy individuals ($p=0.001$), which was in accordance with the literature [489], the discrepancy between iNKT-cell frequency in patients with and without plaque was not explained by age (one of the major differences between SLE-NP and SLE-P patients) (Figure 3.3A&B). In addition, no association was found between iNKT cell frequency and disease duration or systolic blood pressure (Figure 3.3B). A similar trend of increased iNKT cell numbers in SLE-P patients compared to SLE-NP patients was observed when patients were stratified according to patient medication (Figure 3.3C). No significant differences were observed between SLE patients with active (BILAG>5) and inactive (BILAG≤5) disease when separated into SLE-NP and SLE-P groups (Figure 3.3D).

To investigate whether the increased iNKT cell numbers in SLE-P patients remained consistent over time healthy donors, SLE-NP and SLE-P patients were assessed for iNKT cell frequency on multiple visits. Despite clearly being variable between individuals, iNKT cell frequency remained stable for most individuals in the months following ultrasound scans of the patients, with similar observations for healthy donors (Figure 3.4).

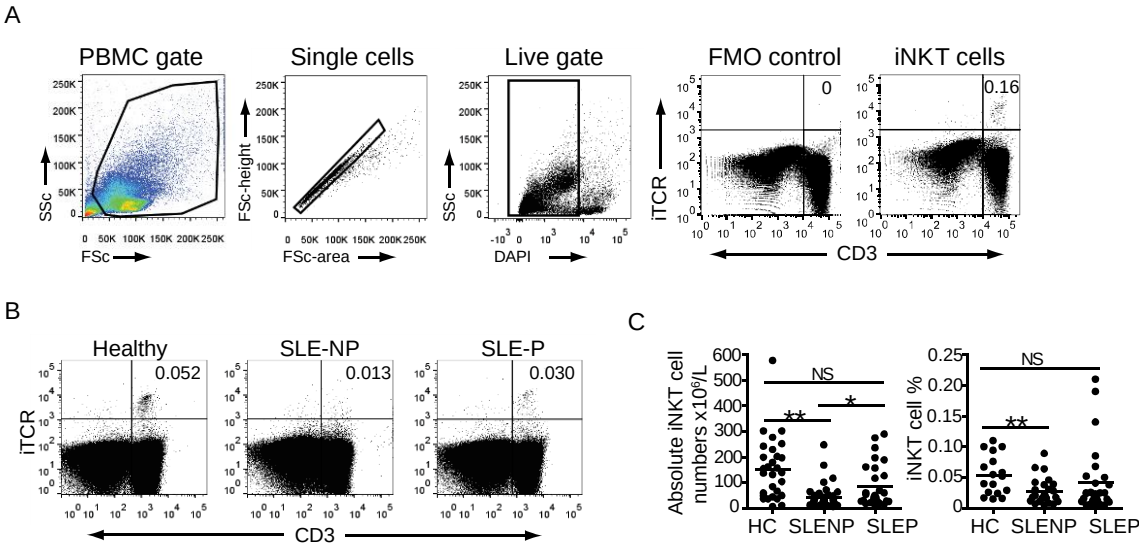


Figure 3.2 Increased iNKT cell frequency in SLE patients with plaque.

PBMC from healthy donors (n=52), SLE-NP (n=30) and SLE-P (n=29) patients were assessed for iNKT cell frequency using antibodies to CD3 and invariant T cell receptor (iTCR). Gating strategy for iNKT cells **(A)** showing gating on total PBMCs, followed by single cells as determined by forward scatter height and area, then live cells which were negative for DAPI. iNKT cell population size was quantified by cells which were positive for both CD3 and the iTCR. $CD3^{+}iTCR^{+}$ populations were gated according to fluorescent minus one (FMO) controls. Representative flow cytometry dot plots **(B)** and cumulative data **(C)** showing iNKT cell frequency (absolute numbers and mean percentage) in healthy (n=52), SLE-NP (n=40) and SLE-P (n=34) populations. One-Way ANOVA and Tukey's multiple comparison post-test; **p=<0.001, *p=<0.05, NS=not significant. HC – healthy controls; SLE-NP – SLE patients without plaque; SLE-P – SLE patients with plaque

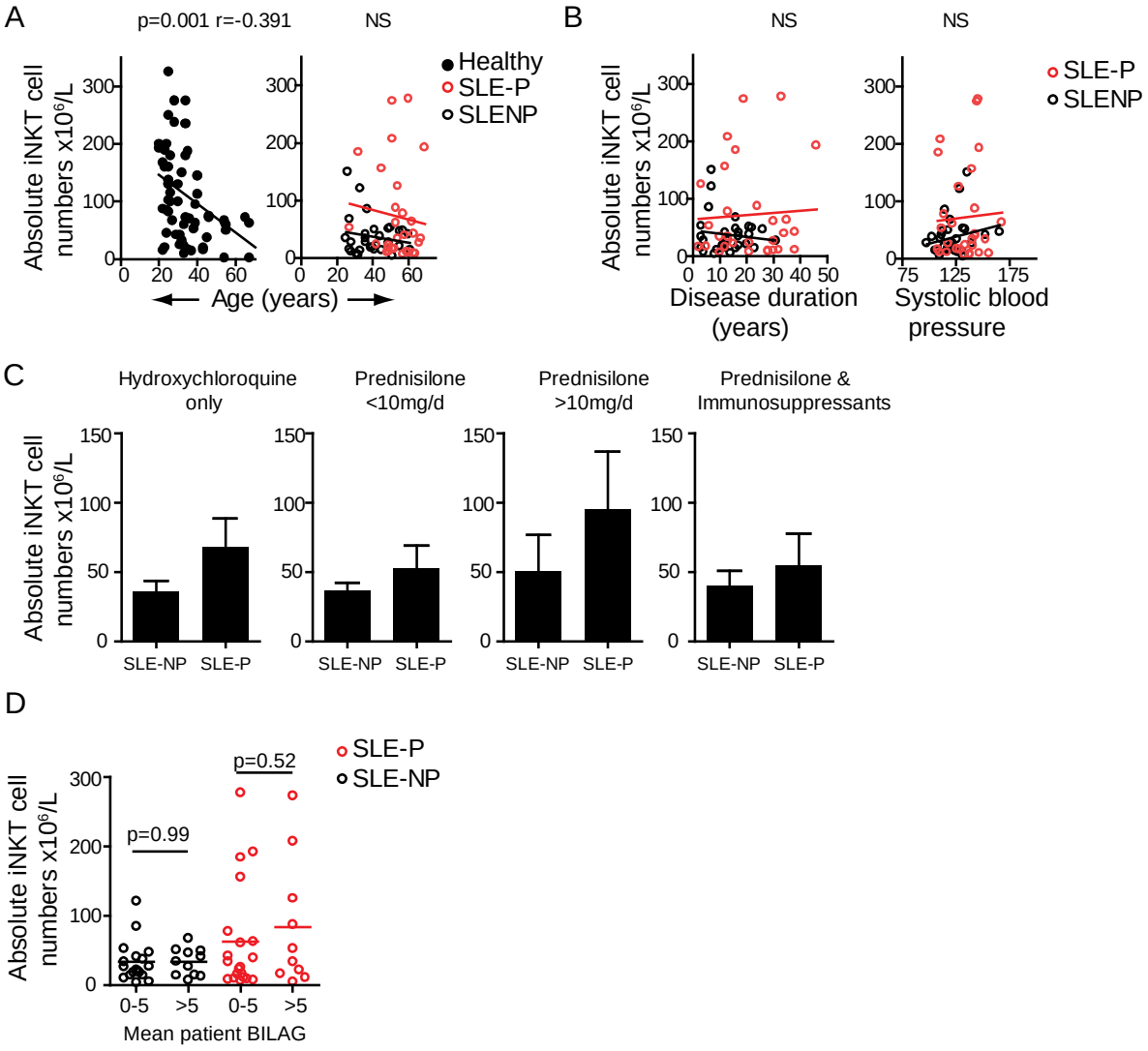


Figure 3.3 Correlation of iNKT cell numbers with age, disease duration, systolic blood pressure, patient medication and disease activity

PBMC from healthy donors (n=52), SLE-NP (n=30) and SLE-P (n=29) patients were assessed for iNKT cell frequency using antibodies to CD3 and iTCR before correlating with age. iNKT cells were analysed according to disease duration, systolic blood pressure, medication and BILAG score (to measure disease activity) in SLE patients. (A) Correlation in healthy donors, SLE-NP and SLE-P patients between iNKT cell absolute number and age. (B) Correlation between iNKT cell absolute number and disease duration and systolic blood pressure in SLE-P (n=22-27) and SLE-NP (n=26-37) patients. Pearson's correlation r= -0.391, *p*=0.001. (C) Graphs showing the influence of patient medication on absolute iNKT cell numbers including hydroxychloroquine, prednisolone and immunosuppressants. Mean ± SE. Students T test *p=<0.05. (D) Stratification of patients according to BILAG score and subsequent analysis of iNKT cell frequency in disease active (BILAG>5) and inactive (BILAG≤5) SLE-NP and SLE-P patients Mean, Students T test *p=<0.05. SLE-NP – SLE patients without plaque; SLE-P – SLE patients with plaque

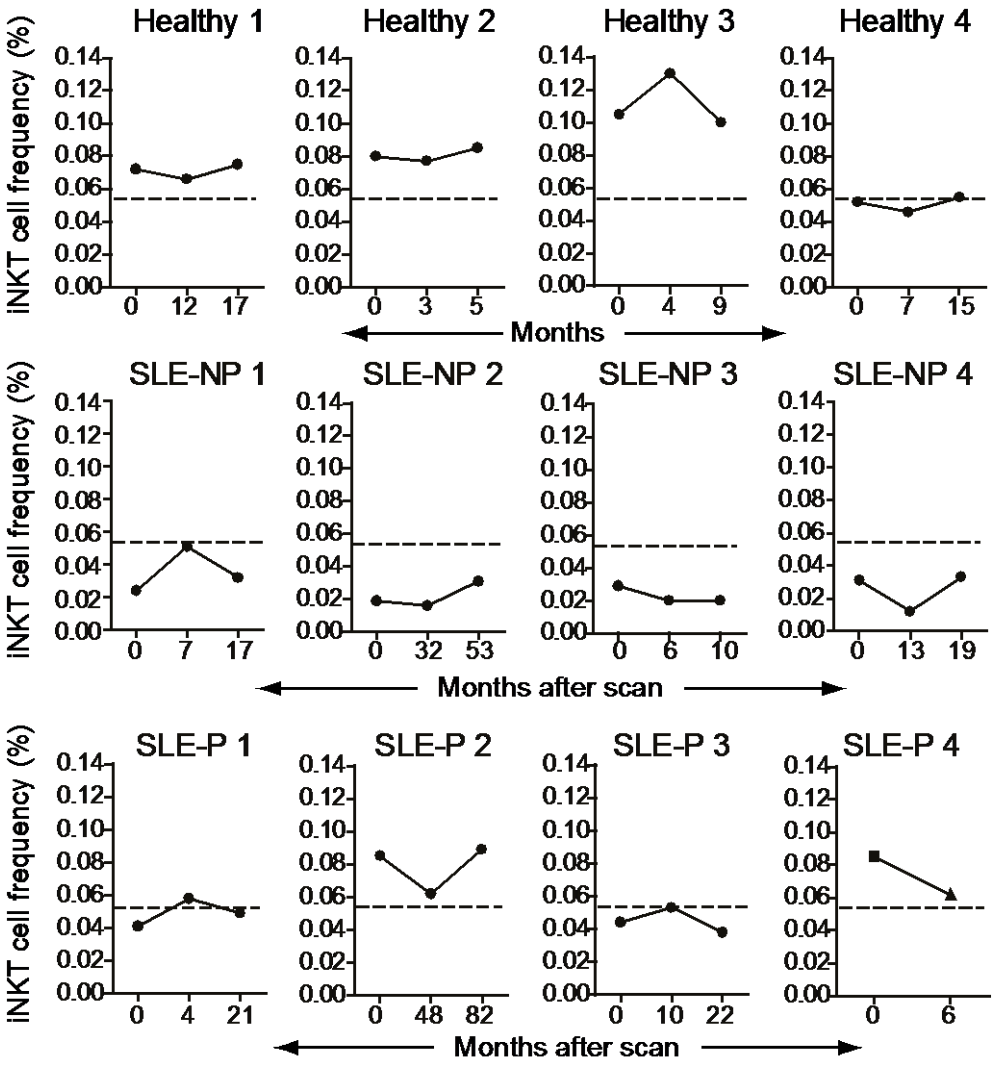


Figure 3.4 Analysis of iNKT cell frequency over time

iNKT cell frequency was assessed in PBMCs collected at different time points in healthy donors or SLE patients after the original scan. Graphs showing longitudinal differences in the frequency of iNKT cells in 4 representative healthy doors, SLE patients with plaque (SLE-P) and SLE patients without plaque (SLE-NP). Dotted line indicates the mean iNKT cell frequency of healthy donors.

3.5 iNKT cells from SLE-P patients are characterised by increased CD4 expression and a more activated phenotype compared to healthy controls

As $CD4^+$ iNKT cells have previously been shown to play a pro-atherogenic role in mouse models of atherosclerosis [465], I decided to investigate whether iNKT cell phenotype is altered in SLE patients with preclinical plaque. $CD4^+$ and $CD8^+$ iNKT cell populations were gated using the distribution of these subsets in $CD3^+iTCR^-$ T cells. SLE-P patients had a distinct phenotype characterised by a switch towards CD4 positivity and a significant decrease in double negative ($CD4^-CD8^-$) iNKT cells compared to SLE-NP patients and healthy donors (Figure 3.5A&B).

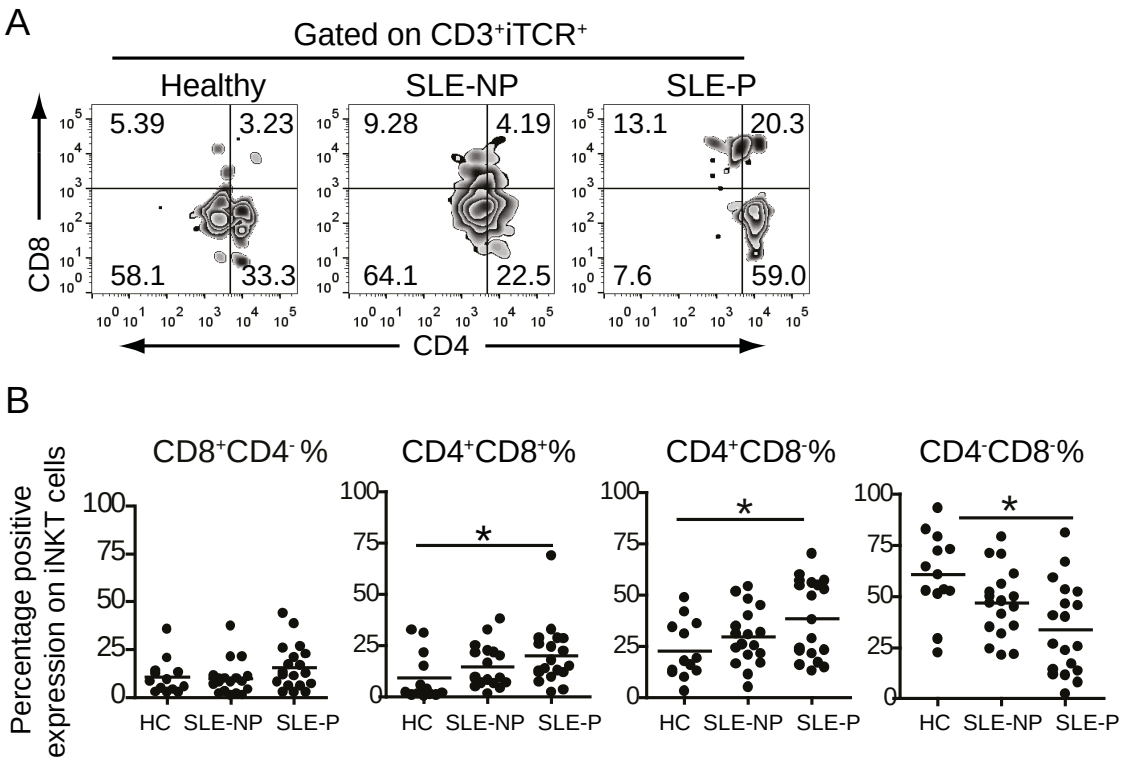


Figure 3.5 iNKT cells from SLE-P patients show increased CD4 expression compared to healthy controls

Analysis of iNKT cell phenotype was performed using polychromatic flow cytometry in healthy (n=13), SLE-NP (n=18) and SLE-P (n=19) populations. CD3, iTCR, CD4 and CD8 labelling was used to identify iNKT cell subsets. **(A)** Representative dot plots and **(B)** cumulative data. One-Way ANOVA and Tukey's multiple comparison post-test; *p=<0.05. HC – healthy controls; SLE-NP – SLE patients without plaque; SLE-P – SLE patients with plaque

3.6 iNKT cells from SLE-P patients have a distinct phenotype compared to healthy individuals and SLE-NP patients

In order to assess whether iNKT cells are differentially activated or are functionally altered in SLE patients with preclinical plaque, I investigated the expression of several surface markers on iNKT cells by flow cytometry by staining for CD25, CD69, CD161, PD-1 and CCR6. Gates were set on healthy donor $CD3^{+}iTCR^{-}$ T cells with reference to fluorescence minus one (FMO) controls. Interestingly SLE-P patients had an altered phenotype compared to SLE-NP patients. Analysis by mean fluorescence intensity revealed a significant reduction in PD-1 expression on iNKT cells from SLE-P patients compared to SLE-NP patients. Subsequent quantification of the percentage of iNKT cells positive for each marker showed that iNKT cells from SLE-P patients had significantly elevated levels of CD69 and reduced expression levels of CD161 (NK marker) and PD-1 (Programmed cell death protein 1, CD279) compared to SLE-NP patients and healthy controls while CD25 and CCR6 expression levels were elevated in iNKT cells from both SLE-P and SLE-NP patients compared to healthy donors (Figure 3.6).

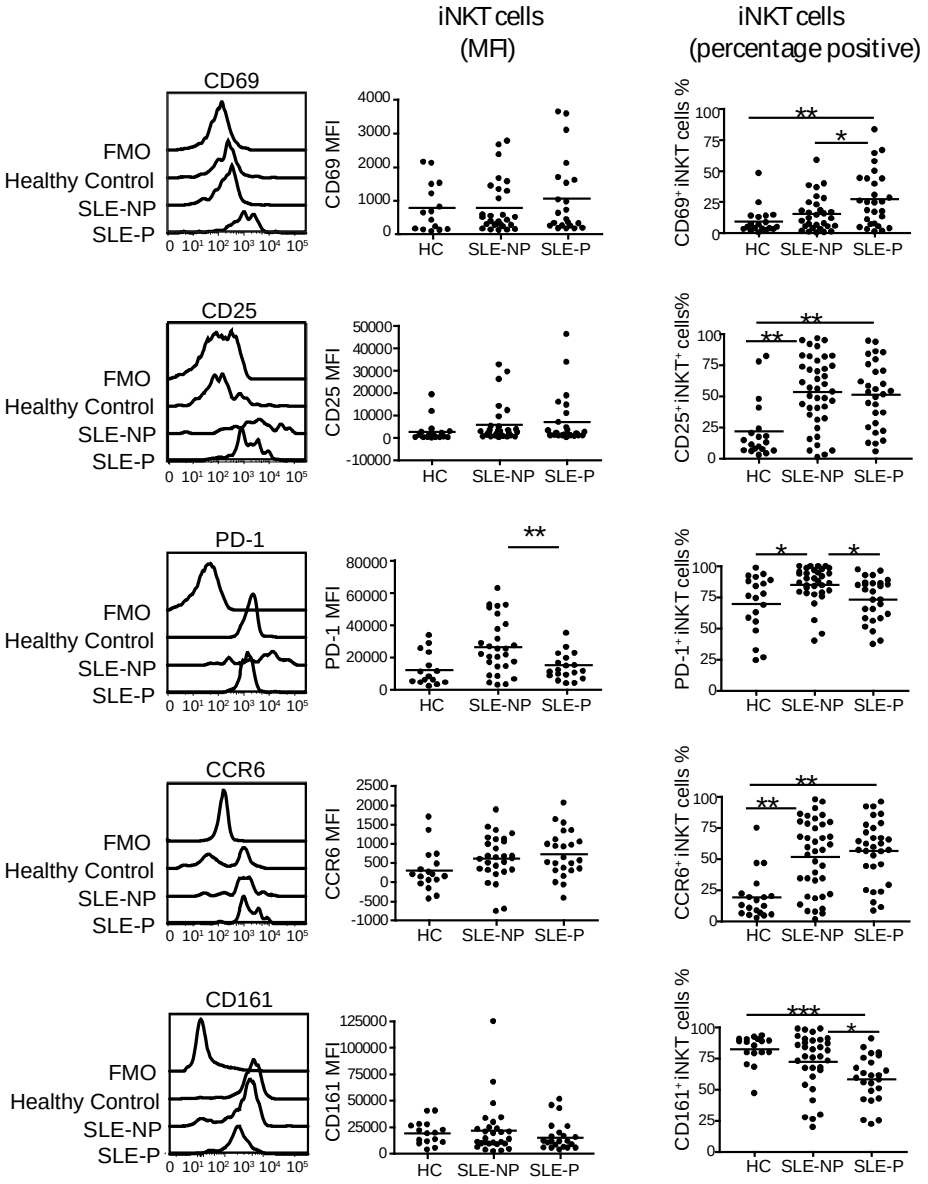


Figure 3.6 iNKT cells from SLE patients with plaque show differential activation marker expression.

iNKT cell phenotype was examined on PBMCs from healthy donors (n=21), SLE-NP (n=29) and SLE-P (n=26) patients, which were stained for CD3, iTCR, CD69, CD25, PD-1, CCR6 and CD161. Representative histograms and cumulative data for MFI (mean fluorescence intensity) and percentage positive expression of CD69, CD25, PD-1, CCR6 and CD161 in iNKT cells are shown. One-Way ANOVA and Tukey's multiple comparison post-test; ***p=0.0001; **p=<0.001, *p=<0.05***. FMO= fluorescence minus one control. HC – healthy controls; SLE-NP – SLE patients without plaque; SLE-P – SLE patients with plaque

3.7 The iNKT cell phenotype observed in SLE-P patients is not observed in conventional CD4+ and CD8+ T cells, and is not explained by differences in expression within CD4 and CD8 iNKT cell populations

In order to investigate whether the phenotype observed in plaque positive iNKT cells was specific to iNKT cells, I investigated the expression of the same surface markers (CD25, CD69, CD161, PD-1 and CCR6) in iTCR$^-$ CD4$^+$ and CD8$^+$ T cells. Importantly many of the surface markers were expressed at much lower levels in CD4+ and CD8+ T cells compared to iNKT cells indicating that they were of lesser importance in conventional T cell subsets. Comparison of healthy and patient groups by one-way ANOVA showed that the iNKT cell phenotype observed in figure 3.6 was unique and not recapitulated in conventional CD3$^+$CD4$^+$ or CD3$^+$CD8$^+$ cells (Figure 3.7). However, increased expression of CD25 and PD-1 and CCR6 in CD4$^+$ conventional T cells from SLE-P patients indicated that conventional T cells as well as iNKT cells were more activated in patients with preclinical plaque.

Investigation of CD4$^+$ and CD8$^+$ iNKT cells showed no differences in surface marker expression (CD25, CD69, CD161, PD-1 and CCR6) between SLE-NP and SLE-P patients when ANOVA was performed (Figure 3.8). Interestingly however, some differences were observed between iNKT cells from healthy donors and SLE patients. This was particularly evident for CCR6 expression which was significantly elevated in SLE-P compared to healthy donors within all CD4 and CD8 populations analysed. Other differences were mainly confined to the CD4$^+$ iNKT cells, and included an increase in expression of the activation markers CD25 and CD69. The differences in CD161 expression observed in Figure 3.6 could in part be explained by the CD8$^+$ iNKT cells where CD161 levels were significantly lower in the SLE-P patients compared to healthy donors.

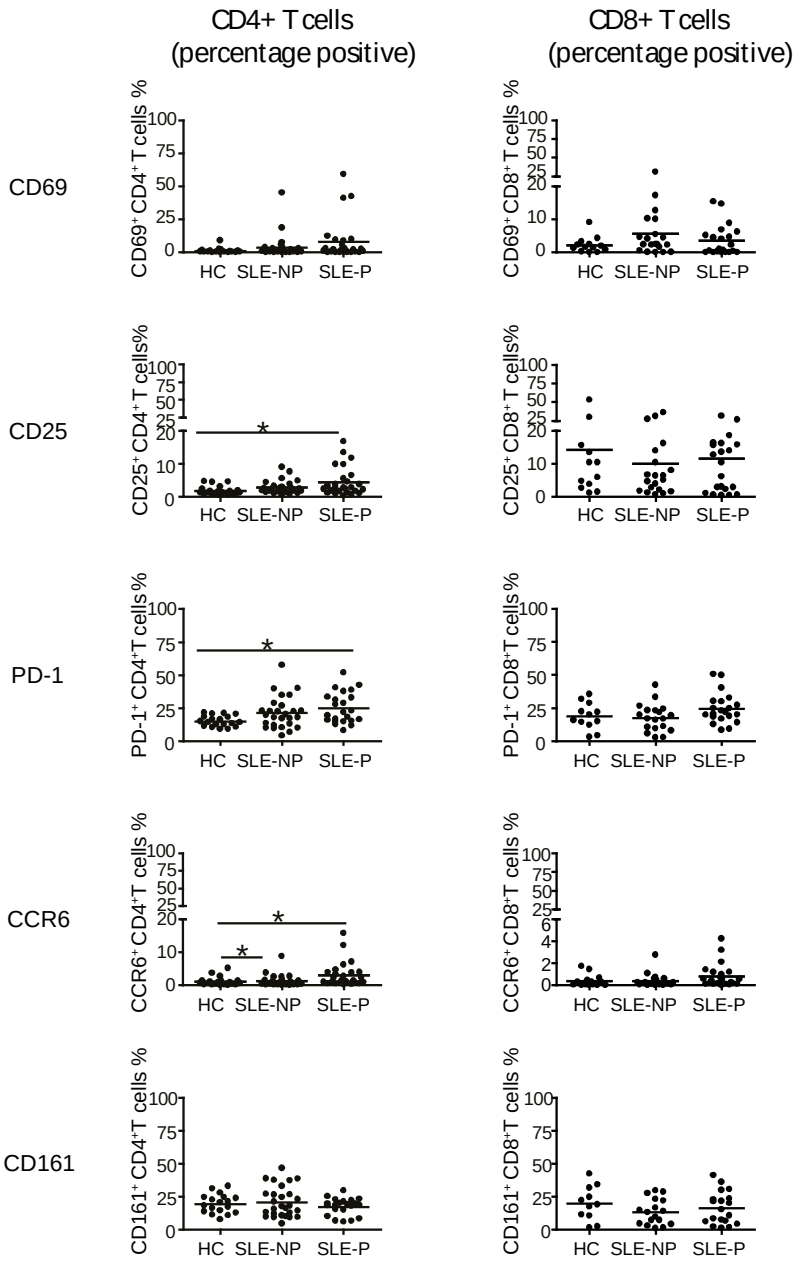


Figure 3.7 Activation marker expression is unaltered on conventional T cells from SLE patients with plaque

$CD4^+$ and $CD8^+$ T cell phenotype was examined on PBMCs from healthy donors (n=21), SLE-NP (n=29) and SLE-P (n=26) patients, which were stained for CD3, iTCR, CD69, CD25, PD-1, CCR6 and CD161. Cumulative data showing the percentage of cells positive for CD69, CD25, PD-1, CCR6 and CD161 on $CD3^+iTCR^-$ $CD4^+$ and $CD8^+$ T cells are shown. One-Way ANOVA and Tukey's multiple comparison post-test; *p=<0.05. HC – healthy controls; SLE-NP – SLE patients without plaque; SLE-P – SLE patients with plaque

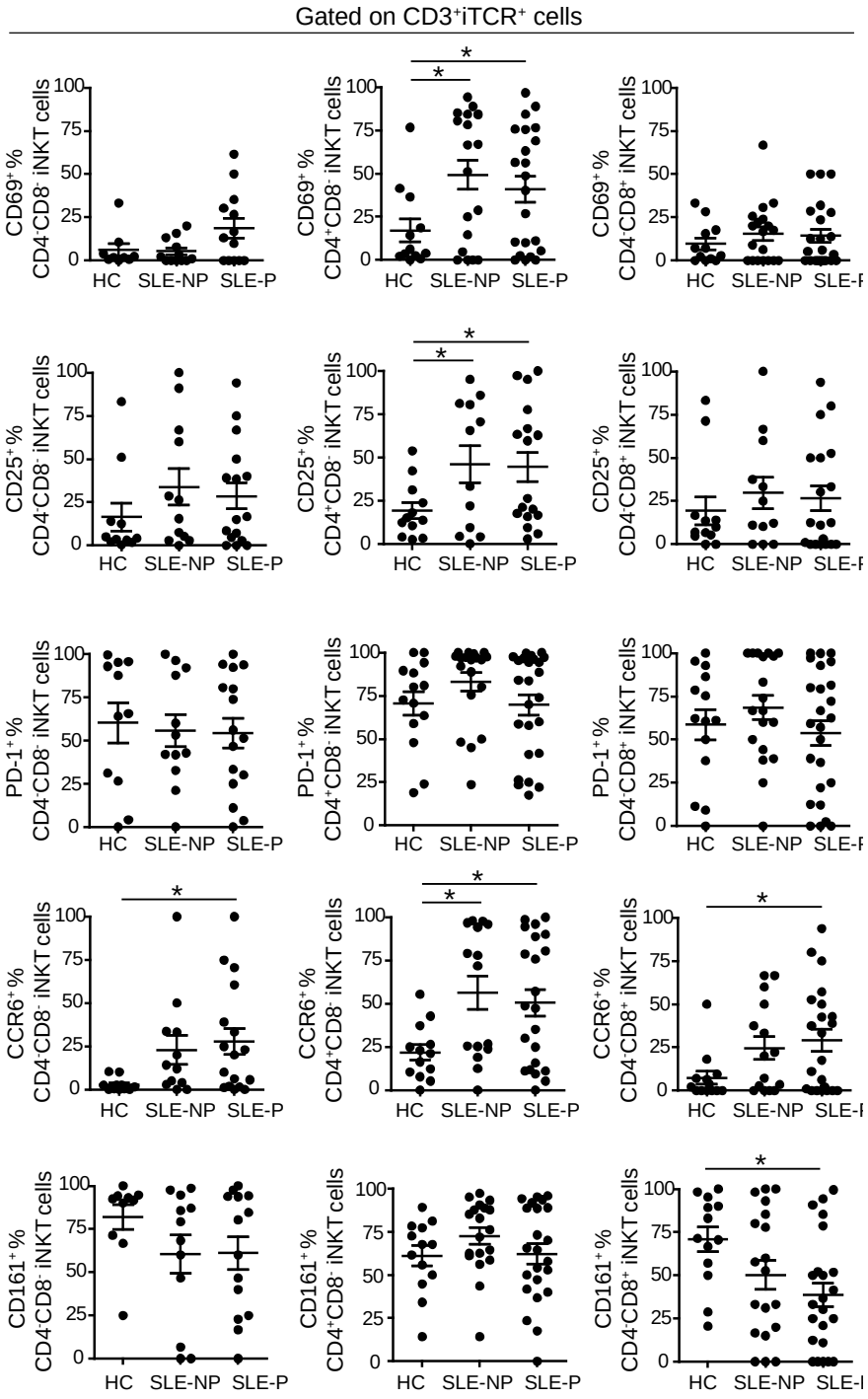


Figure 3.8 Expression of surface markers within CD4+ and CD8+ iNKT cell subsets show no difference between SLE-NP and SLE-P patients

Surface marker expression was examined on $CD4^+$ and $CD8^+$ iNKT cell subsets from healthy donors (n=21), SLE-NP (n=29) and SLE-P (n=26) patients, which were stained for CD3, iTCR, CD4, CD8, CD69, CD25, PD-1, CCR6 and CD161. Cumulative data showing the percentage of cells positive for CD69, CD25, PD-1, CCR6 and CD161 on $CD4^-CD8^-$ $CD4^+CD8^-$ and $CD4^-CD8^+$ iNKT cells. One-Way ANOVA and Tukey's multiple comparison post-test; *p=<0.05. HC – healthy controls; SLE-NP – SLE patients without plaque; SLE-P – SLE patients with plaque

3.8 Low CD161 expression on iNKT cells from SLE patients with preclinical plaque is associated with decreased iNKT cell activation marker expression

CD161 expression is associated with a number of T cell and NK cell functions, most notably activation [222, 490-492], however its role in iNKT cell populations remains uncertain. I questioned whether decreased expression of CD161 in SLE-P patients was associated with decreased expression of activation markers CD25, CD69 and PD-1. I therefore performed correlations between CD161 and other activation markers in healthy individuals, SLE-NP and SLE-P patients (Figure 3.9). Positive correlations were observed between CD161 and PD-1, CD69 and CD25 in SLE-P patients, which were found to be significant, suggesting an association between low CD161 expression on iNKT cells from SLE-P patients and reduced iNKT cell activation.

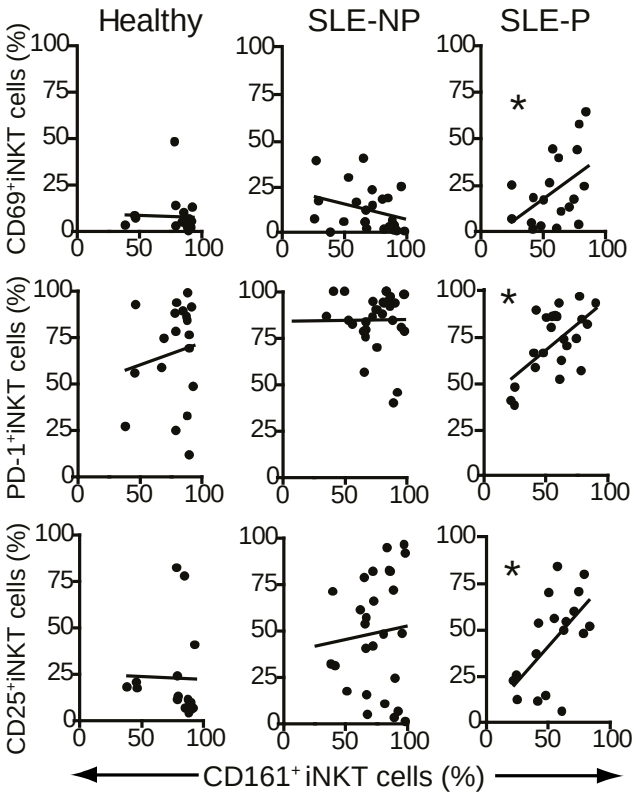


Figure 3.9 CD161 expression is positively correlated with activated increased iNKT cell activation marker expression in SLE-P patients.

PBMCs were assessed for iNKT cell phenotype as described in Figure 3.2-5. Correlation plots relating iNKT cell expression of CD161 with expression of activation markers CD69, PD-1 and CD25 on iNKT cells from healthy donors (n=20), SLE-NP (n=30) and SLE-P (n=27) patients. Pearson's correlation significant only in SLE-P patients; CD69 *p=0.038, r=0.478; PD-1: *p=0.002, r=0.603; CD25 *p=0.01, r=0.587. SLE-NP – SLE patients without plaque; SLE-P – SLE patients with plaque

3.9 iNKT cells from SLE-P patients show increased proliferative capacity compared to SLE-NP patients

I questioned whether the increase in iNKT cell frequency and altered activation marker expression observed in SLE-P patients compared to SLE-NP patients could be associated with functional differences in iNKT cells such as proliferation. To test this, PBMCs from healthy donors, SLE-NP and SLE-P patients were stimulated for 7ds with 100ng/ml α-GalCer (a potent iNKT cell agonist) and 400IU/ml IL-2. At d7 cells were stained for CD3 and the iTCR as well as Ki67 (a marker of cell proliferation) and costimulatory molecules ICOS and CD40L. Cells were also stained for CD4, CD25 and CD127 to identify Tregs.

iNKT cells from healthy donors and SLE-P patients showed increased proliferation as measured by Ki67 expression compared to SLE-NP patients (Figure 3.10A-C) in response to 7 day α-GalCer/IL-2 stimulation. Similar trends were observed when both the MFI of Ki67 expression (Figure 3.10A) and the percentage of Ki67 positive cells (Figure 3.10B) were analysed, although whether there was an increase in proliferation in healthy and SLE-P iNKT cells at day 7 compared to day 0 or whether it simply remained stable is to be determined. Furthermore, at day 7 the significant decrease in Ki67 in SLE-NP iNKT cells compared to SLE-P iNKT cells was associated with proliferative responsiveness, which was found to be low in SLE-NP patients as has previously been reported [233] (Figure 3.10D&E). Analysis of co-stimulatory molecules revealed that ICOS expression on iNKT cells was increased in SLE-P compared to SLE-NP patients, but this wasn't the case for CD40L (Figure 3.10F). No difference was observed in Tregs between SLE-NP and SLE-P patients suggesting that differences in Treg frequency were not responsible for the increased iNKT cell expansion observed in SLE-P patients (Figure 3.10G).

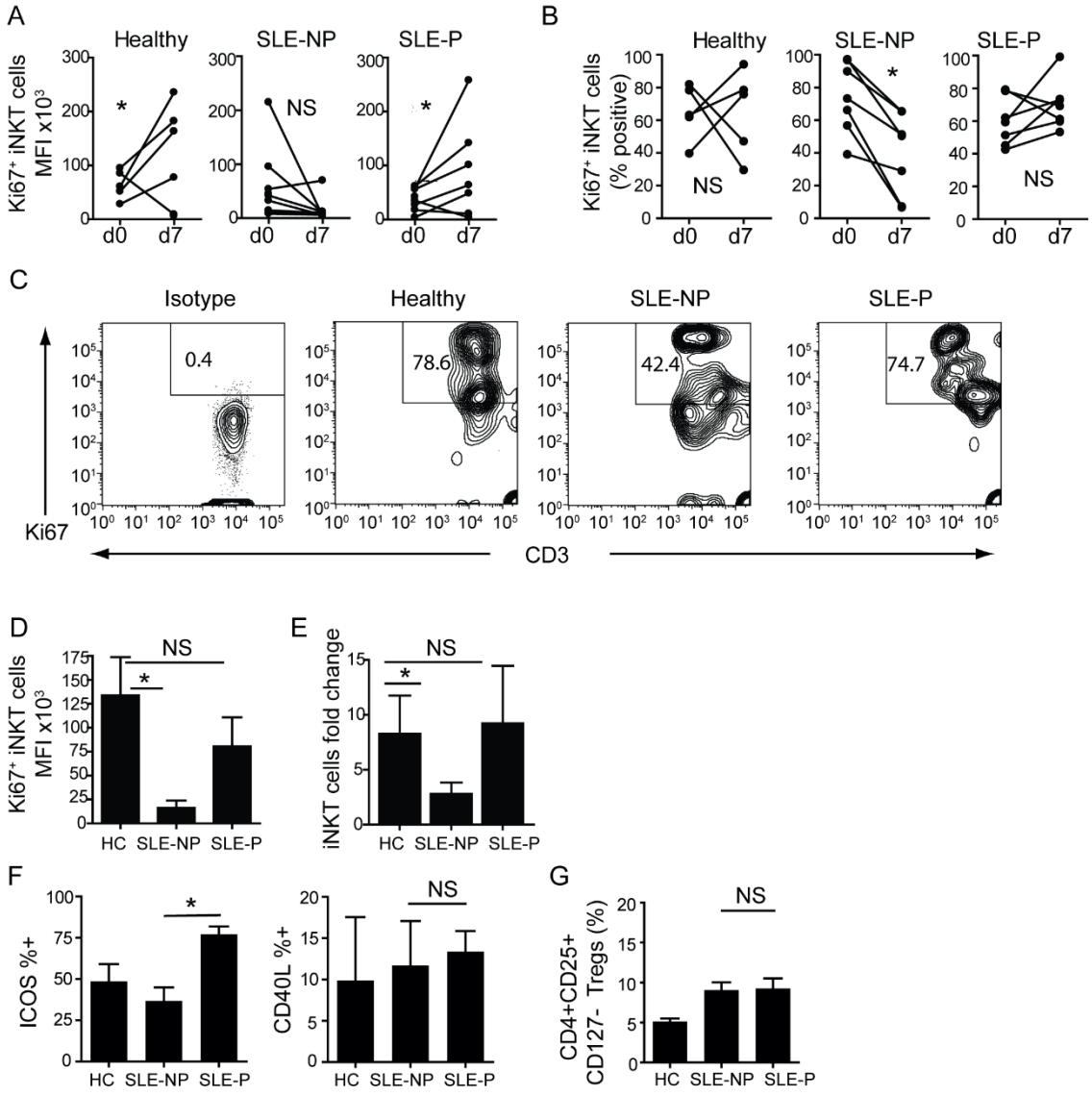


Figure 3.10 iNKT cells from SLE-P patients but not SLE-NP patients undergo proliferation in response to α-GalCer stimulation

PBMC from 5 HC, 7 SLE-NP and 7 SLE-P patients were cultured for 7ds with α-GalCer and IL-2 and assessed for the surface expression of CD3, iTCR and intracellularly for Ki-67 to detect iNKT cell proliferation. iNKT cells were also stained for costimulatory molecules ICOS and CD40L, with a separate panel for CD4, CD25 and CD127 to identify $CD4^+CD25^+CD127^-$ Tregs. Cumulative data showing (A) mean fluorescence intensity (MFI) of Ki67 and (B) percentage of Ki67+ iNKT cells at d0 and 7d culture. (C) Representative plots are shown for day 7. Paired T test; *p=<0.05. (D) iNKT cell Ki67 expression mean fluorescence intensity (MFI) at d7. Mean ± SE. One-way ANOVA and Tukey's multiple comparison post-test; *p=<0.05. (E) Fold change in iNKT cell expression levels at d7 compared to d0. Mean ± SE. One-way ANOVA and Tukey's multiple comparison post-test; *p=<0.05. (F) Percentage of cells positive for ICOS and CD40L expression at d7. (G) Percentage of CD4+CD25+CD127- Tregs at d7. Students T test *p=<0.05. HC – healthy controls; SLE-NP – SLE patients without plaque; SLE-P – SLE patients with plaque

3.10 iNKT cells from SLE-P patients show increased IL-4 production compared to SLE-NP patients

Intracellular cytokine staining was carried out to assess iNKT cell function in SLE-P patients compared to SLE-NP patients and healthy controls. Whilst little change in IFN-γ was observed between patient groups, IL-4 was significantly elevated in iNKT cells from SLE-P patients compared to both SLE-NP patients and healthy donors (Figure 3.11A). Similarly IL-10 was increased in iNKT cells from SLE-P (but not SLE-NP patients) compared to healthy controls (Figure 3.11B), suggesting an immune-regulatory or Th2 cytokine profile. Further analysis of IFN-γ: IL-4 ratio showed that SLE-P patients were skewed towards an IL-4 response (Figure 3.11C).

To determine whether the increased IL-4 production by iNKT cells from SLE-P patients was associated with a Th2 phenotype, PBMC were stained intracellularly for Th1-associated transcription factor T-Bet and the Th2-associated transcription factor GATA-3. Analysis according to percentage positive expression showed increased expression of GATA-3 compared to T-Bet in SLE-P patients (Figure 3.12). This indicated that iNKT cells from SLE-P patients had a Th2-like phenotype and supported the previous observation of increased IL-4 expression in iNKT cells from these patients (Figure 3.11).

Due to my earlier finding of an increase in $CD4^+$ iNKT cells in SLE-P patients, I questioned whether $CD4^+$ iNKT cells were responsible for the Th2 cytokine profile observed in SLE-P patients. Comparison of $CD4^+$ iNKT cells from SLE-NP and SLE-P patients revealed that $CD4^+$ iNKT cells from SLE-P patients produced more IL-4 (but not IFN- γ and IL-10) compared to $CD4^+$ iNKT cells from SLE-NP patients (Figure 3.13A).

To assess whether $CD4^+$ or $CD8^+$ iNKT cells were responsible for the Th2 cytokine profile observed in SLE-P patients, expression of intracellular cytokines IFN-γ, IL-4 and IL-10 were analysed in $CD4^+$ and $CD8^+$ subpopulations of iNKT cells. Data showed that whilst $CD4^+$ iNKT cells were responsible for IFN-γ and IL-10 production, both $CD4^+$ and $CD8^+$ subsets are responsible for IL-4 production in SLE-P patients (Figure 3.13B).

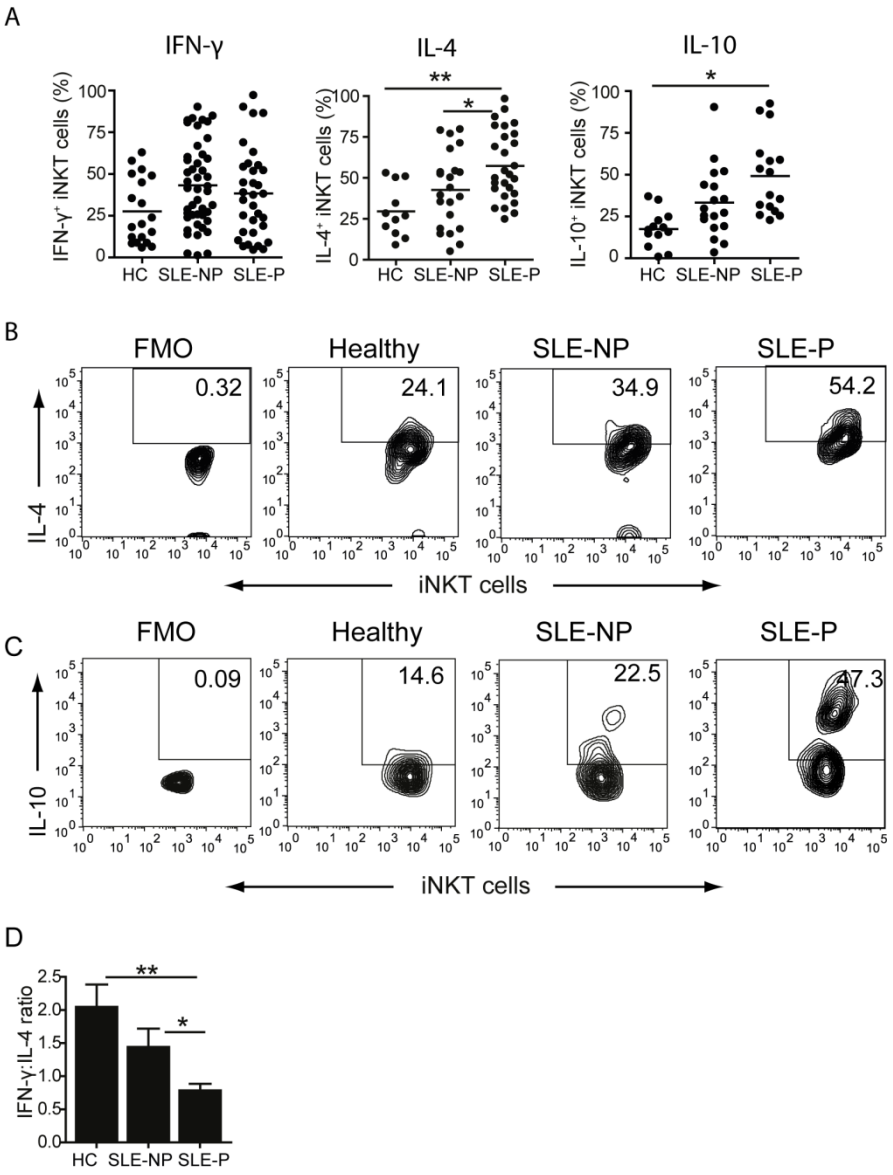


Figure 3.11 SLE-P iNKT cells produce more IL-4 compared to SLE-NP and healthy donors

Ex vivo PBMC from 21 SLE-NP and 21 SLE-P patients were cultured for 4 hours with PMA, ionomycin and Golgi plug then surface stained for CD3, iTCR and intracellularly for IFN-γ, IL-4 and IL-10 expression. **(A)** Cumulative data showing number (%) of iNKT cells positive for intracellular IFN-γ, IL-4 and IL-10 in healthy, SLE-NP and SLE-P patients. One-way ANOVA and Tukey's multiple comparisons post-test. *p=<0.05; **p=<0.001. Representative dot plots showing number (%) of iNKT cells positive for intracellular (B) IL-4 and (C) IL-10 in healthy, SLE-NP and SLE-P patients gated according to FMO controls. **(C)** Ratio of iNKT cells producing IFN-γ compared to IL-4. Mean ± SE. One-way ANOVA and Tukey's multiple comparison post-test; *p=<0.05; **p=<0.001. HC – healthy controls; SLE-NP – SLE patients without plaque; SLE-P – SLE patients with plaque; FMO – fluorescence minus one control.

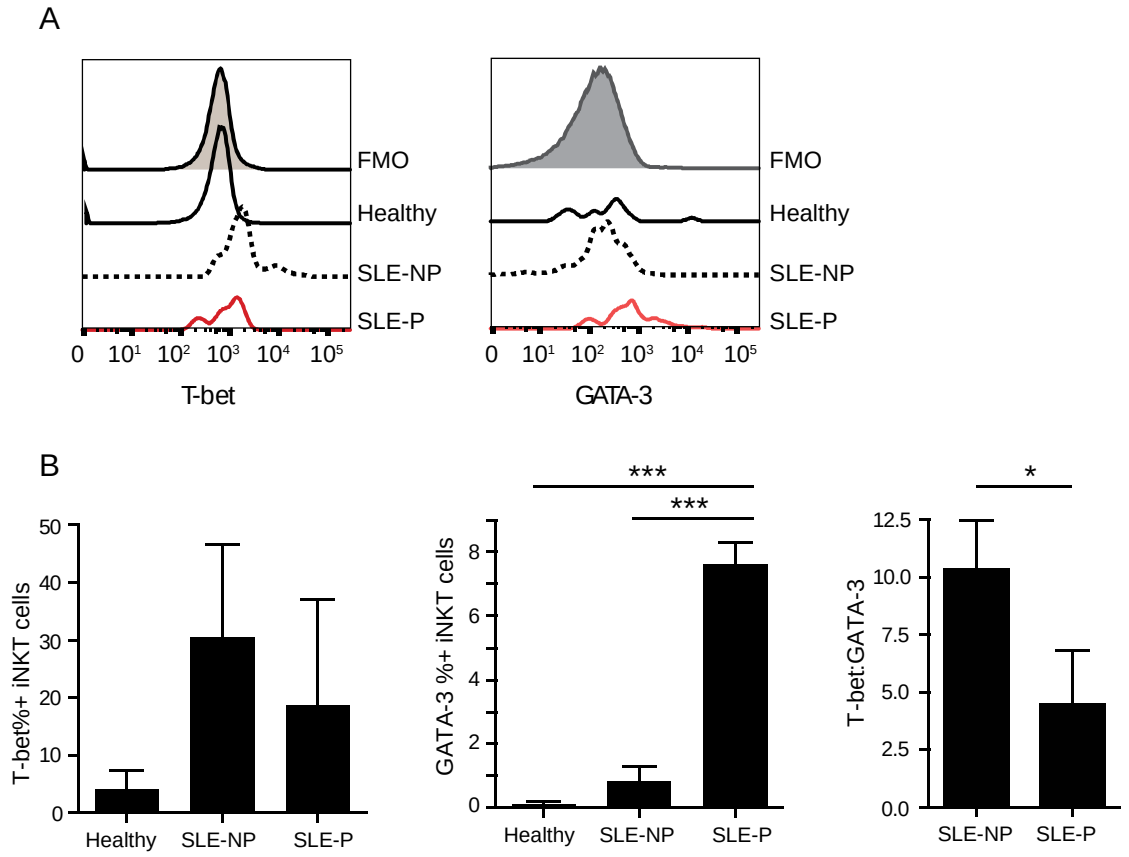


Figure 3.12 SLE-P patients have a decreased T-bet: GATA-3 ratio

PBMCs from 5 healthy donors, 5 SLE-P and 5 SLE-NP patients were surface stained with antibodies against CD3, iTCR and intracellularly with either T-Bet or GATA-3. **(A)** Representative histograms illustrating the differences in T-bet and GATA-3 expression between SLE-NP and SLE-P patients. Cumulative data showing **(B)** Intracellular expression of T-bet and GATA-3 in healthy, SLE-NP and SLE-P patients. Mean ± SE. One-way ANOVA and Tukey's multiple comparison post-test; ***p<0.001. **(C)** T-bet: GATA-3 ratio. Mean ±SE**,** Students T test *p=<0.05. FMO – fluorescence minus one control; HC – healthy controls; SLE-NP – SLE patients without plaque; SLE-P – SLE patients with plaque

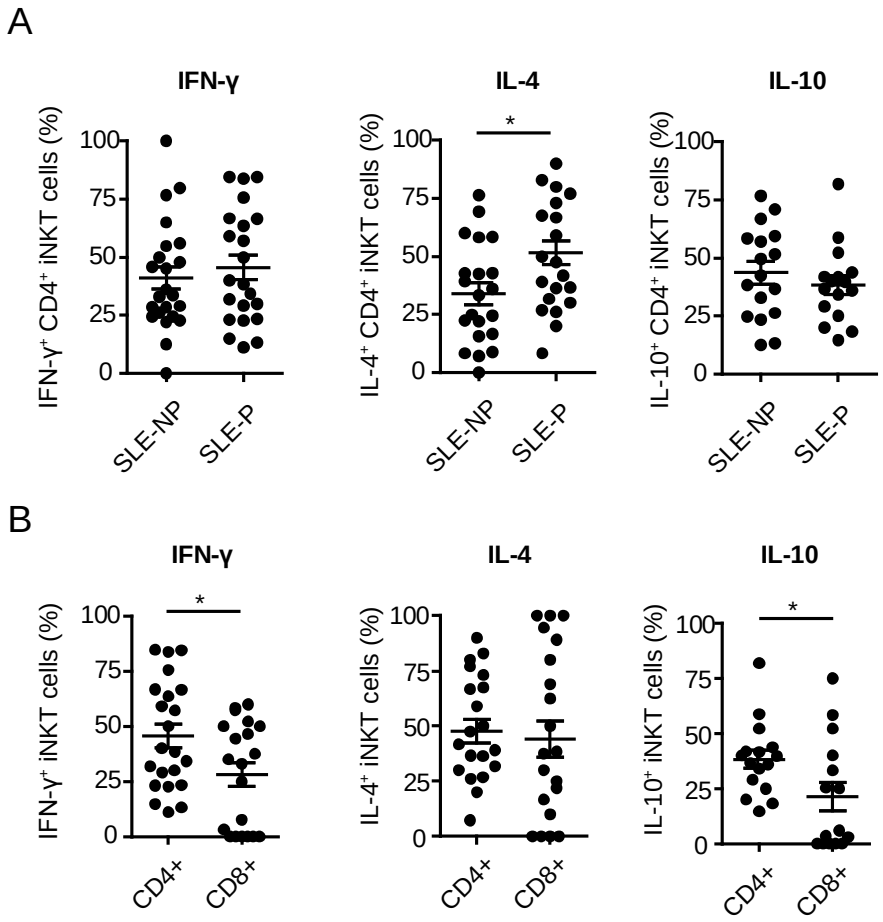


Figure 3.13 Expression of intracellular cytokines in CD4+ and CD8+ iNKT cells in SLE-P patients

Ex vivo PBMC from 22 SLE-NP and 21 SLE-P patients were cultured for 4 hours with PMA, ionomycin and Golgi plug then surface stained for CD3, iTCR, CD4, CD8 and intracellularly for IFN-γ, IL-4 and IL-10 expression. (A) Graphs showing percentage (%) of $CD4^+$ iNKT cells positive for intracellular IFN-γ, IL-4 or IL-10. (B) Comparison of CD4+ and CD8+ iNKT cells from SLE-P patients according to percentage positive expression of intracellular IFN-γ, IL-4 and IL-10. Mean ± SE. Students T test *p=<0.05.

3.11 SLE patients who have suffered a cardiovascular event are characterised by decreased iNKT cells and decreased intracellular IFN- γ and IL-4 expression compared to SLE-P patients

The finding that iNKT cells from SLE-P patients were characterised by IL-4 phenotype led me to hypothesise that iNKT cells could be playing a protective role in the early or preclinical stages of atherosclerosis in SLE patients. To investigate whether iNKT cell phenotype was altered in the later stages of atherosclerosis, I divided SLE patients according to the number of sites with plaque as determined by ultrasound scan. In addition, I examined a group of SLE patients who had been identified as having previously suffered a cardiovascular event (SLE-CV) (Table 3.2). iNKT cell phenotyping was carried out on all patients as before to assess iNKT cell frequency, absolute iNKT cell number, CD4, CD8 and CD69 expression and the intracellular cytokines IFN-γ, and IL-4.

Stratification according to the number of sites with plaque revealed that SLE patients with 3 or 4 plaque sites had significantly fewer iNKT cells compared to patients with 1 or 2 plaque sites, suggesting that the increase in iNKT cells in SLE patients was associated with early plaque development only. Analysis of SLE-CV patients confirmed this hypothesis as indicated by the significant reduction in iNKT cells compared to patients with 1-2 sites with plaque (Figure 3.14A&B). Interestingly iNKT cells from SLE-CV patients also had a reduction in CD69 as well as increases in $CD8^{+}CD4^{-}$ iNKT cells suggesting differences in iNKT cell activation in SLE-CV patients (Figure 3.14C-E). Interestingy however, no differences were observed in $CD4^{+}CD8^{-}$ iNKT cells when analysed according to plaque sites, suggesting that CD4+ iNKT cells remain similar at different stages of atherosclerosis (Figure 3.14E).

Furthermore, the finding that iNKT cells from SLE-CV patients expressed significantly lower intracellular IL-4 compared to patients with 1-2 sites with plaque, suggested that the Th2 response in SLE-P patients was associated with pre-clinical plaque and not more advanced lesions (Figure 3.15A&B). A reduction in IFN-γ^{+} iNKT cells in SLE-CV patients may suggest that iNKT cells in the peripheral blood of these patients have already been activated and are no longer efficient at producing cytokines (Figure 3.15A&B).

When absolute iNKT cell numbers were calculated using patients' lymphocyte counts was there a significant increase in iNKT cells between SLE-NP and SLE-P patients. Furthermore, iNKT cell frequency was significantly inversely correlated with Grey Scale Median (GSM) (Figure 3.16, and methods section for description of GSM measurement), which is quantified according to plaque echogenicity and has been shown to relate to plaque stability, whereby low GSM values are indicative of a propensity to rupture [493]. No relationship however was found with other plaque measures (plaque thickness and total plaque area). Thus patients with more echolucent (unstable and lipid rich) plaque formation had increased iNKT cell frequency suggesting increased iNKT cell activation [241].

I questioned whether IL-4 production by iNKT cells was associated with different types of plaque in SLE-P patients. Grey scale median (GSM), plaque thickness and plaque area were each correlated with the percentage of iNKT cells positive for intracellular IL-4 staining. Interestingly, a significant positive correlation ($p<0.05$) was observed for GSM, whilst trends towards a negative association between iNKT cell IL-4 and plaque area and thickness were observed (Figure 3.16A and C).

	SLE-CV patients (n=11)
Mean Age (range)	50 (20-78)
Years since SLE diagnosis (range)	18 (1-34)
Sex: female: male (%)	9: 2 (82/18)
Ethicity: C/A/AC/O (%)	8/1/1/1 (73/9/9/9)
Smoking (%)	1 (9.09)
Statins (%)	3 (27.27)
Anti-hypertensives (%)	6 (54.54)
Aspirin (%)	1 (9.09)
Hydroxychloroquine (%)	5 (45.45)
Pred ≥10mg/day (%)	1 (9.09)
Pred <10mg/day (%)	7 (63.63)
HCQ + disease modifying agents (%)	3 (27.27)
Pred + disease modifying agents (%)	7 (63.63)
Rituximab (%)	0 (0)
Global BILAG score (range)	4.17 (0-10)
Mean Total Cholesterol mmol/L (range) NR<5.2	4.50 (3.6-5.2)
Mean Triglycerides mmol/L (range) NR<2.2	1.56 (0.4-2.6)
Mean HDL mmol/L (range) NR >1.0	1.43 (0.7-2.1)
Mean LDL mmol/L (range) NR <2.0	2.37 (1.7-3.1)
Cholesterol: HDL ratio (range) NR <3.5	3.43 (2.4-5.1)

Table 3.2 SLE cardiovascular event (SLE-CV) patient clinical characteristics

SLE patients who had previously suffered a cardiovascular event were identified from a cohort of over 500 SLE patients at the University College Hospital London SLE clinic who fulfilled the revised classification criteria for SLE [36]. Patients donated blood at the time of the visit where they were assessed for smoking, medication, serum lipids and disease activity, which was measured by British Isles Lupus Assessment Group index (BILAG) score, which accounts for disease activity in 9 organs/systems. Patients with active SLE disease have a BILAG global score >6. Unlike SLE-NP and SLE-P patients, SLE-CV patients were not scanned for plaque.

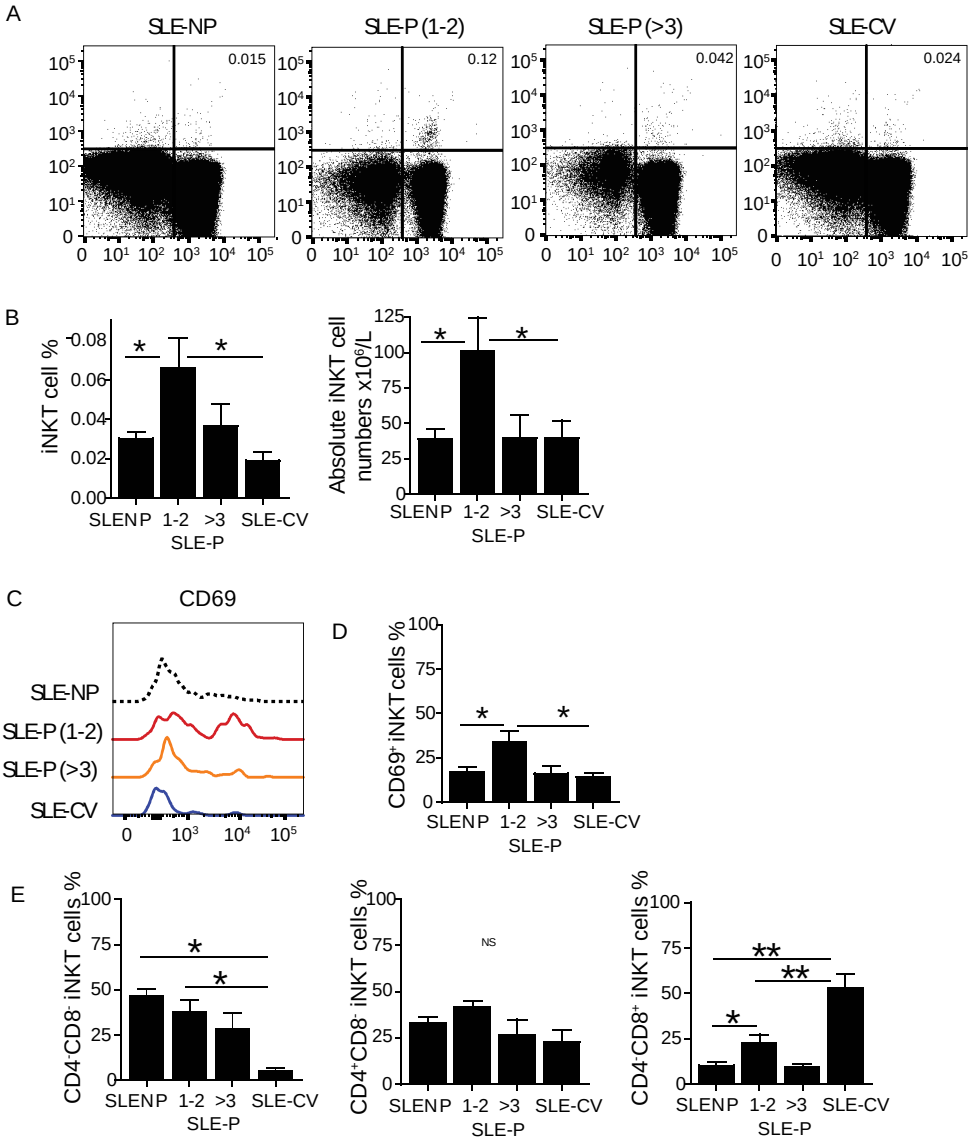


Figure 3.14 SLE patients who have suffered a cardiovascular event have a different iNKT cell phenotype compared to SLE-NP and SLE-P patients

Ex vivo PBMC from 46 SLE-NP, 27 SLE-P patients (stratified according to plaque sites) and 11 SLE-CV patients who had previously suffered a cardiovascular event were surface stained for CD3, iTCR, CD4, CD8 and CD69 expression. (A) Representative dot plots and (B) cumulative data showing iNKT cell frequency and absolute number in SLE patients with 0, 1-2 and 3-4 sites with plaque and SLE-CV patients. (C) Representative histograms and (D) cumulative data showing percentage of iNKT cells positive of CD69 in SLE patients with 0, 1-2 and 3-4 sites with plaque and SLE-CV patients. (E) Frequency of $CD4^{-}CD8^{-}$, $CD4^{+}CD8^{-}$ and $CD8^{+}CD4^{-}$ iNKT cells in SLE patients with 0, 1-2 and 3-4 plaque sites and SLE-CV patients. Mean± SE, One way ANOVA and Tukey's multiple comparisons post-test. **p<0.01, *p<0.05 HC – healthy controls; SLE-NP – SLE patients without plaque; SLE-P – SLE patients with plaque

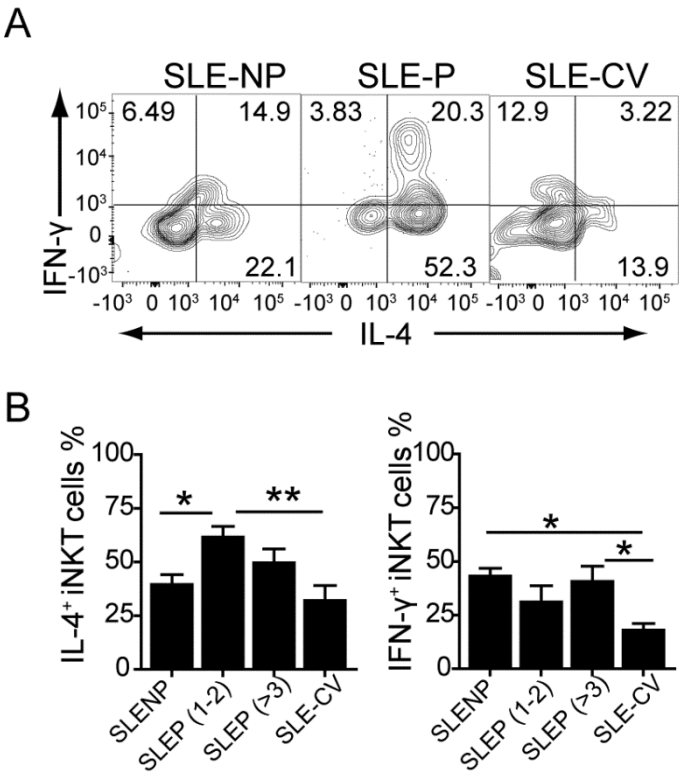
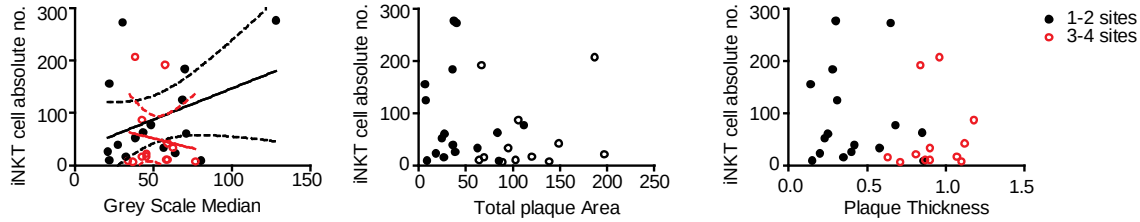


Figure 3.15 SLE patients who have suffered a cardiovascular event have a different iNKT cell cytokine profile compared to SLE-NP and SLE-P patients

Ex vivo PBMC from 46 SLE-NP, 27 SLE-P patients (stratified according to plaque sites) and 11 SLE-CV patients who had previously suffered a cardiovascular event were cultured for 4 hours with PMA, ionomycin and Golgi plug then surface stained for CD3 and iTCR, then stained intracellularly for IFN-γ and IL-4 expression. (A) Representative dot plots and (B) cumulative data showing intracellular IFN-γ and IL-4 expression in the different patient groups. Mean± SE, One way ANOVA and Tukey's multiple comparisons post-test. **p<0.01, *p<0.05 HC – healthy controls; SLE-NP – SLE patients without plaque; SLE-P – SLE patients with plaque

A



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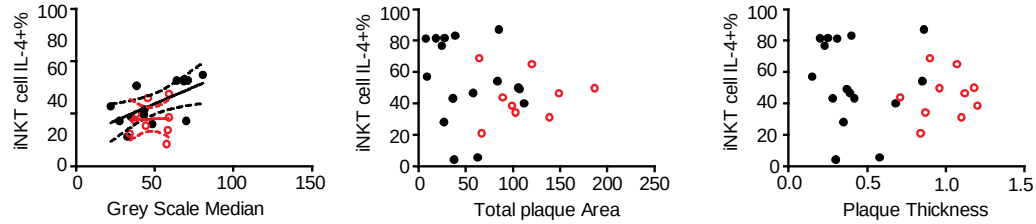


C

Sites with plaque	GSM vs iNKT cell freq.	GSM vs IL-4 $^{+}$ iNKT cells
1-2	r=0.38	r=0.62
	p=0.14	p=0.02
3-4	r=-0.13	r=0.04
	p=0.66	p=0.91

Figure 3.16 Correlations between different plaque measures and iNKT cell frequency or IL-4+ iNKT cells

Ex vivo PBMC from 25 SLE-P patients were cultured for 4 hours with PMA, ionomycin and Golgi plug then surface stained for CD3 and iTCR, then intracellularly for IL-4. Patients were stratified according to the number of sites with plaque: 1-2 (n=15) or 3-4 (n=10). Correlations of (A) iNKT cell numbers and (B) intracellular IL-4 with grey scale median (GSM), total plaque area and plaque thickness. Linear regression was carried out to assess correlation strength. (C) Pearson's correlation coefficient (r) and p-value of correlations between GSM and iNKT cell absolute number or intracellular IL-4.

CHAPTER IV: Results 2

Results 2 – The role of serum lipids in SLE patients with atherosclerosis

4.1 Introduction and aims for this chapter

Previous studies have demonstrated that SLE patients are characterized by altered lipid metabolism, although whether patients are dyslipidaemic is a contentious issue. Evidence suggests that the presence of dyslipidemia in SLE patients ranges from 36% at diagnosis to 60% after 3 years, although this can vary by definition and may depend on patients' disease activity [114]. Whilst not all patients fulfill the criteria for dyslipidaemia there is evidence to suggest that serum lipids are altered in SLE patients, which are characterised by increased VLDL and triglycerides and decreased HDL compared to healthy donors [127]. Since iNKT cells are activated by lipid presentation, I decided to explore the role of serum lipids in SLE patients with and without plaque.

Dyslipidaemia has previously been associated with significantly decreased numbers of iNKT cells in a study by Nakou et al. who showed that treatment with simvastatin lead to an increase in iNKT cells in these patients in addition to a normalised lipid profile [494]. Furthermore, animal models have shown that dyslipidaemia is associated with increased iNKT cell anergy characterised by reduced iNKT cell responsiveness to α-GalCer and a reduction in both IFN-γ and IL-4 [495]. However, the role of serum lipids in SLE and how they may influence iNKT cell function remains largely unknown.

I therefore hypothesised that altered serum lipids could be contributing to defective iNKT cell responses in SLE patients as well as the distinct iNKT cell phenotype observed in SLE patients with preclinical plaque. In addition to measuring serum lipids at the time of the scan using the standard methods at UCLH, metabolomics analysis was carried out to investigate the serum lipid profile in more detail. I correlated my results with the iNKT cell phenotyping data from Chapter 3, and cultured healthy PBMCs with patient serum in an attempt to recapitulate the iNKT cell phenotypes observed in SLE-NP and SLE-P patients.

4.2 SLE-NP and SLE-P patients show minimal differences in traditional measures of serum lipids and other biomarkers

I first decided to investigate differences in lipids and other biomarkers of inflammation by comparing the clinical measures from SLE-NP and SLE-P patient serum, taken at UCLH at the time of the scan. Mean levels of total cholesterol, triglycerides, LDL and HDL (including cholesterol: HDL ratio) as well as C-reactive protein, vitamin D and homocysteine were within the normal clinical ranges used by UCLH to monitor patients' lipids (Table 4.1). Furthermore, out of total cholesterol, triglycerides, HDL, LDL and Cholesterol: HDL ratio, only 9.4% of SLE-NP and 33.3% of SLE-P patients had 3 or more lipid measures outside of normal range with only 9.4% of SLE-NP and 18.9% of SLE-P patients being prescribed statins at the time of the scan (Table 3.1). Both groups of patients in this study would therefore not typically be classified as dyslipidemic since mean levels of cholesterol, HDL, LDL and triglycerides are far lower compared to studies involving patients with hypercholesterolemia [496-498]. Whilst lipid abnormalities were more common in SLE-P patients, analysis of the two groups of patients by student's t tests found very few differences between SLE-NP and SLE-P patients groups in serum lipid measurements. Only triglyceride levels and total cholesterol: HDL ratios were significantly elevated in SLE-P patients compared to those without plaque (Table 4.1).

Table 4.1: SLE patient serum lipids and biomarkers

	SLE–NP (n=64)	SLE–P (n=36)	P value
Mean CRP mg/dL (range) NR <5	6 (0.6-7.7)	3.44 (0-15.5)	p=0.261
Mean Homocysteine µmol/L (range) NR 6-15	13.98 (9-17.5)	16.92 (9-38)	p=0.055
Mean Creatinine µmol/L (range) NR 53-115	71.28 (42-227)	55.79 (40-184)	p=0.311
Mean Vitamin D nmol/L (range) NR 25-137	63.06 (14-228)	55.79 (7-114)	p=0.619
Mean Total Cholesterol mmol/L (range) NR<5.2	4.67 (2.5 – 7.3)	5.04 (2.9 – 6.5)	p=0.067
Mean Triglycerides mmol/L (range) NR<2.2	1.01 (0.4 – 2.3)	1.31 (0.6 – 2.8)	p=0.002[a]
Mean HDL mmol/L (range) NR >1.0	1.71 (0.8 – 2.7)	1.60 (0.8 – 3.0)	p=0.289
Mean LDL mmol/L (range) NR <2.0	2.49 (0.8 – 4.8)	2.84 (1.2 – 4.2)	p=0.084
Cholesterol: HDL ratio (range) NR <3.5	2.87 (1.6 – 5.2)	3.38 (1.8 – 5.6)	p=0.008[a]

Table 4.1 SLE patient serum lipids and biomarkers

Patient blood samples from the time of the scan were assessed for a range of biomarkers including C reactive protein (CRP), homocysteine, creatinine, vitamin D and serum lipids. For creatinine, only 11 patients (5/64 SLE-NP and 6/36 SLE-P) had creatinine levels outside the normal range for UCLH. Data were analysed by a two-tailed T test for significance using a 95% confidence interval. [a] denotes significant results. SLE-P – SLE patients with plaque; SLE-NP – SLE patients without plaque. NR – normal range.

4.3 SLE patients have altered serum metabolites compared to healthy individuals

To better characterise differences in serum lipids and other biomarkers in SLE patients compared to healthy donors proton nuclear magnetic resonance spectroscopy was carried out on serum [499]. This enabled the characterisation of 233 metabolic variables including the size, density and composition of lipoprotein classes as well as other information on the types of lipids and amino acids present. Data were analysed by multiple student's t tests and threshold for significance was adjusted using a Bonferroni correction. The most significant differences were observed for measures of very low density lipoprotein (VLDL), small high density lipoprotein (S-HDL), glutamine and acetate (Figure 4.1A). In particular, differences in lipoprotein content was observed whereby the total cholesterol content of medium and large VLDL and small HDL was increased in SLE patients compared to healthy controls (Figure 4.2B and C).

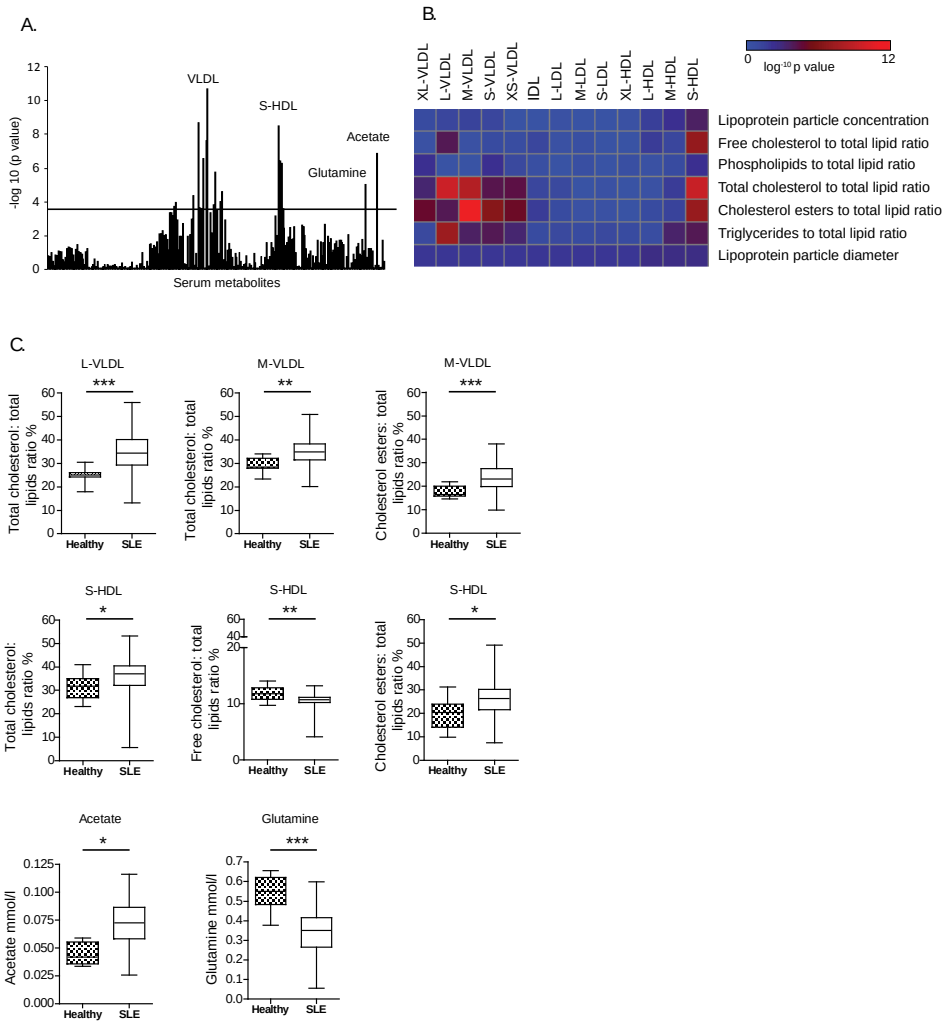


Figure 4.1 SLE patients are characterised by altered serum metabolites compared to healthy donors

Serum from 10 healthy donors and 86 SLE patients was analysed by proton nuclear magnetic resonance spectroscopy to characterize serum lipids and metabolites. Results from healthy individuals and SLE patients were compared using unpaired students t tests and carrying out a logarithmic transformation of the p values to facilitate graphical presentation. (A) Histogram showing the metabolites most significantly different between the two groups, including a Bonferroni correction (y=3.66) to account for the number of comparisons. (B) Heat map depicting most significant comparisons in terms of VLDL, IDL, LDL and HDL particle size and composition, with higher $\log^{-10}$ p values (red colour) representing greater differences between metabolites in healthy donors compared to SLE patients. (C) Comparison of lipoprotein concentration between healthy individuals and SLE patients including VLDL, IDL, LDL and HDL as well as acetate and glutamine, which were also significantly altered. Student's t test: *p<0.05, ** p<0.01, ***p<0.001. VLDL; very large density lipoprotein, IDL; intermediate density lipoprotein, LDL; low density lipoprotein, HDL; high density lipoprotein.

4.4 SLE patients with preclinical plaque have an altered serum metabolite profile compared to SLE patients without plaque

Data from SLE patients were also analysed to identify differences between SLE-NP and SLE-P patients by carrying out multiple student's t tests and using a Bonferroni correction to calculate the level for significance. Whilst none of the metabolites reached significance according to the Bonferroni correction, several differences were observed between SLE-NP and SLE-P patients with a significance of $p<0.05$ (Figure 4.2A, 4.3A and Table 4.2). Analysis of lipoprotein groups showed that most of the differences were in VLDL, in particular the free cholesterol and phospholipid content of VLDL (Figure 4.2B). Furthermore SLE-P had consistently higher concentrations of all forms of VLDL compared to SLE-NP patients, which was not observed for other lipoprotein classes including IDL, LDL and HDL particles (Figure 4.3B).

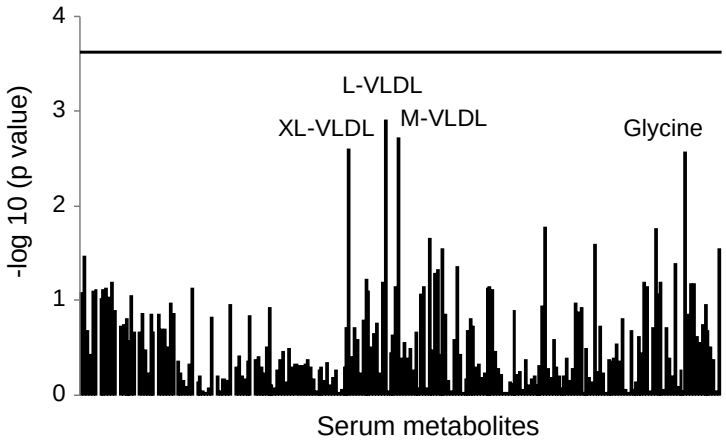
Since triglycerides were previously identified as being increased in SLE-P patients compared to SLE-NP patients, I correlated patients' clinical measures of triglycerides with the metabolites which were identified as most significant between SLE-NP and SLE-P patients (Figure 4.4). However, only triglyceride: phosphoglyceride ratio and glycoprotein aceytls were identified as being significantly correlated with triglycerides. This suggests that the level of triglycerides is not associated with differences in VLDL concentration and composition, suggesting that they should be regarded as independent measures.

Metabolite	SLE-NP mean (range)	SLE-P mean (range)	*p* value
Free cholesterol to total lipids ratio in medium VLDL	10.89 (7.61-13.38)	11.61 (9.18-13.67)	0.00113
Glycine	0.297 (0.196-0.438)	0.265 (0.198-0.347)	0.0027
Free cholesterol to total lipids ratio in large VLDL	9.82 (1.54-15.98)	12.01 (2.72-17.58)	0.00231
Phospholipids to total lipids ratio in chylomicrons and extremely large VLDL	9.31 (2.96-12.12)	10.87 (6.48-22.33)	0.00403
Triglycerides to total lipids ratio in very small VLDL	17.37 (7.75-25.29)	19.44 (12.96-29.48)	0.0103
Mean diameter for LDL particles	24.06 (23.55-25.13)	23.89 (23.47-24.46)	0.0129
Ratio of omega-6 fatty acids to total fatty acids	33.65 (26.17-41.2)	32.15 (26.49-38.67)	0.0162
Triglycerides to total lipids ratio in IDL	17.37 (5.22-18.73)	12.24 (7.61-24.59)	0.0165
Ratio of triglycerides to phosphoglycerides	0.51 (0.25-1.21)	0.61 (0.32-1.27)	0.0183
Ratio of 22:6 docosahexaenoic acid to total fatty acids	1.24 (0.54-1.97)	1.42 (0.49-2.54)	0.0217
Glycoprotein acetyls (mainly a1-acid glycoprotein)	0.074 (0.039-0.1332)	1.52 (1.09-2.43)	0.029
Ratio of monounsaturated fatty acids to total fatty acids	22.9 (17.92-30.05)	24.2 (17.9-29.3)	0.0307
Triglycerides to total lipids ratio in large LDL	9.54 (5.18-19.92)	11.23 (7.25-26.01)	0.0317
Cholesterol esters to total lipids ratio in IDL	46.66 (38.18-62.11)	44.54 (21.71-49.66)	0.0338
Cholesterol esters to total lipids ratio in very small VLDL	43.6 (33.5-61.0)	41.4 (21.5-51.7)	0.0358
Citrate	1.42 (1.02-2.01)	0.05 (0.036-0.103)	0.045

Table 4.2 Serum metabolomics analysis in SLE patients with and without plaque

Serum samples taken at the time of femoral and carotid ultrasound scan from 33 SLE patients with carotid or femoral plaque (SLE-P) and 53 patients with no plaque (SLE-NP) were assessed for lipid distribution in lipoprotein subclasses by proton nuclear magnetic resonance (NMR) spectroscopy. Proportions of total serum cholesterol, phospholipids and triglycerides carried by each lipoprotein subclass were determined [499]. Table lists the markers showing significant differences between SLE patients with and without plaque using Students T test a 95% confidence interval (no significance was seen using the Bonferoni correction). HDL, high density lipoprotein; LDL, low density lipoprotein; IDL intermediate density lipoprotein; VLDL, very low density lipoprotein; SLE-P, SLE patients with plaque; SLE-NP, SLE patients without plaque.

A



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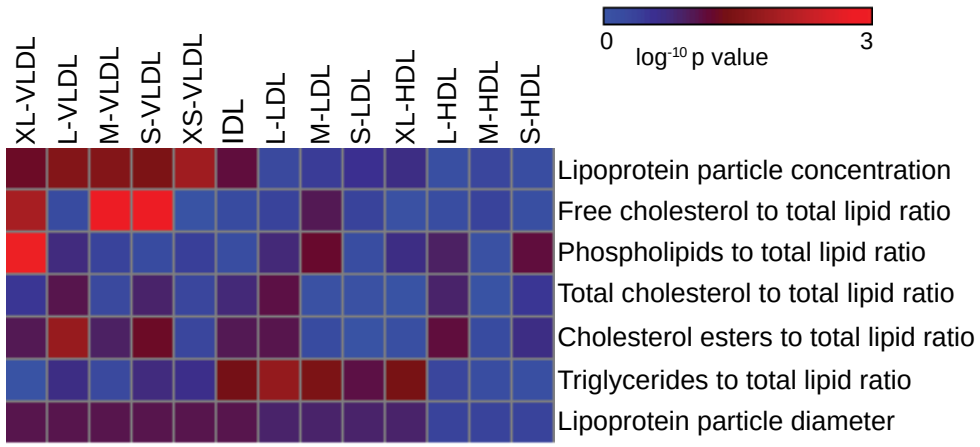

Figure 4.2 SLE-P patients are characterised by altered serum metabolites compared to SLE-NP patients

Serum from 30 SLE-P and 56 SLE-NP patients were analysed by proton nuclear magnetic resonance spectroscopy for 233 serum lipid metabolites. Results were compared using unpaired Students T tests and carrying out a logarithmic transformation of the p values for plotting. (A) Histogram showing the most significant metabolites between the two patient groups, including a Bonferroni correction (y=3.66) to account for the number of comparisons. (B) Heat map depicting most significant comparisons in terms of VLDL, IDL, LDL and HDL particle size and composition, with higher $\log^{-10}$ p values (red colour) representing greater differences between metabolites in SLE-NP compared to SLE-P patients.

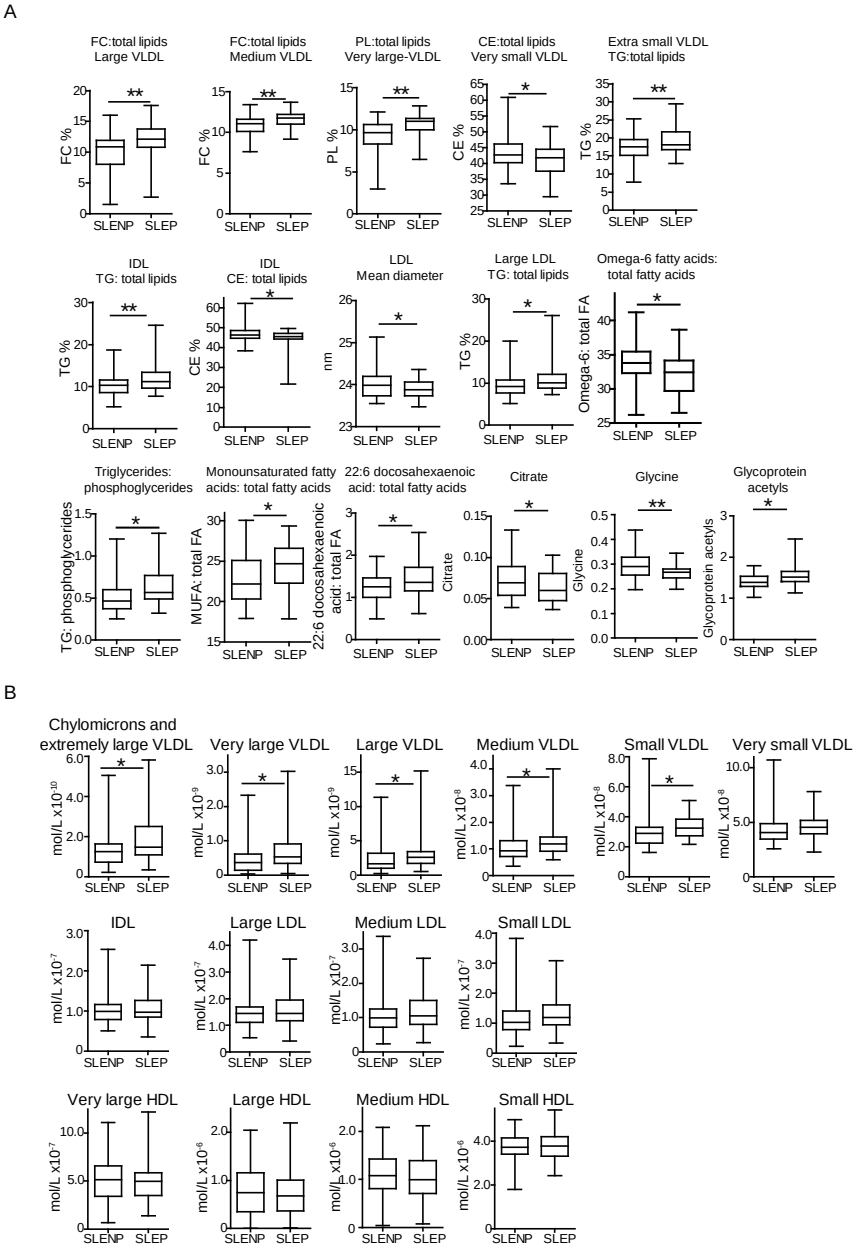

Figure 4.3: Serum metabolomics analysis in SLE patients with and without plaque.

Serum samples taken at the time of femoral and carotid ultrasound scan from 36 SLE patients with carotid or femoral plaque (SLE-P) and 64 patients with no plaque (SLE-NP) were assessed by proton nuclear magnetic resonance spectroscopy for 332 serum lipid metabolites. **(A)** Box and whisker plot showing the markers with significant differences between SLE patients with and without plaque using multiple Students T tests, a 95% confidence interval (no significance was seen using the Bonferoni correction). (**B)** Proportions of total serum cholesterol, phospholipids and triglycerides carried by each lipoprotein subclass were determined [499] HDL, high density lipoprotein; LDL, low density lipoprotein; IDL intermediate density lipoprotein; VLDL, very low density lipoprotein; SLE-P, SLE patients with plaque; SLE-NP, SLE patients without plaque. TG, triglycerides; FA, fatty acid; PL, phospholipid; CE, cholesterol esters; FC, free cholesterol; MUFA, monounsaturated FA. Mann Whitney test, *p=<0.05, **p=<0.001.

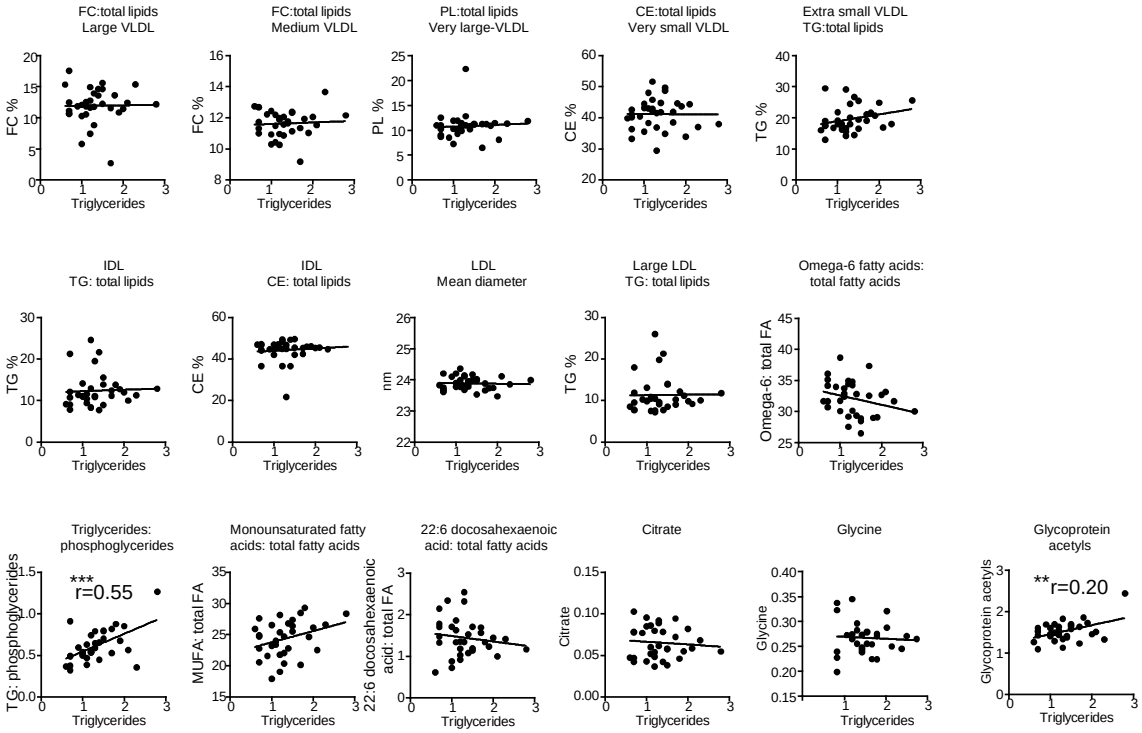

Figure 4.4: Triglycerides are positively associated with phosphoglycerides and glycoprotein acetyls in SLE-P patients

Serum samples taken at the time of femoral and carotid ultrasound scan from 36 SLE patients with carotid or femoral plaque (SLE-P) and 64 patients with no plaque (SLE-NP) were assessed by proton nuclear magnetic resonance spectroscopy for 332 serum lipid metabolites. Significant metabolites were correlated against SLE-P patient serum metabolites and linear regression was carried out to determine any relationship. ***p<0.001, ** p<0.01

4.5 The iNKT cell phenotype in SLE-P patients is associated with altered serum lipids, in particular with VLDL.

Whilst triglycerides represent a good diagnostic measure for dyslipidemia, more specific analysis more specific analysis of metabolites could prove useful in determining the relationship between serum lipids and iNKT cell function. In light of the differences observed between serum metabolites (e.g. VLDL content) in SLE-NP compared to SLE-P patients, I questioned whether they were associated with the differential iNKT cell phenotype described in Results 1. The most significant variables reported in Table 4.2 were correlated with iNKT cell frequency, CD69, CD161 and intracellular IL-4 expression. In all cases, correlations were found to be significant for SLE-P patients only, with most correlations for iNKT cell frequency, followed by CD161 then IL-4. In particular, these correlations mostly involved VLDL particle composition, including phospholipid and free cholesterol content (Figure 4.5 and Table 4.3). Other metabolic markers found to correlate with iNKT cell markers included mono-unsaturated fatty acids, omega 6 fatty acids and docosahexaenoic acid (DHA) (Figure 4.5 and Table 4.3)

Metabolite	Correlation with iNKT cell absolute number: Pearson's correlation coefficient (r) and (p value)		Correlation with CD161+ iNKT cells: Pearson's correlation coefficient (r) and (p value)		Correlation with iNKT cell intracellular IL-4: Pearson's correlation coefficient (r) and (p value)	
	SLE-NP	SLE-P	SLE-NP	SLE-P	SLE-NP	SLE-P
Free cholesterol to total lipids ratio in medium VLDL	0.0729 (0.7350)	-0.4950 (0.0087)**	-0.006318 (0.9745)	0.2819 (0.2285)	-0.3646 (0.1502)	0.1873 (0.4039)
Glycine	0.2747 (0.1940)	-0.1742 (0.3660)	-0.1135 (0.6150)	0.04907 (0.8467)	-0.1342 (0.5956)	-0.2539 (0.2543)
Free cholesterol to total lipids ratio in large VLDL	-0.1169 (0.5684)	-0.5135 (0.0350)*	-0.0063 (0.5384)	0.6572 (0.0016**)	-0.05266 (0.8356)	0.3801 (0.0810)
Phospholipids to total lipds ratio in chylomicrons and extremely large VLDL	-0.1275 (0.5528)	-0.5948 (0.0092)**	-0.1204 (0.5417)	0.4750 (0.0398*)	-0.1193 (0.6485)	-0.05080 (0.8137)
Triglycerides to total lipids ratio in very small VLDL	0.01406 (0.9480)	-0.3285 (0.1832)	-0.05913 (0.7650)	-0.3180 (0.1600)	-0.2211 (0.3779)	-0.01551 (0.9426)
Mean diameter for LDL particles	0.07357 (0.7326)	-0.2659 (0.2862)	0.06292 (0.7504)	0.05822 (0.8021)	0.2961 (0.2328)	-0.1291 (0.5477)
Ratio of omega-6 fatty acids to total fatty acids	0.2004 (0.3478)	0.5610 (0.0154)*	0.1720 (0.3814)	0.1495 (0.5177)	0.1217 (0.6305)	-0.3340 (0.1288)
Triglycerides to total lipids ratio in IDL	0.06820 (0.7515)	-0.2858 (0.2661)	0.06698 (0.7349)	-0.3025 (0.2081)	-0.3136 (0.2051)	-0.01551 (0.9426)
Ratio of triglycerides to phosphoglycerides	-0.04249 (0.8437)	-0.3621 (0.1398)	-0.1377 (0.4934)	-0.2560 (0.2759)	-0.3255 (0.2023)	0.7954 (<0.0001)*
Ratio of 22:6 docosahexaenoic acid to total fatty acids	0.2976 (0.1578)	0.3857 (0.1139)	0.2889 (0.1360)	-0.2826 (0.2146)	-0.00268 (0.9916)	-0.5275 (0.0116)*
Glycoprotein acetyls (mainly a1-acid glycoprotein)	-0.1798 (0.4005)	0.1861 (0.3628)	-0.08732 (0.6920)	-0.01568 (0.9477)	-0.0944 (0.7095)	0.3720 (0.0734)
Ratio of monounsaturated fatty acids to total fatty acids	-0.03111 (0.8853)	-0.5570 (0.0163)*	0.06407 (0.7460)	0.4901 (0.039*)	-0.3282 (0.1837)	0.4555 (0.0332)*
Triglycerides to total lipids ratio in large LDL	0.02350 (0.9132)	-0.2727 (0.2896)	0.03345 (0.8658)	-0.2433 (0.3154)	-0.3136 (0.2051)	-0.1364 (0.5252)
Cholesterol esters to total lipids ratio in IDL	-0.1044 (0.6274)	0.005076 (0.9841)	-0.1361 (0.4897)	0.6572 (0.0016**)	0.3029 (0.2373)	0.2659 (0.2092)
Cholesterol esters to total lipids ratio in very small VLDL	-0.04726 (0.8264)	-0.2168 (0.3874)	-0.01703 (0.9315)	0.1047 (0.6514)	0.3486 (0.1562)	0.3179 (0.1393)
Citrate	0.3126 (0.1370)	-0.0478 (0.8054)	-0.0279 (0.8894)	-0.2297 (0.3300)	-0.5179 (0.0277)*	-0.1229 (0.5672)

Table 4.3 Serum metabolomics analysis in SLE patients with and without plaque

Serum samples taken at the time of femoral and carotid ultrasound scan from 36 SLE patients with carotid or femoral plaque (SLE-P) and 64 patients with no plaque (SLE-NP) were assessed for lipid distribution in lipoprotein subclasses by proton nuclear magnetic resonance (NMR) spectroscopy as carried out by Kettunen et al. [499]. Proportions of total serum cholesterol, phospholipids and triglycerides carried by each lipoprotein subclass were determined. Table lists the markers identified as most significant between SLE patients with and without plaque using student's t test a 95% confidence interval (no significance was seen using the Bonferoni correction). Serum metabolite expression was correlated with iNKT cell frequency and percentage of iNKT cells positive for CD161 and intracellular IL-4 expression using Pearson's correlation coefficient (r), significance was determined using a 95% confidence interval. * denotes significant correlations between iNKT cell frequency and serum metabolites.

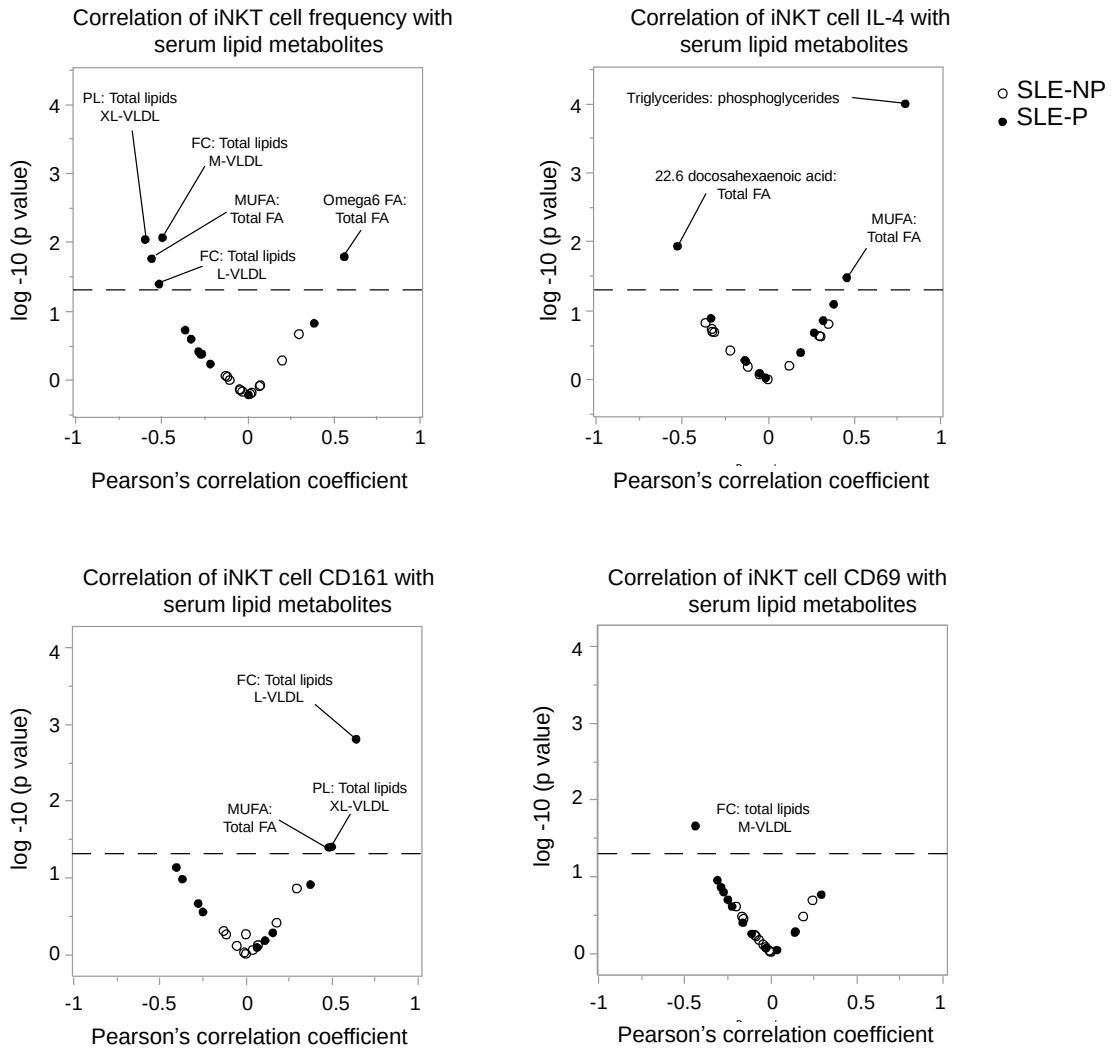

Figure 4.5 The iNKT cell phenotype observed in SLE-P patients is associated with differences in serum metabolites including VLDL

Serum from 30 SLE-P and 56 SLE-NP patients were analysed by proton nuclear magnetic resonance spectroscopy for 233 serum lipid metabolites. Results were compared using an unpaired Students T test and the most significant metabolites were correlated against iNKT cell frequency and the percentage of iNKT cells positive for CD69, CD161 and intracellular IL-4 as shown in Figure 3.5 and 3.9 respectively. Volcano plots depicting Pearson's correlation coefficient (r) against –log10 (p value) are shown for correlations between iNKT cell frequency, CD69, CD161 or IL-4 and all metabolites identified as significant between SLE-NP and SLE-P. HC – healthy controls; SLE-NP – SLE patients without plaque; SLE-P – SLE patients with plaque

4.6 The iNKT cell phenotype in SLE-P patients is not mediated by cytokines in the serum

In addition to activation by CD1d-mediated lipid antigen presentation, iNKT cells can also be activated by cytokines; IL-12 for example has been shown to activate iNKT cells directly [286]. To investigate whether there were differences in serum cytokines between healthy controls, SLE-NP and SLE-P patients a cytometric bead array was carried out for cytokines IL-2, IL-4, IL-6, IL-10, IL-12, IL-13, IL-17a, IFN-α, IFN-γ and TNF-α (Figure 4.4). However, no significant difference was observed for any of the cytokines, suggesting that differences in cytokines were not responsible for the altered iNKT cell phenotype in healthy donors, SLE-NP and SLE-P patients. Therefore I hypothesised that differences in the serum lipids were driving the altered iNKT cell phenotype in SLE-P patients.

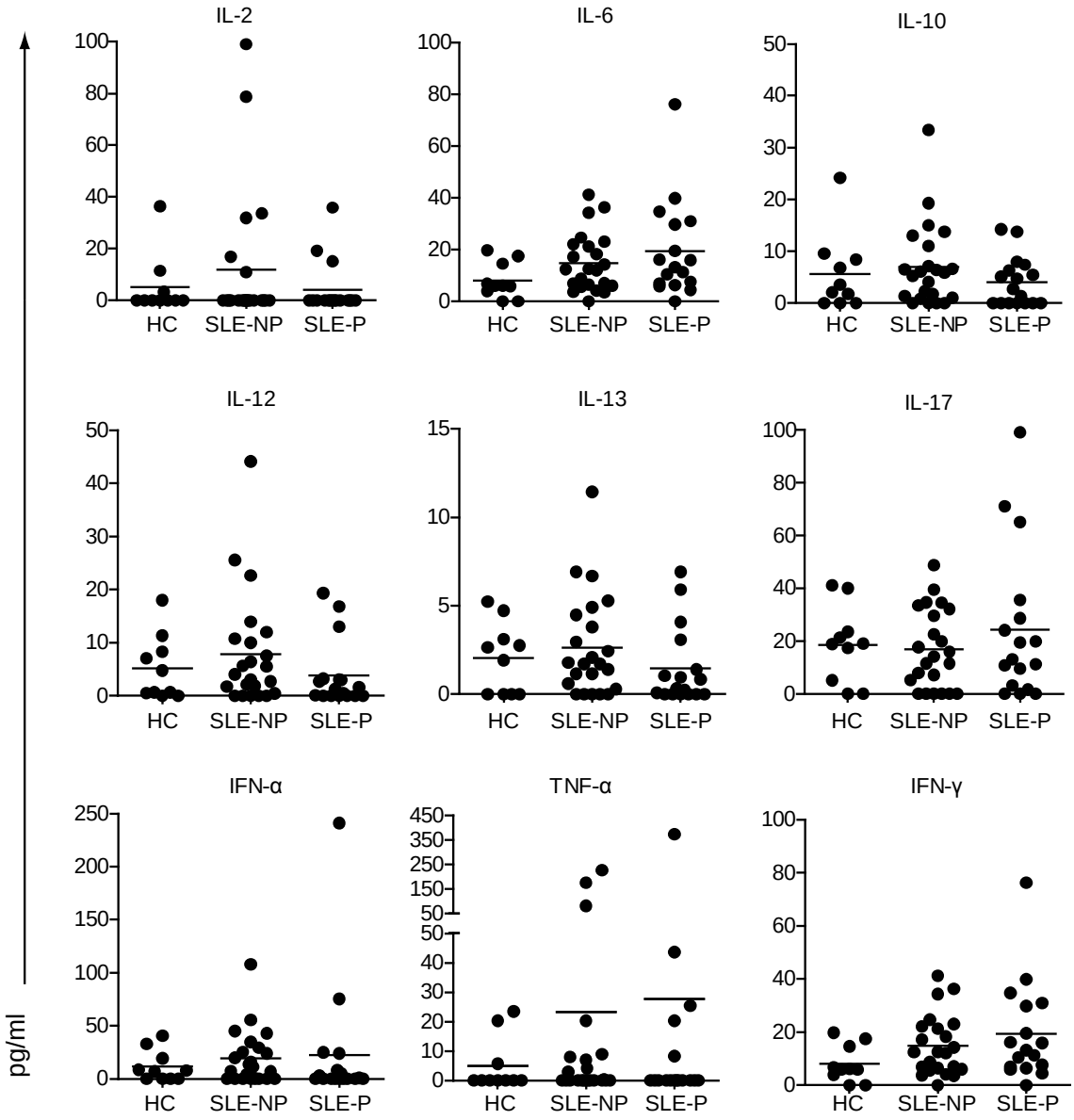

Figure 4.6 Serum cytokine expression levels are similar in SLE patients with and without plaque.

Serum from 10 healthy donors, 23 SLE-NP and 17 SLE-P patients taken at the time of the carotid/femoral ultrasound scan were analysed by Cytometric Bead Array (CBA, BD Bioscience) for the following cytokines: IL-2, IL-4, IL-6, IL-10, IL-12, IL-13, IL-17a, IFN-α, IFN-γ and TNF-α according to manufacturer's instructions. No signal was detected for the presence of IL-4 using this method (data not shown). Scatterplot showing mean. No significant differences in cytokine expression levels were detected when all groups were compared using ANOVA, or when SLE-NP and SLE-P patients were compared using a Mann Whitney test. HC – healthy controls; SLE-NP – SLE patients without plaque; SLE-P – SLE patients with plaque

4.7 Altered lipid presentation drives the iNKT cell phenotype associated with SLE-P patients

To determine whether the differential iNKT cell phenotype observed between SLE-NP and SLE-P patients in “Results 1” was associated with serum factors, PBMCs from healthy donors were cultured with serum from non-autologous healthy controls, SLE-NP and SLE-P patients in an attempt to recapitulate the SLE-P phenotype in healthy PBMCs. Only serum from SLE-P patients induced increased iNKT cell expansion (Figure 4.5A-C), which was associated with increased IL-4 production compared to SLE-NP serum in a similar way to the *ex vivo* iNKT cell phenotype seen in SLE-P patients (Figure 4.5E). The development of this phenotype was blocked by the addition of blocking anti-CD1d antibody (Figure 4.5F) indicating that CD1d-mediated lipid antigen presentation was required for the differential iNKT cell phenotype observed upon treatment with SLE-P serum.

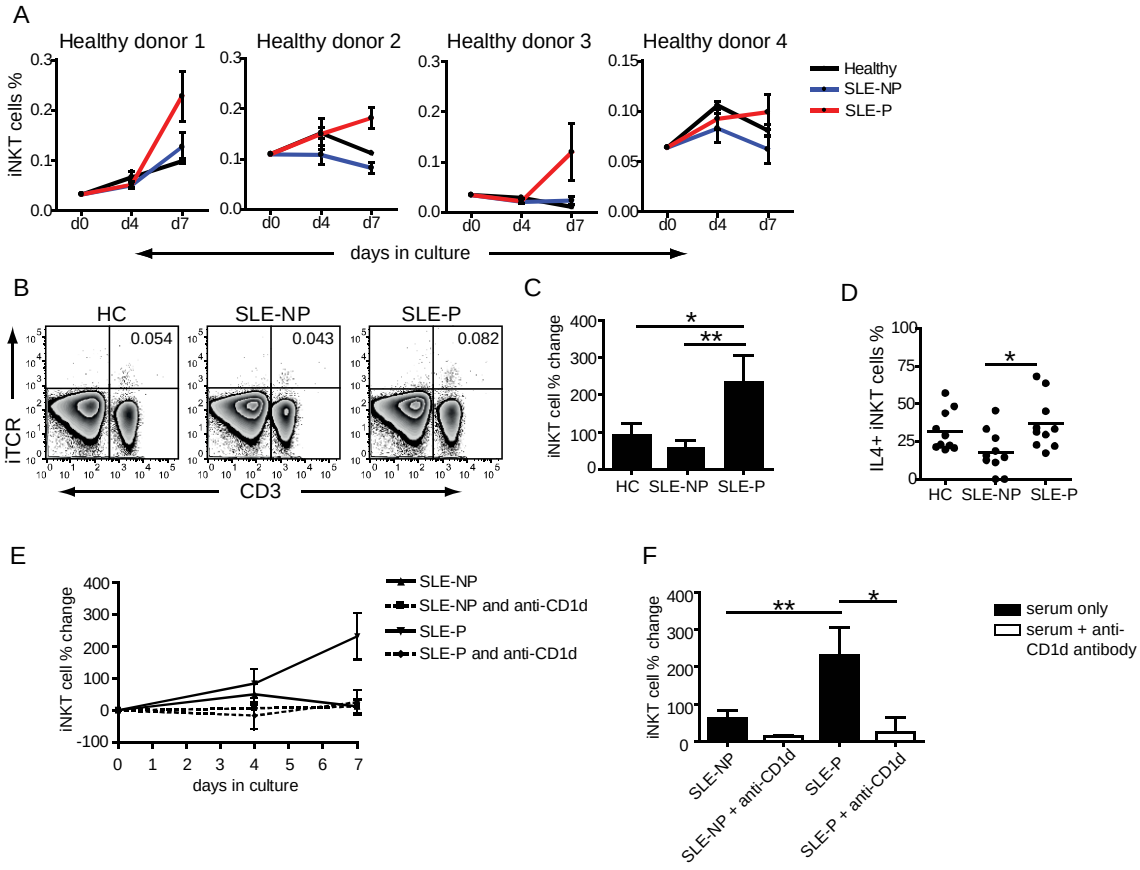

Figure 4.7 Serum from SLE-P patients drives an altered iNKT cell phenotype

PBMCs isolated from 4 different healthy donors were each cultured ($5x10^6$/well) for 7d in RPMI containing 50% serum from 3 different healthy donors, SLE-NP or SLE-P patients with the addition of anti-CD1d as a control. At d0, d4 and d7 PBMCs were labelled with CD3 and iTCR and stained intracellularly for IFN-γ and IL-4 before analysing by flow cytometry. (A) iNKT cell frequency over time as per gating strategy (Figure 3.2), showing results from 4 different healthy donors. (B) Representative plots at d7 and (C) cumulative data showing percentage change in iNKT cell frequency at d7 compared to d0. (D) Percentage of iNKT cells positive for intracellular IL-4 at d7 culture. (E) iNKT cell percentage change at d4 and d7 from *ex vivo* PBMC frequency at day0 upon treatment with SLE-NP and SLE-P serum, and the influence of blocking the iTCR using anti-CD1d (F) Cumulative data at d7, showing the effect of anti-CD1d blocking antibody. Mean ±SE. One-way ANOVA and Tukey's multiple comparison post-test, **p=<0.001, *p=<0.01. HC – healthy controls; SLE-NP – SLE patients without plaque; SLE-P – SLE patients with plaque

CHAPTER V: Results 3

Results 3 – CD1d-mediated lipid presentation in SLE patients with atherosclerosis

5.1 Introduction and aims for this chapter

CD1d is an MHC-class I-like molecule expressed by antigen presenting cells including monocytes, macrophages, dendritic cells and B cells. It is necessary for presentation of lipid antigens to iNKT cells via binding to the iTCR and was first characterised by its ability to bind the marine sponge-derived iNKT cell agonist α-GalCer [268].

In addition, the plasma membrane distribution of CD1d with lipid microdomains (so called lipid rafts) can also influence iNKT cell responses, and can direct iNKT cell function [377]. In particular, a study by Im et al showed that co-localisation between CD1d and lipid rafts in a JAWS II cell line resulted in a Th1 cytokine response, whereas CD1d exclusion from lipid rafts induced in a Th2 response. Lipid rafts are defective in B and T cells from SLE patients [367, 368], however the role of CD1d-lipid raft interactions on iNKT cell function is humans has not been previously investigated, and such experiments are extremely difficult to carry out since modulating CD1d-lipid raft interactions would likely interfere with lipid metabolism in general.

The aim of this chapter was to investigate whether CD1d expression and its association with lipid rafts was altered in SLE patients with and without plaque and whether this could be due to differences in CD1d recycling, which is required for the loading of different lipids for CD1d-mediated antigen presentation to iNKT cells. Furthermore, I explored whether altered antigen presenting cells in SLE patients could induce differences in conjugate formation with iNKT cells in response to α-GalCer and the effect on signalling downstream of the iTCR.

5.2 CD1d and lipid raft expression on B cells is altered in SLE patients compared to healthy controls

CD1d expression on B cells, monocytes and pDCs was characterised by flow cytometry. Whilst no significant differences were observed for monocytes and pDCs, B cells from both SLE-NP and SLE-P patients were found to have significantly decreased CD1d expression compared to healthy controls in line with data previously published by Bosma et al [233] (Figure 5.1A, C and D).

Since membrane lipids are thought to play a role in CD1d-mediated antigen presentation I decided to investigate expression of lipid rafts on B cells, monocytes and pDCs using CTB which has previously been shown to be a surrogate marker for glycosphingolipids, a major component of lipid rafts [380]. Analysis of flow cytometry data revealed increased lipid raft expression in B cells from both SLE-NP and SLE-P patients compared to healthy controls. Whilst no significant differences were detected in monocytes (Figure 5.1A&B), there was a trend towards increased lipid raft expression on pDCs (Figure 5.1D).

Correlations between the MFI of CD1d and lipid rafts (CTB) were performed for B cells and monocytes. Healthy donors were compared to both SLE-NP and SLE-P patients. Interestingly, I observed significant strong positive correlations in healthy individuals for B cells but not monocytes, which was lost in both SLE-NP and SLE-P patients (Figure 5.2A). Thus the results indicated that while significant differences in CD1d and lipid raft expression were observed in B cells from SLE patients compared to healthy donors, this was not associated with the presence of preclinical plaque.

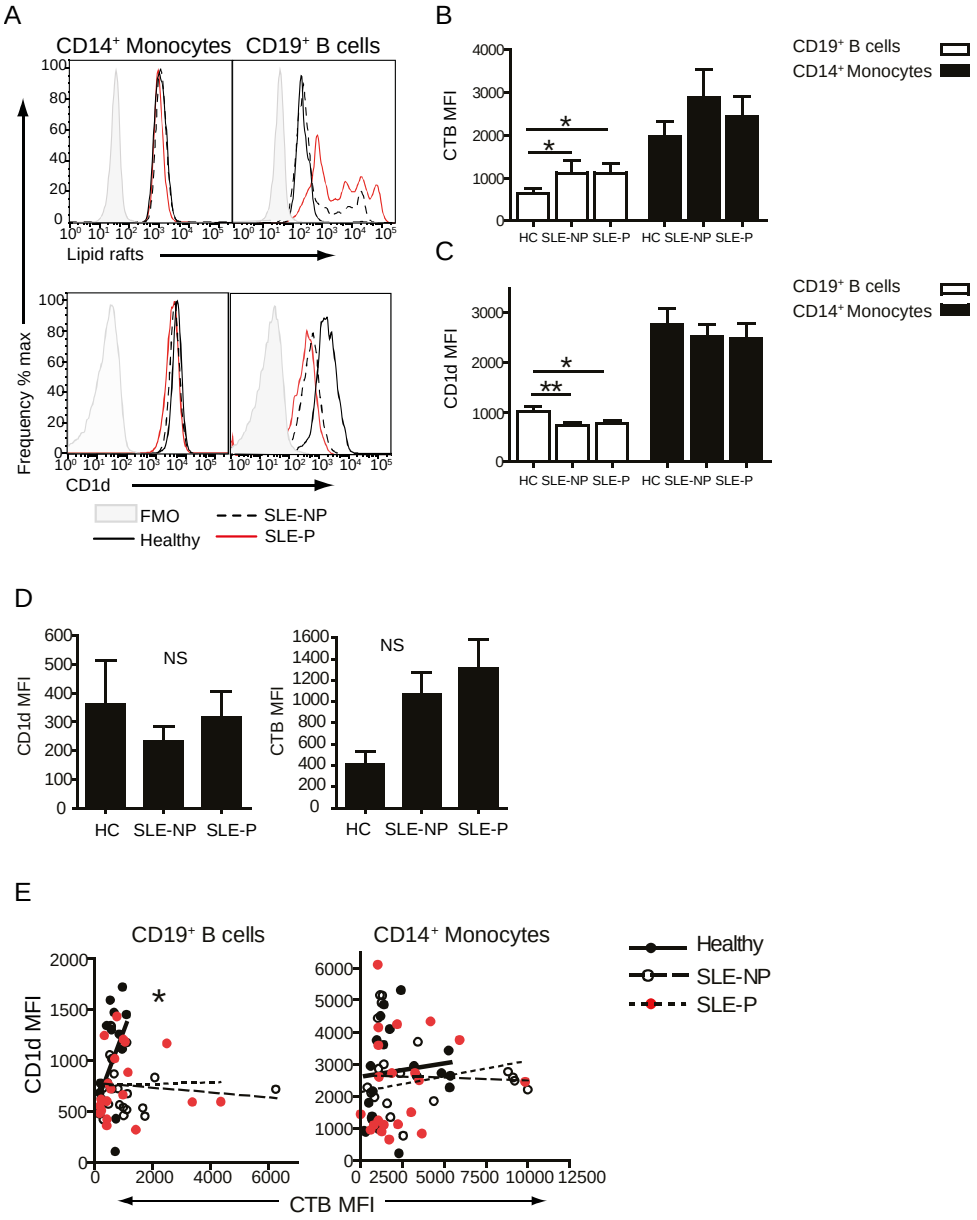

Figure 5.1 B cells and monocyte CD1d and lipid raft expression in healthy donors, SLE-NP and SLE-P patients

10^6 PBMCs from 14 healthy donors, 10-32 SLE-NP and 10-23 SLE-P patients were surface stained *ex vivo* for CD14, CD19, CD303, CD1d and Cholera toxin B (lipid rafts). Data were analysed by flow cytometry. **(A)** Representative histograms showing lipid raft and CD1d expression in B cells and monocytes from healthy donors, SLE-NP and SLE-P patients. **(B)** Cumulative data showing mean fluorescence intensity (MFI) of lipid raft and **(C)** CD1d expression on B cells and monocytes from healthy donors, SLE-NP and SLE-P patients. **(D)** CD1d and lipid raft expression on pDCs. Mean ± SE. One-way ANOVA and Tukeys multiple comparisons test. *p=<0.05, **p<0.01 **(E)** Correlation between CD1d and CTB (lipid raft) expression in B cells and monocytes from healthy donors and SLE patients. Linear regression was used to determine the strength of correlation given. *p=<0.05, r=0.6892; HC – healthy controls; SLE-NP – SLE patients without plaque; SLE-P – SLE patients with plaque

5.3 SLE patients show differences in CD1d and lipid raft co-localisation

Differences in the level of CD1d expression alone, perhaps due to increased recycling may influence iNKT cell proliferation [233]. Whilst this may contribute to the reduced numbers of iNKT cells observed in SLE patients in general, CD1d expression alone cannot account for differences in the iNKT cell cytokine profile observed between SLE-NP and SLE-P patients.

CD1d association with lipid rafts has previously been shown to influce iNKT cytokines responses, whereby CD1d incorporation into lipid rafts promoted an NKT1 response and CD1d exclusion from lipid rafts promted an NKT2 response [377]. However, such work has been carried out using cell lines and may not represent the situation in human cells.

Due to the pathogenic role of lipid defects in SLE and based on work by Im et al [377], I decided to investigate whether differences in CD1d and lipid raft colocalisation could influence iNKT cell responses in healthy, SLE-NP and SLE-P patients. Specifically, I questioned whether differences in CD1d and lipid raft co-localisation could be responsible for the Th2 iNKT cell phenotype observed in iNKT cells from SLE patients with preclinical plaque (Figure 3.10). ImageStream cytometry was used to quantify co-localisation between CD1d and lipid rafts, by means of the similarity feature in IDEAS analysis software for ImageStream (Amnis). Importantly, monocytes were found to have more CD1d and lipid raft co-localisation compared to B cells, as can be seen from the representative images (Figure 5.2A); a finding which was proved significant upon analysis using the similarity feature, which compares the fluorescence of CD1d and CTB across different regions of each cell. Importantly, B cells but not monocytes from SLE patients were also found to have significantly increased CD1d-CTB co-localisation (Figure 5.2B), with no difference between SLE-NP and SLE-P patients. These results were confirmed by confocal microscopy performed by a graduate student in the Jury Lab (data not shown).

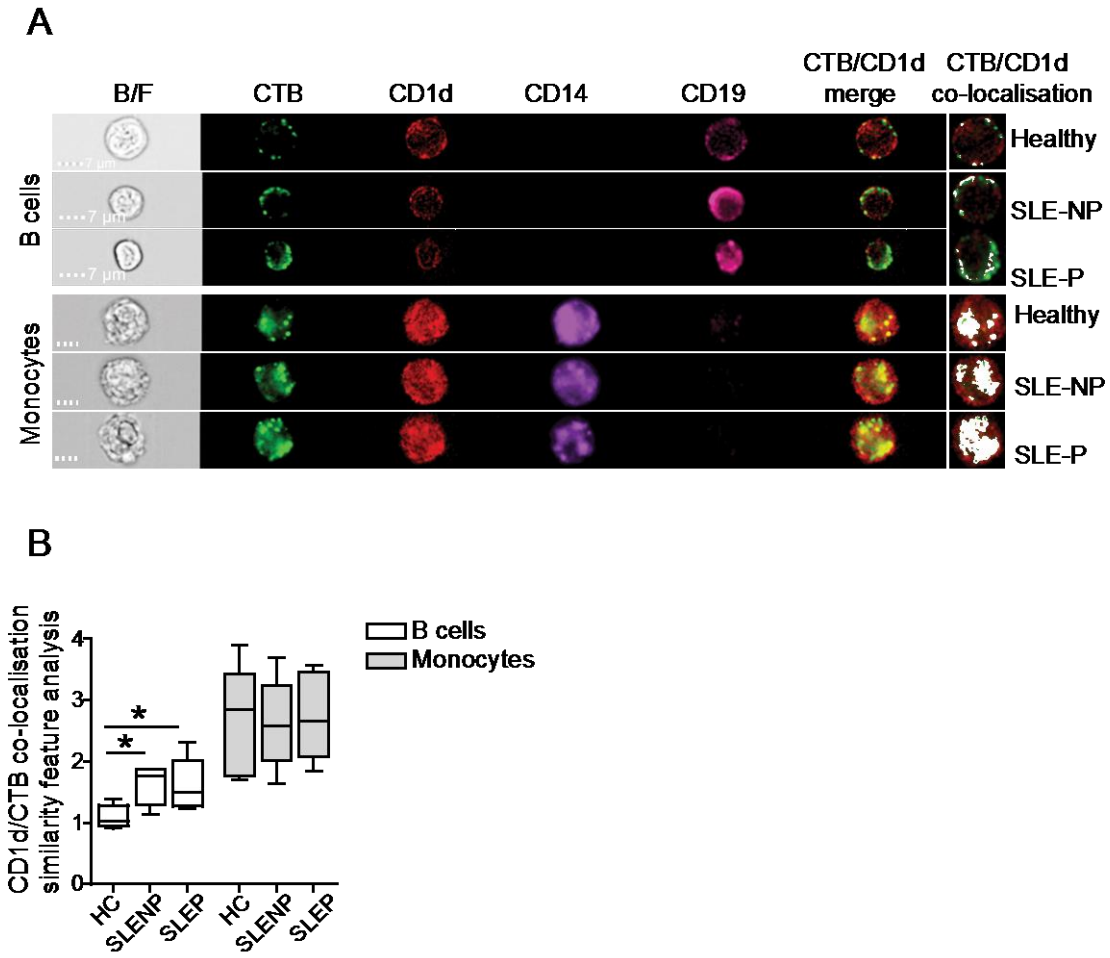

Figure 5.2 B cells and monocytes show differences in colocalisation between CD1d and lipid rafts in SLE patients compared to healthy donors.

10^6 PBMCs from 5 healthy donors, 5 SLE-NP and 6 SLE-P patients were surface stained *ex vivo* for CD14, CD19, CD1d and CTB (lipid rafts). Data were acquired by imaging flow cytometry and analysed using IDEAS software for ImageStream to quantify CD1d and CTB co-localisation. Briefly cells were gated on focused and single cells, then either $CD14^+$ or $CD19^+$ populations before applying the similarity feature to measure co-localisation between CD1d and CTB. **(A)** Representative plots showing expression of lipid rafts [CTB] (green) and CD1d (red) on monocytes (purple) and B cells (pink) as well as CD1d-lipid raft co-localisation (merged image) for healthy, SLE-NP and SLE-P patients.**(B)** Cumulative data showing the level of co-localisation in B cells and monocytes from healthy donors, SLE-NP and SLE-P patients. Mean± SE. One-way ANOVA *p=<0.05

5.4 Serum from SLE patients contributes to the decrease in CD1d expression observed on B cells from SLE patients

Since B cells from SLE patients were found to have reduced CD1d expression and increased lipid raft expression compared to healthy individuals [233, 368], I questioned whether serum factors (potentially lipids) could be driving this response by culturing healthy PBMCs with patient serum in an attempt to recapitulate the antigen presenting cell phenotype observed in SLE-NP and SLE-P patients. Interestingly, there was a reduction in CD1d on B cells from SLE patients, which was found to be significant for SLE-NP patients compared to healthy controls after 4 days of culture (Figure 5.3A). Whilst no difference was observed for B cell lipid raft expression, there was a trend towards increased lipid raft expression on SLE-NP monocytes (Figure 5.3B).

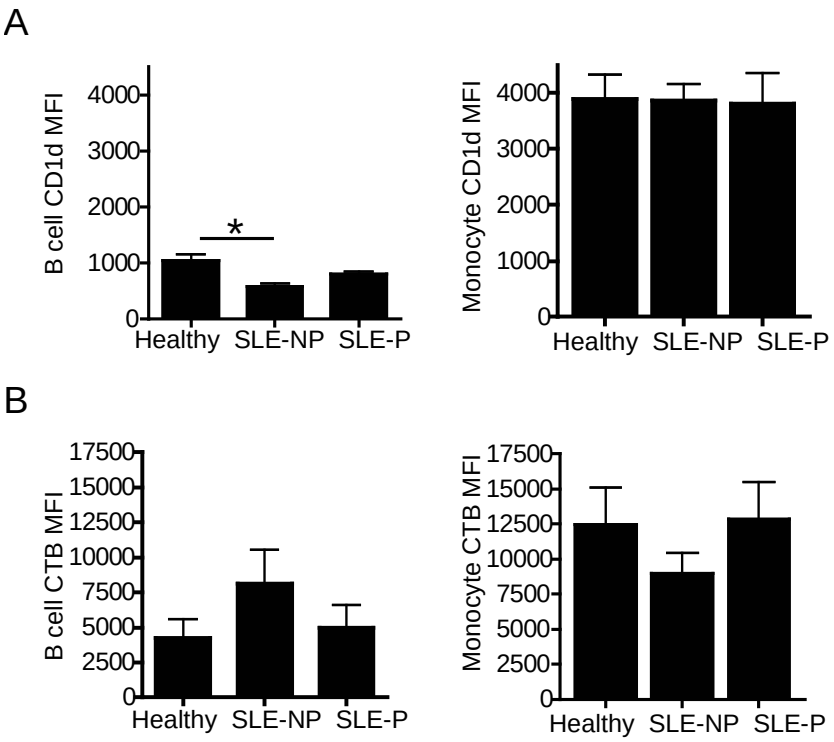

Figure 5.3 CD1d and lipid raft expression in healthy B cells and monocytes in response to 4 day culture with serum from healthy donors, SLE-NP and SLE-P patients

PBMCs isolated from 4 different healthy donors were each cultured ($5x10^6$/well) for 7d in RPMI containing 50% serum from healthy donors (n=3), SLE-NP (n=3) or SLE-P (n=3) patients. At d0, d4 and d7 PBMCs were stained for $CD14^+$ monocytes, CD19+ B cells, CD1d and CTB (lipid rafts), before analysing by flow cytometry. MFI of (A) CD1d Lipid raft and (B) CTB expression on B cells and monocytes after 4 days of culture Mean± SE. One-way ANOVA *p=<0.05. HC – healthy controls; SLE-NP – SLE patients without plaque; SLE-P – SLE patients with plaque

5.4 SLE-NP patients have increased endosome expression compared to SLE-P patients and healthy individuals

Internalisation and CD1d recycling is necessary for CD1d-mediated lipid antigen presentation to iNKT cells. CD1d internalisation occurs via sorting endosomes, which fuse to lysosomes where the loading of either endogenous or exogenous lipids takes place.

Since endosomes and lysosomes are important in this process, I quantified the numbers of B cell and monocyte endosomes and lysosomes in healthy donors, SLE-NP and SLE-P patients using early endosomal marker EEA-1 and lysosomal marker LAMP-1. ImageStream cytometry was used to acquire data, which were analysed using the spot count feature in IDEAS analysis software (Amnis) to identify and count the “spots” with greatest intensity for EEA-1 and LAMP-1.

Lysosome numbers were found to be similar B cells and monocytes in healthy donors and patients with SLE (Figure 5.4A). However, monocytes from healthy donors had significantly increased endosome numbers compared to B cells, (Figure 5.4B). Interestingly, SLE-NP patients had significantly increased numbers of monocyte endosomes compared to both healthy controls and SLE-P patients. However, analysis of CD1d-endosome and CD1d–lysosome co-localisation using the similarity feature showed no difference between SLE-NP and SLE-P patients, despite finding more co-localisation in monocytes compared to B cells (Figure 5.4C). Whether differences in endosome function between SLE-NP and SLE-P monocytes contribute to the differential iNKT cell phenotype observed remains to be seen.

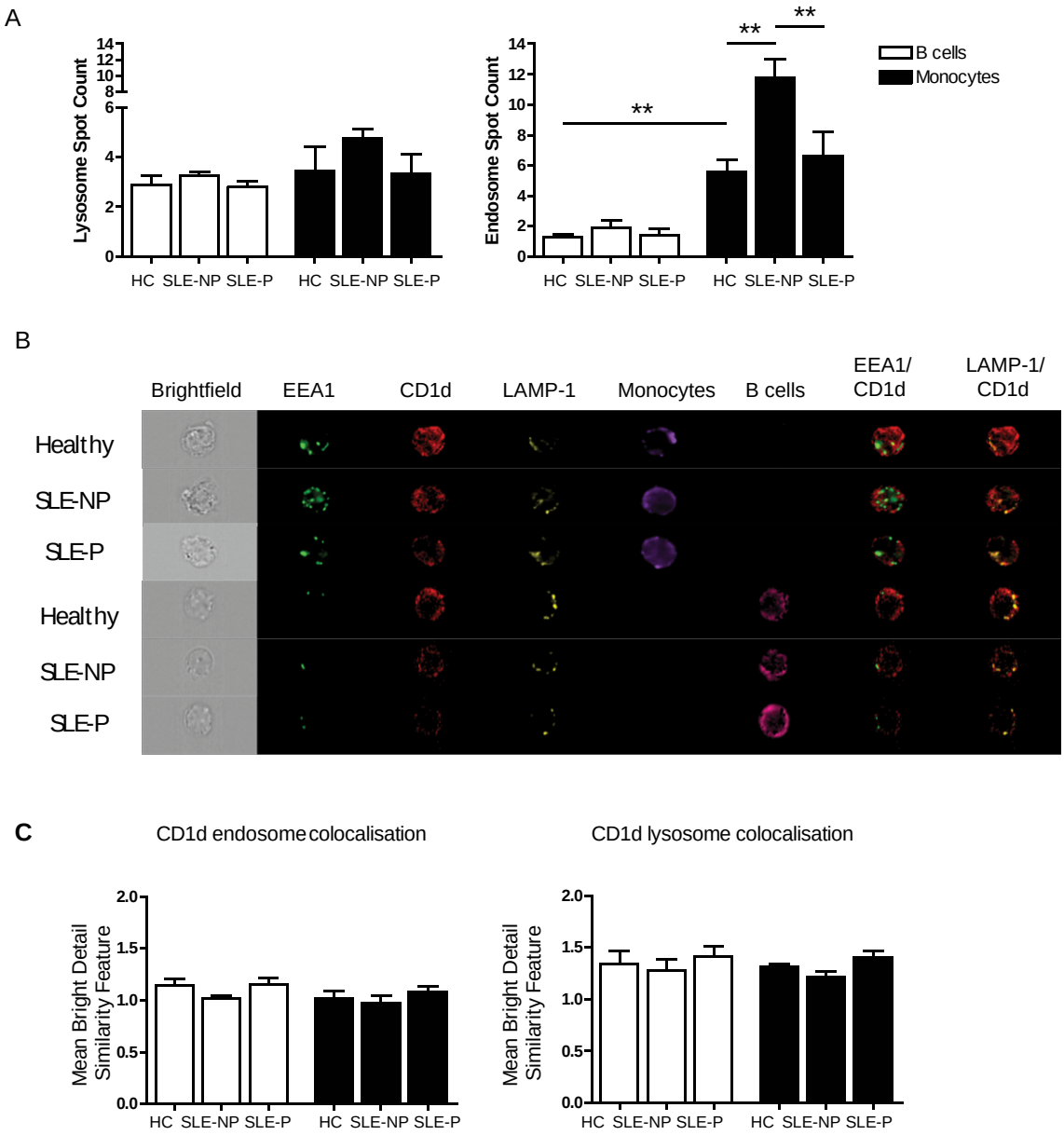

Figure 5.4 Differences in endosome and lysosome expression in SLE patients compared to healthy donors.

$2x10^6$ fresh PBMCs from 4 healthy donors, 5 SLE-NP and 5 SLE-P patients were stained for CD14, CD19, CD1d, early endosome marker EEA-1, and lysosome marker LAMP-1. Cells were acquired by ImageStream cytometry (Amnis) and analysed using IDEAS software to quantify endosome and lysosome frequency and co-localisation with CD1d. (A) Cumulative data for endosome and lysosome spot counts in B cells and monocytes from healthy donors, SLE-NP and SLE-P patients. (B) Representative images for endosome and lysosome frequency showing EEA1 (green), CD1d (red) LAMP-1 (yellow), monocytes (purple) and B cells (pink) and merged images for CD1d co-localisation with endosomes and lysosomes. (C) CD1d co-localisation with endosomes and lysosomes in healthy and SLE patients as determined by the bright detail similarity feature. Mean± SE. One-way ANOVA *p=<0.05

5.5 B cells form fewer stable conjugates with iNKT cells compared to monocytes in healthy donors, SLE-NP and SLE-P patients

I questioned whether differences in lipid raft and CD1d expression in B cells and monocytes were associated with differences in immune synapse formation with iNKT cells and changes in subsequent signalling via iTCR activation. To investigate this, an iNKT cell line was grown using monocyte-derived dendritic cells (MDDCs) as described in Methods section 2.10. Meanwhile, freshly isolated PBMCs from healthy donors, SLE-NP or SLE-P patients were pre-treated with α-GalCer overnight and FACS-sorted to obtain purified B cells and monocytes. These were then co-cultured with purified iNKT cells for 5, 10 and 15 minutes before assessing conjugate formation by flow cytometry. Methods were carried out by Natalya Ellis, a medical student who I supervised.

At all time-points, a greater percentage of iNKT-cells formed conjugates with monocytes compared to B cells (Figure 5.5A-C). B cell-iNKT cell conjugate formation was relatively consistent over time with a trend towards fewer conjugates formed in B cells from SLE-NP and SLE-P patients compared to healthy donors (Figure 5.5A-C). iNKT cell-monocyte interactions were more varied; monocytes from SLE-P patients formed more conjugates with iNKT cells at 5 minutes compared to 15 minutes, in contrast to monocytes from healthy controls where the numbers of conjugates increased over time (Figure 5.5).

These results suggested that iNKT cells formed more transient interactions with B cells compared to monocytes. This observation was investigated further using imaging cytometry to visualise conjugates, which confirmed that monocytes formed more conjugates with iNKT cells compared to B cells (Figure 5.5D).

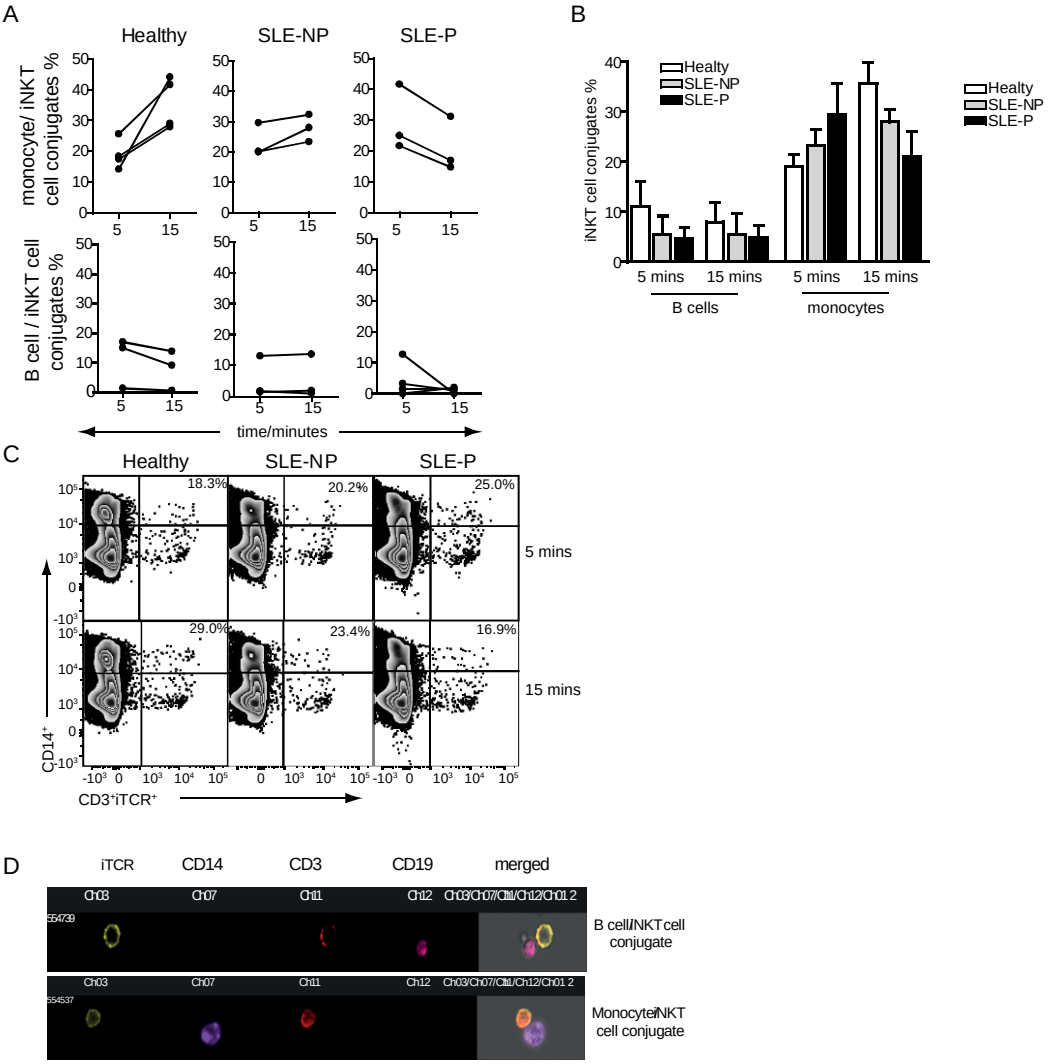

Figure 5.5 Frequency of iNKT cells forming conjugates with healthy, SLE-NP and SLE-P B cells and monocytes in response to α-GalCer.

PBMCs from healthy (n=8), SLE-NP (n=8) and SLE-P (n=10) donors were pre-treated with α-GalCer before staining for CD14+ monocytes and CD19+ B cells, which were isolated by FACS sorting. Meanwhile PBMCs from healthy individuals were stained for live/dead, CD3 and iTCR before isolating iNKT cell by FACS sorting. Isolated B cells or monocytes were subsequently co-cultured with iNKT cells from healthy individuals for 5, 10 and 15 minutes before fixing/permeabilising and staining with antibodies to phosphorylated ERK and pTCRζ. The percentage of iNKT cells forming conjugates with healthy, SLE-NP or SLE-P B cells and monocytes at 5 and 15 was calculated. (A) Line graphs and (B) cumulative data showing the kinetics of B cell- and monocyte-iNKT cell conjugate formation at 5 and 15 minutes. Mean± SE. One-way ANOVA *p=<0.05 (C) Representative FACS plots showing the percentage of monocytes from healthy donors, SLE-NP and SLE-P patients forming conjugates with heterologous healthy iNKT cells at 5 and 15 minutes. Imaging cytometry was used to confirm the presence of conjugates in experiments (D) Representative images of B cell-iNKT cell and monocyte-iNKT cell conjugates showing iTCR (yellow), monocytes (purple), CD3 (red) and B cells (pink).

5.6 B cells and monocytes from healthy donors and SLE patients with and without plaque induce differential iNKT cell signalling

Next I investigated whether changes in B cell and monocyte interactions with iNKT cells were associated with differential signalling via the iTCR. iNKT cells forming conjugates were analysed for pTCRζ and pERK1/2 expression by flow cytometry. As expected iNKT cells forming conjugates with either B cells or monocytes from healthy donors had higher levels of pTCRζ and pERK1/2 compared to unconjugated iNKT cells (Figure 5.6A). Methods here were performed by Natalya Ellis, an iBSc student under my supervision.

Interestingly, in healthy donors despite forming fewer and potentially more transient interactions with B cells compared to monocytes, both TCRζ and ERK1/2 phosphorylation was increased compared to that seen in monocyte/iNKT cell conjugates (Figure 5.6B-E). Further analysis of B cell-iNKT cell conjugates showed that B cells from SLE-NP (but not SLE-P) patients induced stronger pERK upon conjugation with healthy iNKT cells, compared to B cells from healthy donors (Figure 5.6B&C). However, no differences in TCRζ phosphorylation were observed in iNKT cell conjugates with B cells from healthy donors or either SLE groups.

When I considered iTCR signalling in the monocyte-iNKT cell conjugates, monocytes from SLE-NP patients were found to induce lower ERK1/2 and TCRζ phosphorylation compared to monocytes from healthy donors and SLE-P patients (Figure 5.6D&E), which reached significance for TCRζ expression between SLE-NP monocyte- and SLE-P monocyte-iNKT cell conjugates.

Overall, these results show that B cells and monocytes interact differently with iNKT cells in healthy individuals and that this is associated with differences in the activation of iTCR-associated signalling molecules. In B cells and monocytes from SLE patients altered patterns of iTCR signalling are triggered compared to healthy donors, which could be related to changes in lipid raft and CD1d expression driving abnormal iNKT cell activation.

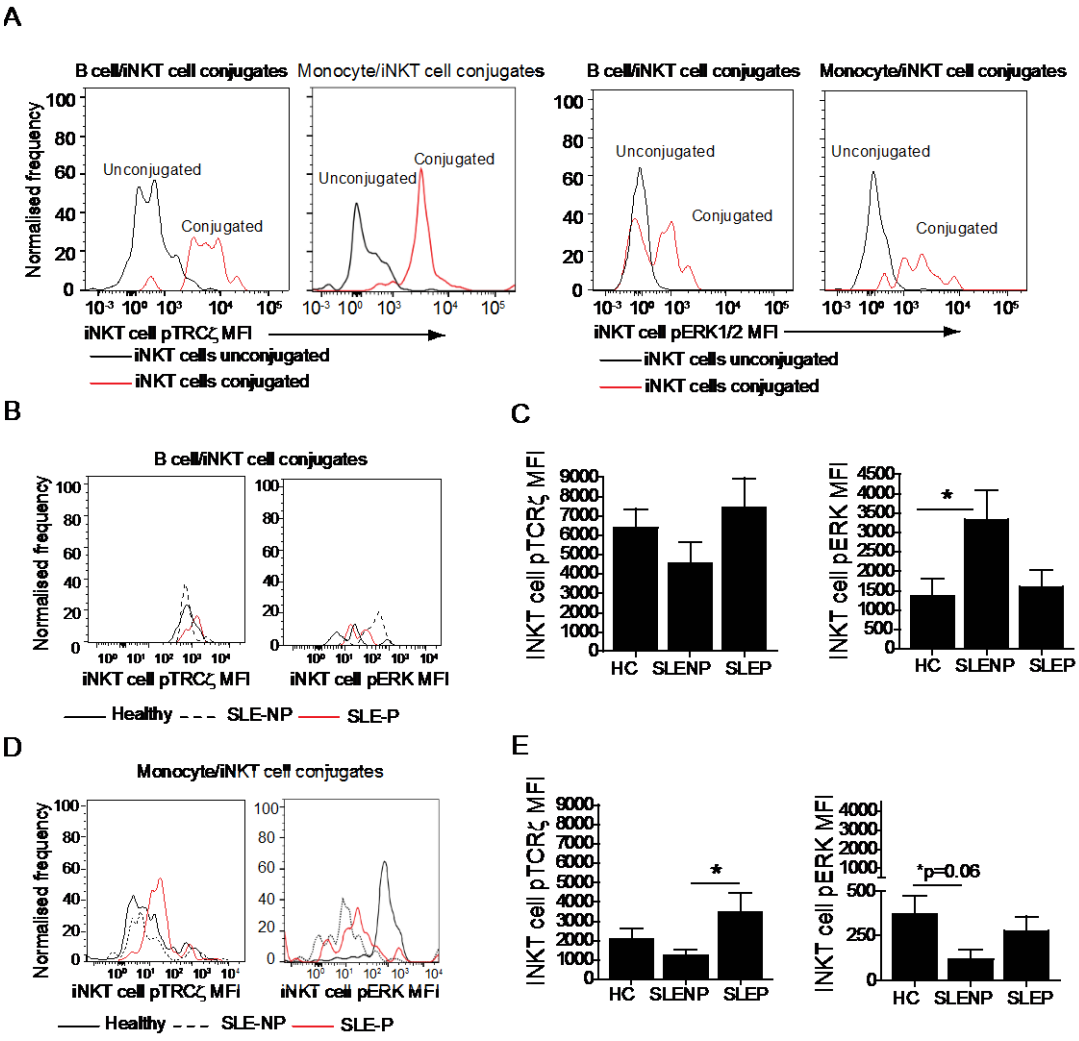

Figure 5.6 ERK and TCR zeta signalling in iNKT cells forming conjugates with B cells and monocytes from healthy, SLE-NP and SLE-P patients

PBMCs from healthy (n=8) , SLE-NP (n=8) and SLE-P (n=10) donors were pre-treated with α-GalCer before staining for CD14+ monocytes and CD19+ B cells, which were isolated by FACS sorting. Meanwhile PBMCs from healthy individuals were stained for live/dead, CD3 and iTCR before isolating iNKT cell by FACS sorting. Isolated B cells or monocytes were subsequently co-cultured with iNKT cells from healthy individuals for 5, 10 and 15 minutes before fixing/permeabilising and staining with antibodies to phosphorylated ERK and pTCRζ. (A) Representative histograms showing pTCRζ and pERK1/2 expression in unconjugated healthy iNKT cells compared to those forming conjugates with healthy B cells and monocytes. (B) Representative histograms and (C) cumulative data showing mean fluorescence intensity of pTCRζ and pERK1/2 expression in iNKT cell conjugates with B cells from healthy, SLE-NP and SLE-P patients at 15 minutes. (D) Representative histograms and (E) cumulative data showing mean fluorescence intensity of pTCRζ and pERK1/2 expression in iNKT cell conjugates with monocytes from healthy, SLE-NP and SLE-P patients at 15 minutes. Mean± SE. One-way ANOVA and Tukey's multiple comparison post-test *p=<0.05

CHAPTER VI: Results 4

Results 4: Role of endogenous lipids in iNKT cell responses and monocyte transactivation

6.1 Introduction and aims for this chapter

The results described in Chapter 3 show that monocytes and B cells interact with iNKT cells in different ways. Furthermore the results suggest that only monocytes show differential effects on iNKT cell activation in SLE-P patients compared to SLE-NP patients and healthy donors.

Little is known about the endogenous lipids which activate iNKT cells despite attempts to characterise them through their ability to bind CD1d. Lipids previously identified include LPC, β-GlcCer and Igb3 although their roles in humans and in the absence of infection remains contentious [310, 314, 315, 318]. Instead most studies have used α-GalCer due to its potency to activate iNKT cells; however since it is derived from a marine sponge such culture experiments do not resemble the normal physiological situation *in vivo* [273].

Therefore the first aim of this chapter was to investigate the ability of lipids isolated from B cells or monocytes from healthy, SLE-NP and SLE-P patients to activate iNKT cells from healthy donors. Lipids were isolated by chloroform: methanol extraction with further purification into several fractions based on their chemical composition

Due to the importance of monocytes in the development of atherosclerosis, I went on to investigate whether monocytes had a different phenotype in SLE patients with pre-clinical plaque, including the frequency of intermediate or $CD14^{++}CD16^{+}$ monocytes, which have been shown to be increased in atherosclerosis [168-170], as well as characterisation of monocytes based on $CD68^{+}CCR2^{+}$ and $CD206^{+}CX_3CR1^{+}$ which have been described as M1-like and M2-like respectively by Fadini et al. who observed altered frequencies in dyslipidaemic patients [497]. Scavenger receptor expression was also determined due to their importance in the uptake of lipids, which I have shown to be different between SLE-NP and SLE-P patients shown in Results 3.

Finally, M2 macrophages are induced by IL-4 and play a protective role in atherosclerosis, where they promote tissue repair [188]. I therefore investigated whether iNKT cells or serum from SLE patients with preclinical plaque could also induce polarisation of M2 macrophages in order to support my previous finding of increased intracellular IL-4 in SLE-P patients shown in Results 1.

6.2 Phospholipids from SLE-P monocytes induce greater iNKT cell expansion compared to those from SLE-NP patients

To determine whether altered endogenous lipids in healthy donors, SLE-NP and SLE-P patients could influence iNKT cell responses cellular lipids were extracted from purified *ex vivo* monocytes and B cells from healthy donors, SLE-NP and SLE-P patients using chloroform: methanol isolation and subsequent fractionation with amino propyl columns (Figure 6.1A for experimental plan). B cell and monocytic lipids (Fractions 1-6) from healthy donors, SLE-NP and SLE-P patients were then co-cultured with PBMCs isolated from healthy donors in the presence of pooled human serum and IL-2 to induce iNKT cell expansion. Initial experiments established that iNKT cells from healthy donors responded more robustly to the lipid fraction 5 (F5) containing phospholipids and sphingomyelin; this was regardless of whether lipids were B cell- or monocyte-derived (Figure 6.1B).

F5 lipids purified from B cells and monocytes isolated from SLE-NP and SLE-P patients and healthy donors were then used to stimulate iNKT cells from healthy donors. Only monocytic F5 lipids isolated from SLE-P patients induced a significant increase in iNKT cell expansion (Figure 6.2 A-C). This was associated with a more anti-inflammatory cytokine environment, characterised by increased expression levels of IL-4, IL-13 and reduced IFN-γ in tissue culture supernatants compared to SLE-NP patients or healthy controls (Figure 6.2D-F). These responses were blocked by the addition of anti-CD1d antibody suggesting that CD1d-mediated presentation of F5 lipids to iNKT cells was required for iNKT cell expansion and the increase in IL-4 and IL-13 and decrease in IFN-γ in response to Fraction 5 (Figure 6.2G and H).

These results suggested that phospholipids and sphingomyelin from monocytes presented via CD1d in SLE-P patients were more effective at inducing iNKT cell expansion compared to those presented by SLE-NP patients, and that this triggered the production of anti-inflammatory and potentially athero-protective cytokines. Whilst cytokine secretion was blocked by anti-CD1d, whether iNKT cells are the source remains under question.

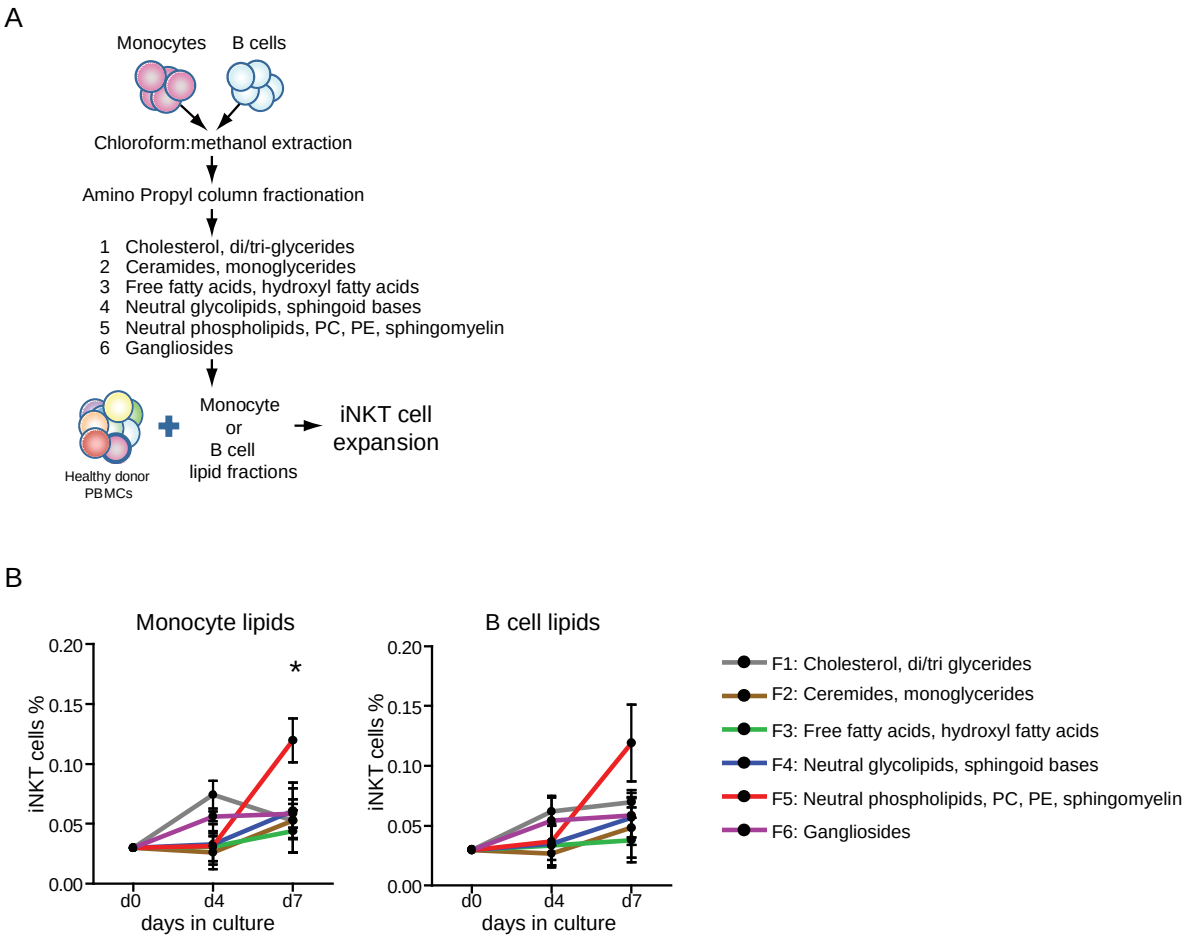

Figure 6.1 Isolation of B cell and monocyte lipid fractions from healthy donors, SLE-NP and SLE-P patients

Cellular lipids from B cells or monocytes from 5 healthy donors were isolated by chloroform: methanol extraction, and separated into 6 different fractions using amino propyl columns before resuspending in 50µl DMSO. $5x10^6$ PBMCs from healthy donors were then cultured with 2µl of each lipid fraction in complete RPMI supplemented with 200IU/ml IL-2. *i*NKT cells were quantified by flow cytometry at d0, d4 and d7. (B) Cumulative data from 3-5 experiments showing iNKT cell expansion upon culture of lipid fractions from B cells or Monocytes with PBMCs from healthy donors. Mean ±SE. One-Way ANOVA and Tukey's multiple comparison post-test; *p=0.05. PC – phosphatidylcholine; PE - phosphatidylethanolamine

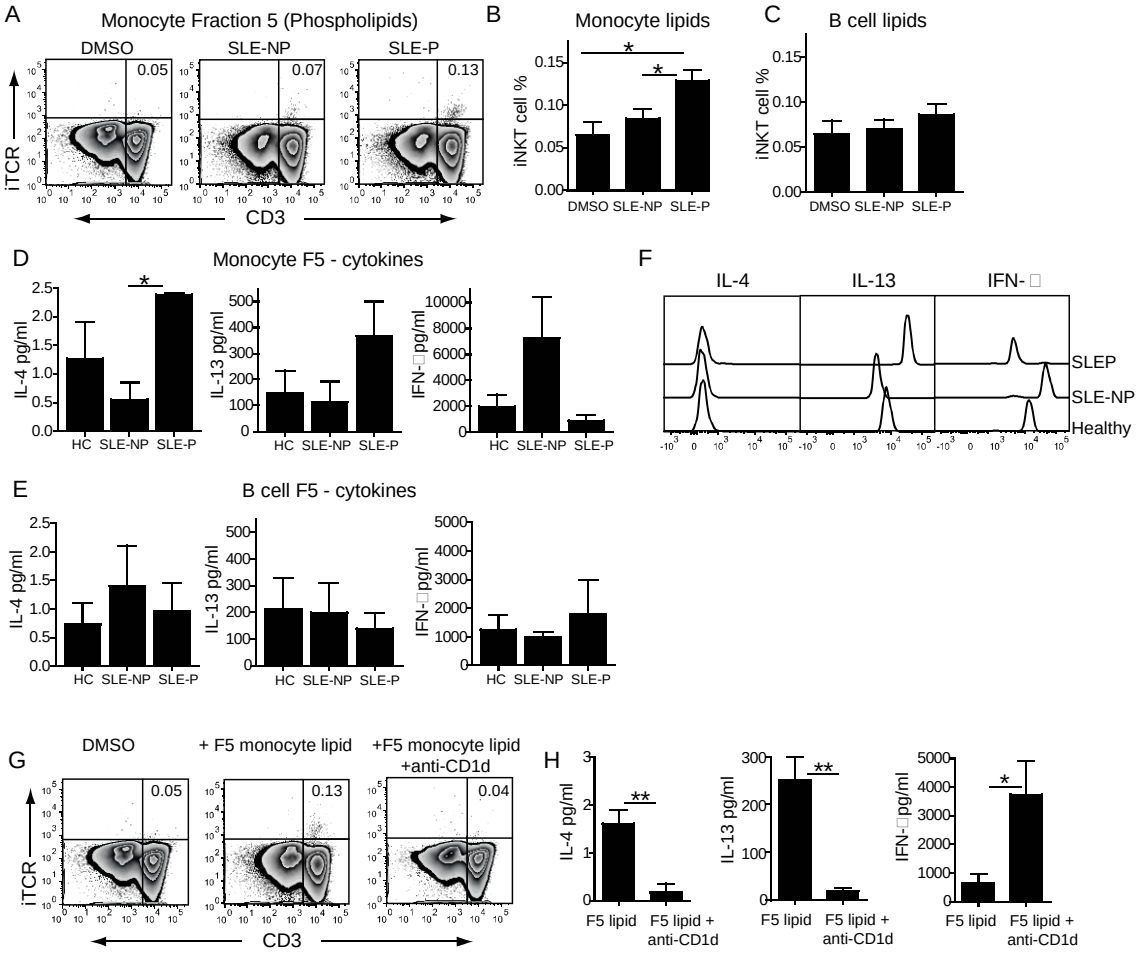

Figure 6.2 The effect of B cell and monocyte lipid fractions on iNKT cells from healthy donors

Monocyte and B cell lipids were purified from 5 healthy donors, 6 SLE-NP and 6 SLE-P patients (10^7 cells/group) by chloroform/methanol isolation and fractionation using amino propyl columns. Fraction 5 (F5) lipids were re-suspended in DMSO and cultured with PBMCs from 3 healthy donors +IL-2 for 7ds. At d7 cell culture supernatants were collected and PBMCs were labelled with CD3 an iTCR. **(A)** Representative flow cytometry dot plots showing INKT cell frequency in response to monocyte derived F5 lipids and cumulative data showing response to **(B)** monocyte derived and **(C)** B cell derived F5 lipids. Mean ±SE. One-way ANOVA and Tukey's multiple comparison test, *p=<0.05. Cell culture supernatants from d7 cultures were assessed for expression of IL-4, IL-13 and IFN-γ by CBA. Cumulative data for **(D)** Monocytes and **(E)** B cells are shown as well as **(F)** representative overlays. Mean ±SE. One-way ANOVA and Tukey's multiple comparison post-test, *p=<0.05. The experiment was repeated ± blocking anti-CD1d antibody or isotype control. **(G)** Representative flow cytometry dot plots showing iNKT cell frequency from cultures with SLE-P monocyte lipids for 7d and **(H)** cumulative CBA data of IL-4, IL-13 and IFN-γ expression in tissue culture supernatants. Mean ±SE. Students t test, **p=<0.01, *p=<0.05.CBA – cytometric bead array; SLE-NP – SLE patients without plaque; SLE-P – SLE patients with plaque

6.3 Expression of B cell and monocyte scavenger receptors is comparable in SLE-NP and SLE-P patients

My observation that phospholipids from SLE-P monocytes could induce differences in iNKT cell expansion and cytokine response suggested that other differences in monocytes could contribute to atherosclerosis in SLE-P patients. I therefore examined monocyte phenotype and function in more detail.

Due to the importance of scavenger receptors in lipid uptake, B cells and monocytes were assessed for CD36, LDLR and LOX-1 expression in healthy individuals, SLE-NP and SLE-P patients as well as a group of SLE patients who had previously suffered a cardiovascular event (Table 3.2). No significant differences were observed between healthy, SLE-NP and SLE-P patients (Figure 6.3), although trends were observed including an increase of CD36 and LDLR expression on B cells and a decrease in CD36 expression on monocytes from SLE-P patients, which could represent changes in scavenger receptor expression in SLE patients with asymptomatic plaques. Interestingly, the major finding was an increase in LOX-1 expression on monocytes from SLE patients who had previously had a cardiovascular event, suggesting that alterations in LOX-1 expression on monocytes occur at later stages of atherosclerosis.

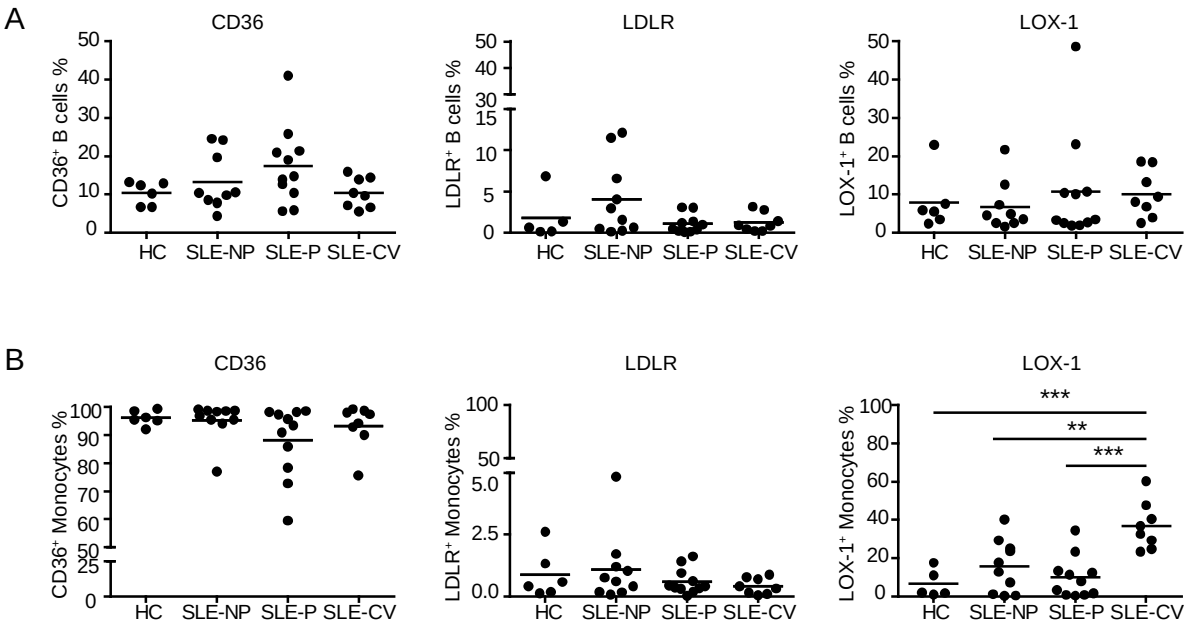

Figure 6.3 Expression of CD36, LDLR and LOX-1 in Monocyte and B cell phenotype in healthy donors, SLE-NP, SLE-P and SLE-CV patients

10^6 PBMCs from 6 healthy donors, 10 SLE-NP, 10 SLE-P and 8 SLE-CV patients were surface stained *ex vivo* for CD14, CD19 plus a combination of CD36, LDLR and LOX-1, or CD1d. Data were analysed by flow cytometry. Percentage of (A) B cells and (B) monocytes positive for CD36, LDLR and LOX-1 Mean, One-way ANOVA and Tukey's multiple comparison post-test, **p=<0.01, ***p=<0.001. HC – healthy controls; SLE-NP – SLE patients without plaque; SLE-P – SLE patients with plaque; SLE-CV – SLE patients who have suffered a cardiovascular event

6.4 SLE-P patients are characterised by increased numbers of anti-inflammatory monocytes

Ex vivo quantification of pro-inflammatory monocytes according to CD14 and CD16 expression revealed that SLE-NP patients had a significantly greater percentage of $CD14^{++}CD16^{+}$ (so called 'intermediate') monocytes compared to healthy individuals and SLE-P patients (Figure 6.4A and B). Interestingly, analysis of SLE patients who had previously suffered a cardiovascular event had a higher frequency of intermediate monocytes in these patients compared to SLE-P patients and healthy controls, with numbers being more similar to SLE-NP patients.

Further *ex vivo* characterisation of monocytes was carried out based on a gating strategy by Fadini et al. which identified M1- or M2-like monocytes ex vivo [497]. Analysis showed that both SLE-NP and SLE-P patients had an increase in frequency of the pro-atherogenic or M1-like $CD14^{+}CD68^{+}CCR2^{+}$ monocytes compared to healthy individuals (Figure 6.4C-F). In contrast, M2-like $CD14^{+}CD206^{+}CX_3CR1^{+}$ monocytes, which are thought to be anti-atherogenic, had a significantly increased frequency in SLE-P patients compared to SLE-NP patients (Figure 6.4C-F).

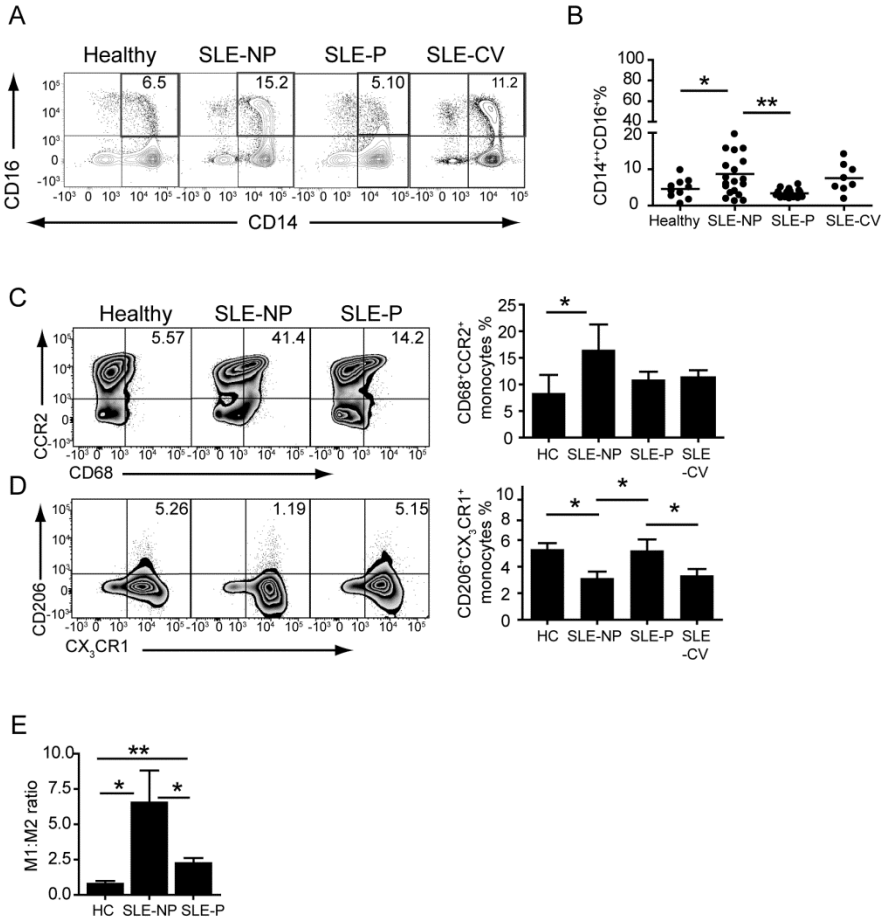

Figure 6.4 Monocyte phenotype in healthy individuals, SLE-NP patients and SLE-CV patients who had previously suffered a cardiovascular event.

PBMCs from 8 healthy donors, 18 SLE-NP, 11 SLE-P and 8 SLE-CV patients were surface stained for CD14, CD16, CD206, CD68, CCR2 and CX_3CR1. Monocyte populations were assessed by flow cytometry. **(A)** Representative dot plots and **(B)** cumulative data for $CD14^{++}CD16^{-}$ classical monocyte, $CD14^{++}CD16^{+}$ intermediate monocyte and $CD14^{+}CD16^{+}$ non-classical monocyte frequency. Mean, One-way ANOVA and Tukey's multiple comparison post-test, **p=<0.001, *p=<0.01. **(C)** Representative dot plots and cumulative data for M1-like ($CD68^{+}CCR2^{+}$) monocytes and **(D)** M2-like ($CD206^{+}CX_3CR1^{+}$) monocytes. **(E)** M1:M2 ratio in healthy, SLE-NP and SLE-P patients. Mean ±SE. One-way ANOVA and Tukey's multiple comparison post-test, **p=<0.01, *p=<0.05. HC – healthy controls; SLE-NP – SLE patients without plaque; SLE-P – SLE patients with plaque; SLE-CV – SLE patients who have suffered a cardiovascular event

6.5 iNKT cells are required for polarisation of M1/M2 macrophages

I questioned whether iNKT cells themselves could influence monocyte/macrophage polarisation, and therefore determine the immune response within the atherosclerotic plaque. To test this purified monocytes were co-cultured with T cells from healthy donors with serum from SLE-NP, SLE-P patients and healthy donors for 7d. SLE-P serum preferentially induced the differentiation of M2 macrophages detected by CD206 (mannose receptor), which is regulated by IL-4 [188]. In monocyte only cultures there was no significant difference in M2 macrophage differentiation in cultures with healthy, SLE-NP and SLE-P serum (Figrue 6.5B&C). In monocyte and T cell co-cultures there was a greater amount of M2 macrophage differentiation, with nearly al the cells positive for CD206, although no differences were observed between healthy, SLE-NP and SLE-P serum cultures (Figure 6.5D). The addition of anti-CD1d blocking antibody was able to inhibit M2 macrophage differentiation on each occasion (Figure 6.5D).

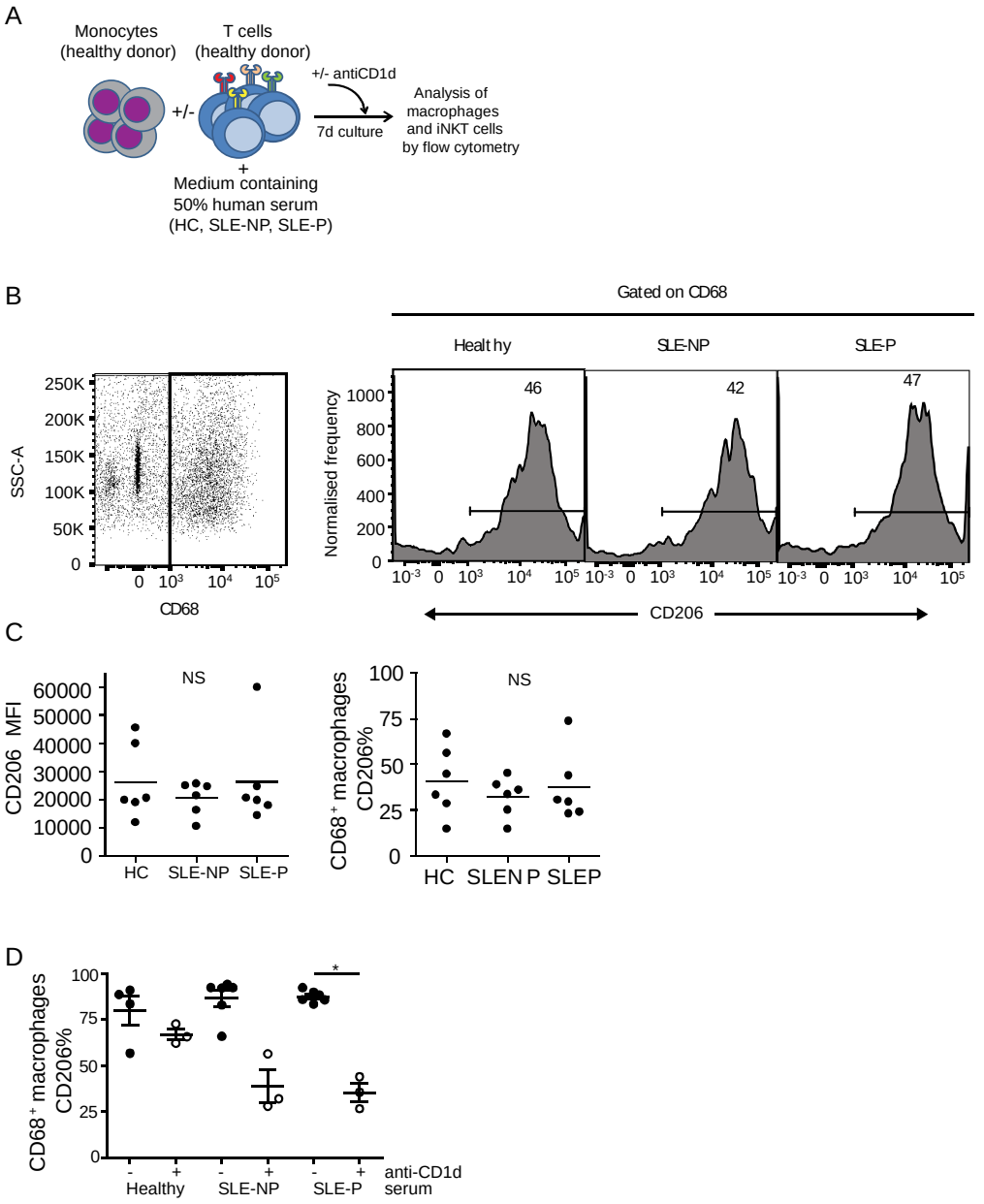

Figure 6.5 Macrophages fail to differentiate to M2 phenotype in the absence of iNKT cells

(A) Experimental plan: Purified monocytes (10^5/well) either with or without T cells ($7.5x10^5$/well) from 4 healthy donors were co-cultured in the presence of 50% serum from six SLE-NP, SLE-P patients and heterologous healthy donors for 7ds. At d7 adherent differentiated macrophages were recovered and surface stained for CD68 and CD206. (B) Gating strategy showing $CD68^+$ gating, followed by representative plots for CD206. (C) Cumulative data from monocyte only cultures showing frequency and MFI of CD206 on $CD68^+$ macrophages. Mean ±SE. One-way ANOVA and Tukey's multiple comparison post-test, *p=0.05. (E) Cumulative data showing percentage of $CD206^+$ macrophages from monocyte and T cell co-cutures. Mean ±SE. One-way ANOVA and Tukey's multiple comparisons post-test. HC – healthy controls; SLE-NP – SLE patients without plaque; SLE-P – SLE patients with plaque; NS – not significant

6.6 iNKT cells from SLE-P patients influence monocyte and macrophage phenotype

To establish the role of iNKT cells in driving monocyte and macrophage polarisation iNKT cells were differentiated with SLE-P and SLE-NP serum to induce the characteristic SLE-NP or SLE-P iNKT cell phenotype. Pre-conditioned iNKT cells were then co-cultured with the human THP-1 macrophage cell line (in order to rule out differences between macrophages derived from healthy donor PBMCs). Expression of M1 and M2 macrophage genes was then assessed by qPCR, which was carried out by Kirsty Waddington.

Genes analysed were the M1-associated gene for Pro-platelet basic protein (*PPBP*) [500] and several M2-associated genes, namely *CD163, CD206, CD200R* and the Stabilin-1 (*STAB-1*) gene [501]. Such genes were chosen based on transcriptional profile analysis studies of GM-CSF- and M-CSF-differentiated macrophages, which led to the identification of several genes including *PPBP*, *CD163, CD206, CD200R* and *STAB-1* which could discriminate between M1 and M2 macrophages respectively [500, 502]. Furthermore, Roma-Lavisse et al. showed that M2 macrophage genes CD206, CD200R and STAB-1 were positively associated with plaque stability in atherosclerosis patients [502].

Only iNKT cells exposed to SLE-P serum induced upregulation of the CD206 and CD200R genes associated with M2 macrophage polarisation [501] while no differential effect was observed with expression of PPBP an M1 macrophage-associated gene [500] (Figure 6.6A for experimental strategy, 6.6B and data not shown).

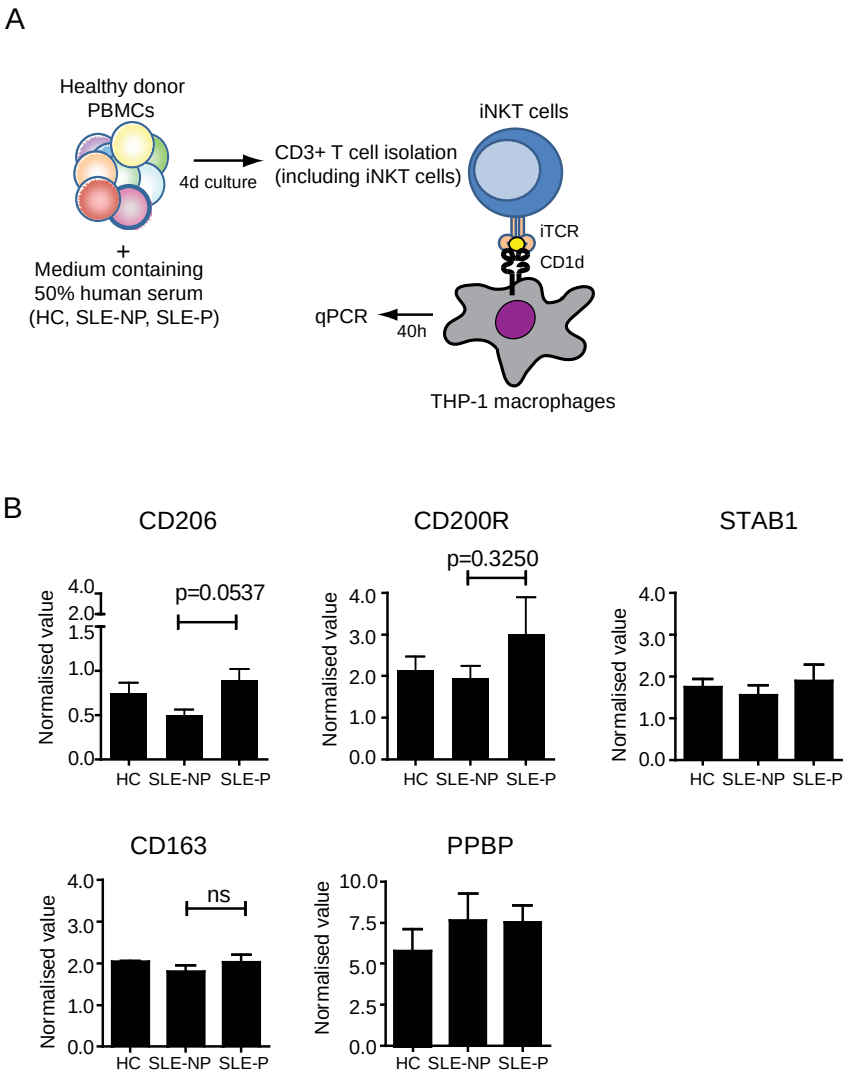

Figure 6.6 iNKT cells from SLE-P patients support M2 macrophage differentiation

PBMCs from 2 healthy donors were each cultured with 50% serum from three different healthy donors, 3 SLE-NP and 3 SLE-P patients for 7ds to induce iNKT cell differentiation. **(A)** Experimental plan: $CD3^+$ T cells were isolated and co-cultured ($1.5x10^6$/well) with differentiated THP-1 human macrophage cell line ($1x10^6$/well) for 40hs. Macrophages were recovered and RNA analysed for expression of CD206 and CD200R gene expression by qPCR. **(B)** cumulative data showing CD206, CD200R, STAB1, CD163 and PPBP gene expression in THP-1 macrophages normalised to the level of cyclophilin [503]. Mean ±SE. One-way ANOVA and Tukey's multiple comparison post-test *p=0.05. HC – healthy controls; SLE-NP – SLE patients without plaque; SLE-P – SLE patients with plaque

CHAPTER VII: Results 5

Results 5: Preliminary data - lipid metabolism in antigen presenting cells and its effect on iNKT cells

7.1 Introduction and aims for this chapter

The results presented in the previous chapters suggest that serum factors influence differential iNKT cell phenotypes in SLE patients with and without plaque and that this is associated with interactions with monocytes rather than B cells. However, previous work performed in the lab has shown that B cells also influence iNKT cell defects in patients with SLE.

Lipid metabolism is important for many aspects of immune cell function. These include the formation of lipid rafts which are regulated through cholesterol biosynthesis, and the production of cytokines in order to modulate immune responses [374]. Previous work from the laboratory has shown that lipid biosynthesis is altered in SLE patients whereby T cells have increased expression of LXRβ, a gene which controls lipid biosynthesis [380]. This was subsequently found to contribute to the defects in lipid rafts in SLE patients, which could be corrected by normalising glycolipid metabolism [380]. Similar defects in lipid biosynthesis through LXR, as well as PPARγ have been described in cardiovascular disease [504, 505], which is of relevance to this study due to the increased cardiovascular risk in SLE patients.

Furthermore, there is evidence that lipid biosynthesis can regulate CD1d-mediated lipid antigen presentation to iNKT cells. For example, in dendritic cells PPARγ stimulation has been shown to upregulate CD1d expression [383], whilst defective expression of both CD1d and PPARγ on adipocytes has been shown to contribute to the lack of iNKT cells observed in obesity [384]. It is unknown, however whether LXR also plays a role in this process.

I therefore decided to investigate whether altering lipid biosynthesis through LXR and PPARγ could explain the differences in CD1d and lipid raft expression in B cells described in Chapter 5 that was observed in all SLE patients regardless of whether they had plaque.

7.2 Differences in lipid biosynthesis between healthy, SLE-NP and SLE-P patients

To question whether there were differences in cholesterol biosynthesis between B cells and monocytes from healthy donors, SLE-NP and SLE-P patients FACS staining for filipin (cholesterol) and ANE (lipid order) was carried out with MSc student Shaheer El-Bardisy [364]. I observed an increase in both lipid order and cholesterol expression in monocytes compared to B cells, indicative of increased plasma membrane stability (Figure 7.1A and B). Interestingly, cholesterol expression was increased in B cells and monocytes from SLE-NP, but not SLE-P patients compared to healthy individuals (Figure 7.1B).

Membrane lipids are regulated by several mechanisms including de novo lipid biosynthesis, uptake and recycling pathways and cholesterol efflux via channels such as ATP binding cassette A1 (ABCA1)[374]. Flow cytometry analysis of ABCA1 expression found increased ABCA1 on B cells compared to monocytes (Figure 7.1C), indicating differences in lipid metabolism. However, no significance was observed due to the low numbers of samples in each group.

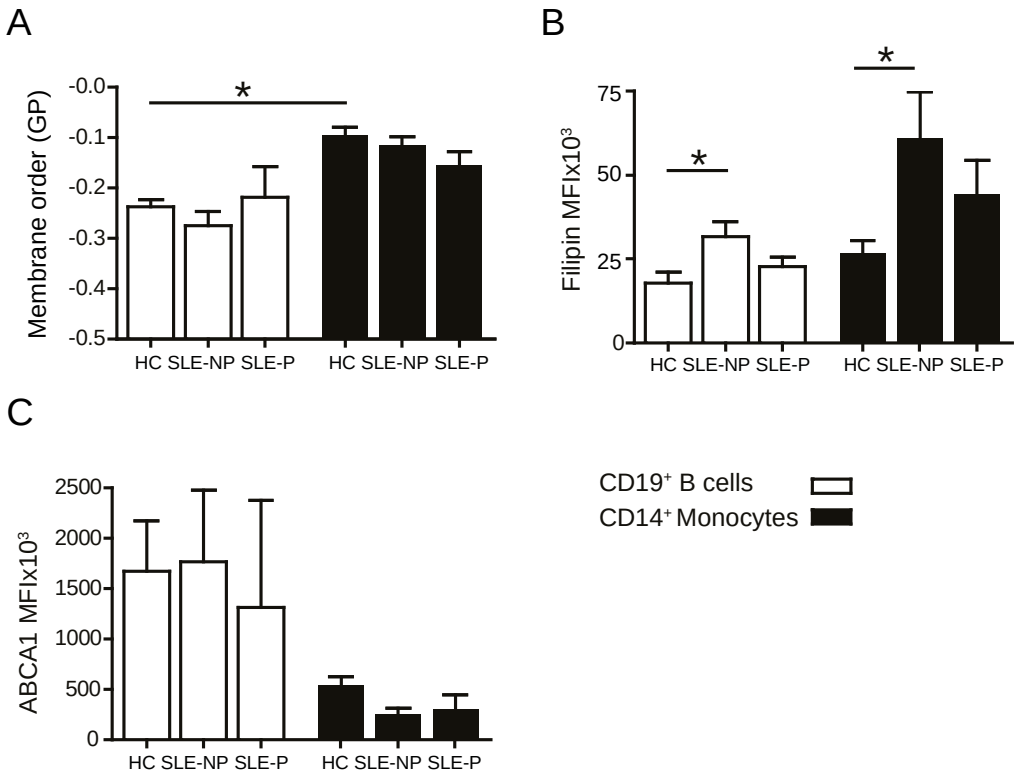

Figure 7.1 Differences in membrane order, cholesterol and ABCA1 transporter expression between healthy donors, SLE-NP and SLE-P patients

10^6 PBMCs from 3-15 healthy donors, 3-15 SLE-NP and 3-15 SLE-P patients were surface stained *ex vivo* for CD14, CD19 plus filipin (cholesterol), ABCA1 or ANE (to measure membrane order). Data were analysed by flow cytometry. **(A)** Membrane order of B cells and monocytes, as determined by formula in methods section 2.5. **(B)** MFI of Filipin (cholesterol) expression on B cells and monocytes **(C)** MFI of ABCA1 channel expression on B cells and monocytes. Mean ± SE One-Way ANOVA was used to test for significance. *p<0.05; HC – healthy controls; SLE-NP – SLE patients without plaque; SLE-P – SLE patients with plaque; ABCA1- ATP Binding Casette A1; ANE- di-4-ANEPPDHQ.

7.3 Liver X receptor agonist induces altered lipid biosynthesis in B cells from healthy individuals

I then questioned whether the differences in CD1d and CTB expression observed from *ex vivo* staining of B cells and monocytes (Results Chapter 5) could be explained by altered lipid biosynthesis. To investigate how altered lipid biosynthesis could influence the expression of different glycosphingolipid species in B cells from healthy individuals, PBMCs from healthy donors were cultured for 24h with either 1μM LXR agonist GW3965 or vehicle (DMSO). Whole PBMCs, rather than B cells were cultured with LXR agonist GW3965 in order to preserve B cell viability, whilst reflecting the situation *in vivo*.

B cells were subsequently labelled with CD19 and sorted by FACS before pooling $1x10^7$ B cells for each condition. Lipids were subsequently isolated from B cells by chloroform: methanol extraction before being assessed for glycosphingolipids by high performance liquid chromatography (HPLC), which was carried out by Dr Dominic Alonzi at the Glycobiology Institute, Department of Biochemistry University of Oxford.

Interestingly, treatment of PBMCs with LXR agonist GW3965 resulted in the upregulation of several glycosphingolipid species in B cells in comparison to treatment with vehicle alone (DMSO). Namely, GW3965 treatment resulted in augmented GA2 expression as well as the induction of GD3, GD1a and GT3 (Figure 7.2).

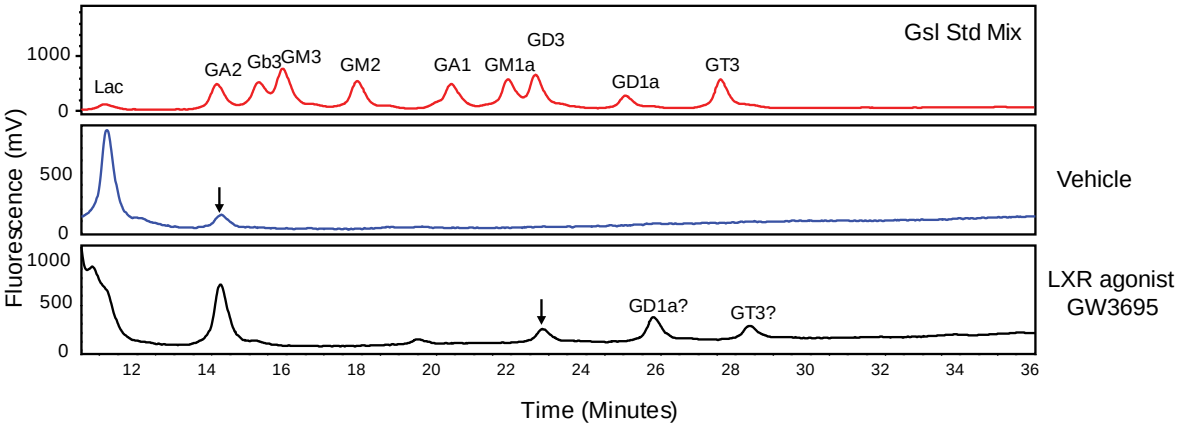

Figure 7.2 The effect of LXR agonist on B cell glycosphingolipid species

PBMCs from 3 healthy donors were cultured for 24h with1μM LXR agonist GW3965 or vehicle (DMSO). Cells were labelled with CD19 and B cells were isolated using FACS sorting before pooling healthy donors and carrying out chloroform: methanol extraction on $1x10^7$ B cells. The total cellular GSL profile was analysed by HPLC following glycanase digestion to release the GSL sugar head groups. Data is represented as qualitative HPLC plots showing the position of known GSL standards and GSL species in B cells from healthy donors treated with vehicle (DMSO) or LXR agonist GW3695.

7.4 Glycolipid content in B cells and monocytes from SLE patients is similar to healthy controls

Previous work from the laboratory has identified increased expression of certain glycosphingolipids in T cells from SLE patients compared to healthy donors, which were associated with defective T cell signalling and function [367, 380]. I therefore decided to investigate whether glycolipid expression was altered in antigen presenting cells from SLE patients with and without plaque by carrying out HPLC on lipids isolated from B cells and monocytes (Figure 7.3). Interestingly, GM3 was found to be absent in B cells from SLE-NP patients, although it is unknown whether this was due to poor sample quality. Analysis of monocytes showed a slight reduction in GM3 in SLE-NP monocytes, and potentially increased levels of GD3 in SLE-P monocytes. This work is part of an ongoing collaboration with Dr Alonzi, Glycobiology Inst. Oxford University.

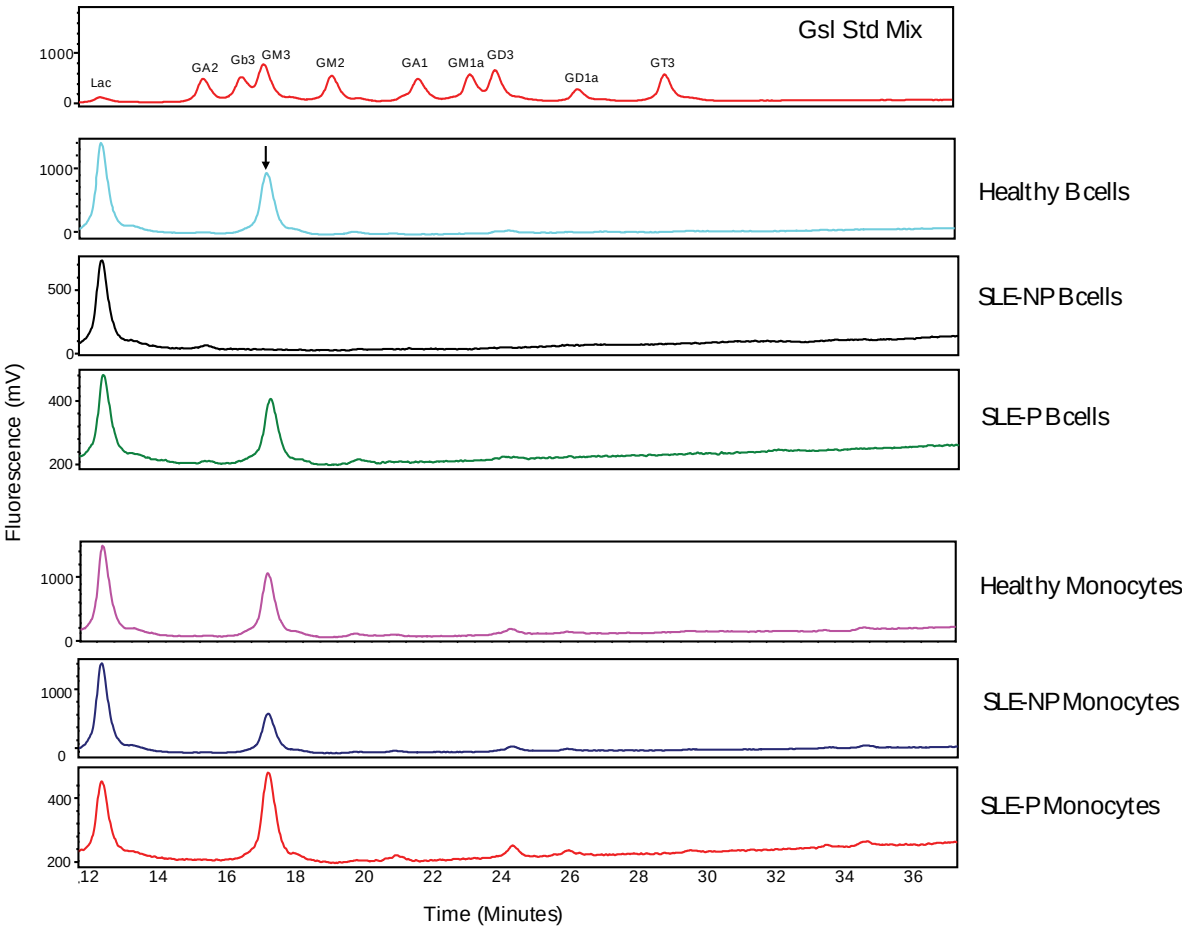

Figure 7.3 Differences in glycolipid content in B cells and monocytes from healthy, SLE-NP and SLE-P patients

$1x10^7$ B cells and monocytes from healthy (n=7), SLE-NP (n=6) and SLE-P (n=7) patients were labelled with CD14 and CD19 then isolated by FACS sorting before carrying out chloroform: methanol extraction. The total cellular GSL profile was analyzed by HPLC following glycanase digestion to release the GSL sugar head groups. Data is represented as qualitative HPLC plots showing the position of known GSL standards and GSL species in B cells and monocytes from healthy controls, SLE-NP and SLE-P patients. SLE-NP – SLE patients without plaque; SLE-P – SLE patients with plaque

7.5 Altered lipid metabolism in B cells and monocytes of SLE patients

Previous work from the lab has shown that treatment of $CD4^+$ T cells with LXR agonist induced increased CTB expression in cells from healthy donors and induce lipid raft-associated defects similar to those identified in in SLE patients [380]. I therefore sought to determine whether stimulation of lipid biosynthesis pathways (LXR or PPARγ) could influence B cells and monocyte membrane lipids as well as CD1d expression, and whether this had any subsequent influence on iNKT cells. To achieve this, freshly isolated PBMCs were cultured *in vitro* with LXR agonist (GW3965), PPARγ agonist (Rosiglitazone) or vehicle (DMSO) for 4, 24 and 96h, before staining for B cells (CD19), monocytes (CD14), CD1d, lipid rafts (CTB) and cholesterol (filipin). Experiments were performed by MSc student Shaheer El-Bardisy under my supervision.

Whilst LXR and PPARγ agonist failed to influence CD1d expression in both B cells and monocytes when compared to vehicle-treated (data not shown), trends were observed for lipid raft expression whereby LXR agonist was found to increase lipid raft expression on B cells over time, whilst PPARγ agonist treatment was found to increase lipid raft expression on monocytes. However, neither of these trends reached significance when paired t tests were used to compare vehicle with LXR or PPARγ treatments, although this was probably due to low sample numbers.

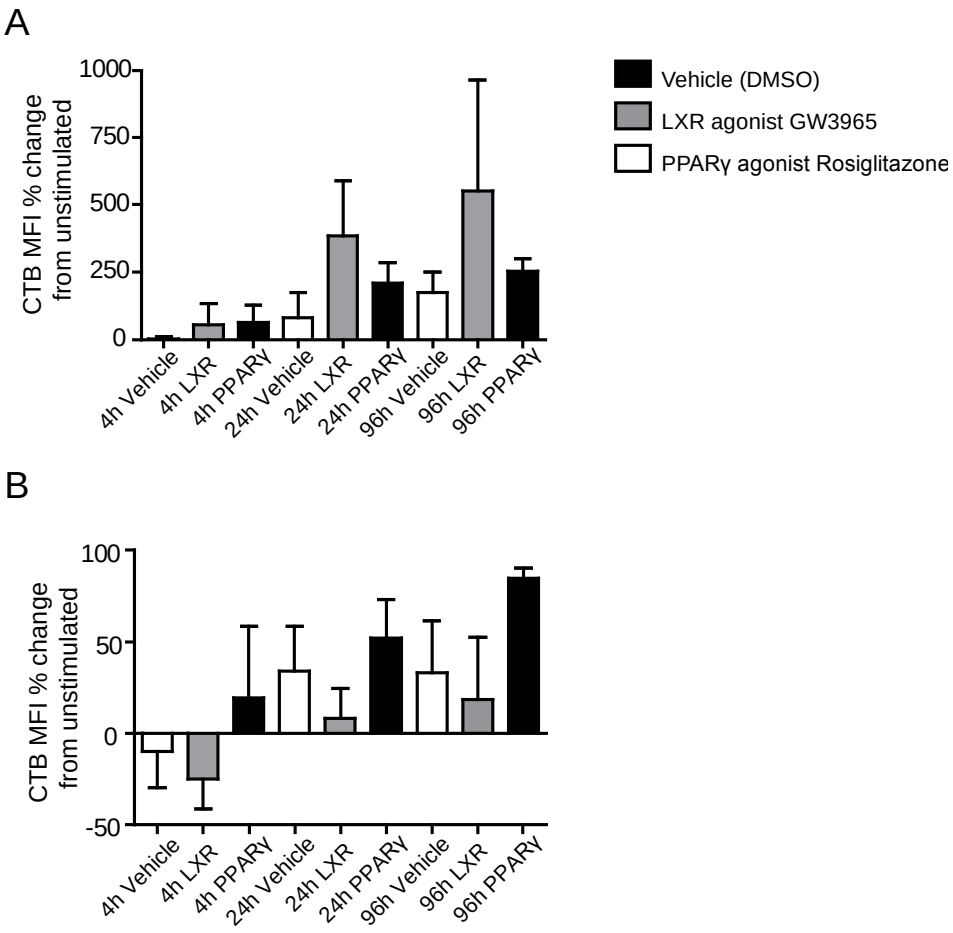

Figure 7.4 LXR agonist GW3965 and PPARγ agonist Rosiglitazone stimulation influences B cell and monocyte lipid raft expression

PBMCs from healthy donors (n=3-4) were cultured in complete medium at a concentration of 10^7/ml in the presence of 1μM of LXR agonist GW3965, 2.5 μM PPARγ agonist Rosiglitazone or equivalent concentrations of DMSO (vehicle) for 4, 24 and 96h, At each time point cells were stained for CD14 (Monocytes), CD19 (B cells), and CTB (lipid rafts) and expression quantified by flow cytometry. Percentage change in MFI for CTB in (A) B cells and (B) monocytes for each treatment, calculated relative to ex vivo CTB expression from the same healthy donors.

7.6 Altered lipid biosynthesis in B cells (but not monocytes) influences iNKT cell expansion and cytokine responses

To determine whether LXR or PPARγ agonist stimulation could influence iNKT cell responses, potentially via changes in CD1d and lipid raft expression, an iNKT cell line was generated over 7 days as described in methods section 2.10. Meanwhile, PBMCs were cultured with 1μM LXR agonist (GW3965), 2.5μM PPARγ agonist (Rosiglitazone) or vehicle (DMSO) overnight before negatively isolating B cells or monocytes and co-culturing for 48h with the expanded iNKT cell line ($CD3^+$ cells isolated by FACS sorting or by negative selection kits). Cells were subsequently surface stained for CD3 and iTCR, and intracellularly for IFN-γ, IL-4 and IL-10. iNKT cell phenotype was analysed by flow cytometry. Experiments were performed by Shaheer El Bardisy under my supervision.

Data was plotted relative to unstimulated (vehicle/DMSO). Interestingly, iNKT cells were expanded when cultured with B cells which had been pre-treated with PPARγ agonist, whilst LXR agonist had the opposite effect (Figure 7.5A). Both these responses were inhibited with anti-CD1d antibody, demonstrating that this effect was due to alterations in CD1d-mediated antigen presentation. No effect however was observed for co-cultures with monocytes (Figure 7.5B).

Analysis of cytokines by intracellular staining revealed that LXR (but not PPARγ)-stimulated B cells could induce a Th1 cytokine response in iNKT cells, characterised by increased intracellular IFN-γ and decreased IL-4 (Figure 7.6A and B). This was inhibited in cultures with anti-CD1d antibody, suggesting that this was due to altered CD1d-mediated antigen presentation. No effect was observed on intracellular IL-10 in co-cultures with either LXR- or PPARγ-stimulated B cells (Figure 7.6C), whilst stimulation of monocytes with LXR or PPARγ agonist failed to have any effect on iNKT cell cytokine production (data not shown).

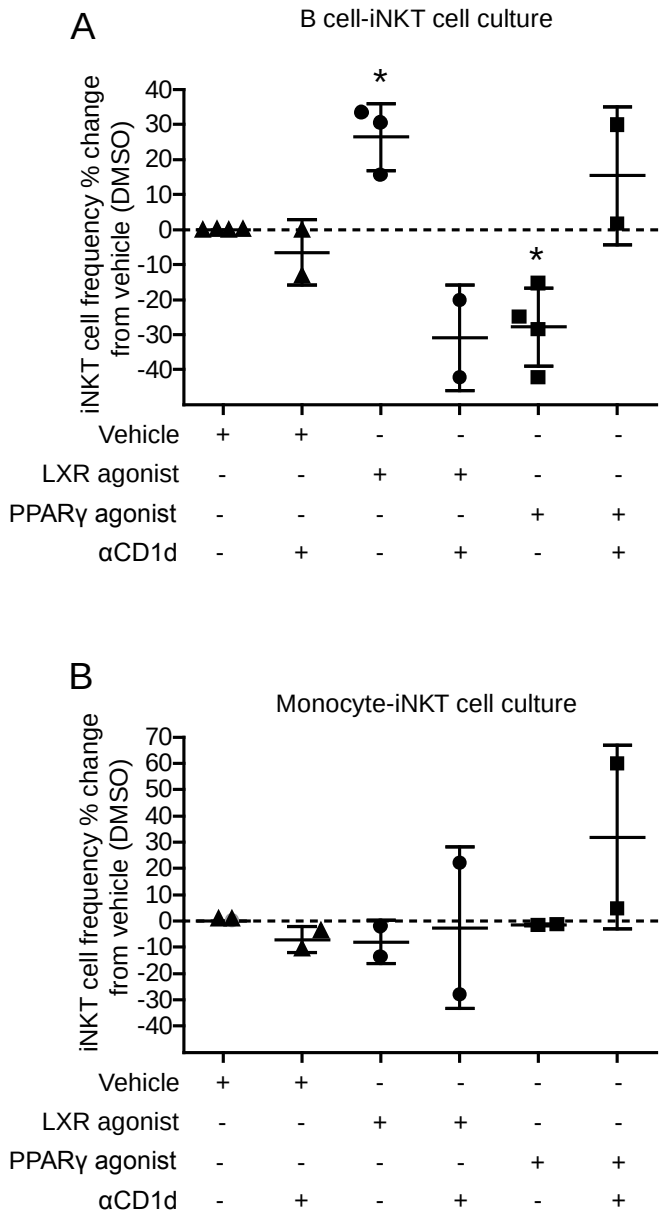

Figure 7.5 LXR and PPARγ stimulation of B cells but not monocytes influences CD1d-mediated presentation and subsequent activation of iNKT cells

PBMCs from healthy donors (n=2-4) were cultured in complete medium at a concentration of 10^7/ml in the presence of 1μM of LXR agonist GW3965 or 2.5μM PPARγ agonist Rosiglitazone for 24h before isolating B cells or monocytes by negative selection. These were co-cultured for 48h with purified iNKT cells from a cell line expanded over 7 days ±αCD1d antibody. Cells were stained for CD3 and iTCR before analysing by flow cytometry. Percentage change in iNKT cell frequency was calculated for (A) B cell and (B) monocyte co-cultures relative to unstimulated (DMSO) control. Data were analysed by student's t test whereby each treatment was compared to anti-CD1d. *$p<0.05$

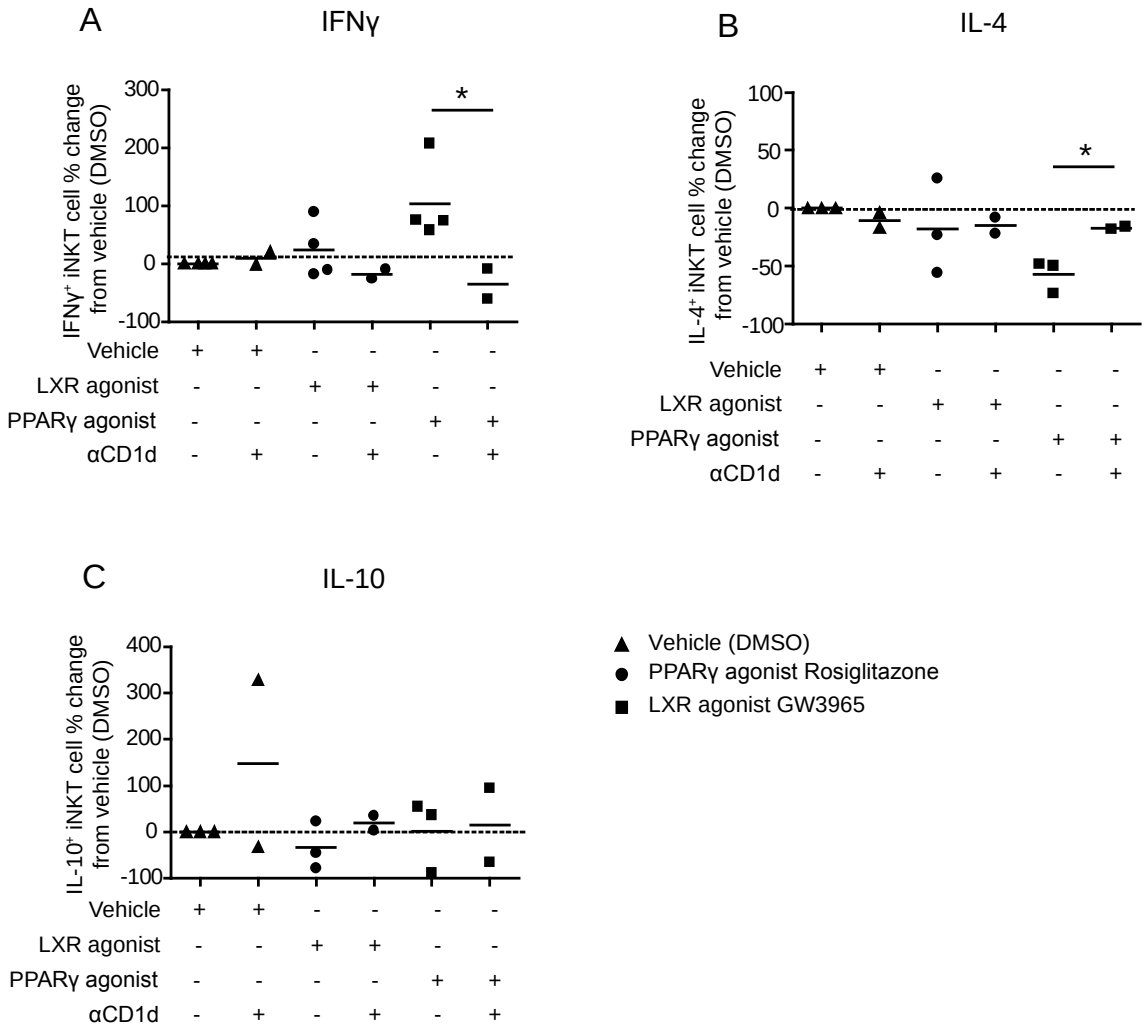

Figure 7.6 LXR stimulation of B cells induces a Th1 response upon subsequent co-culture with iNKT cells

PBMCs from healthy donors (n=2-4) were cultured in complete medium at a concentration of 10^7/ml in the presence of 1μM of LXR agonist GW3965 or 2.5μM PPARγ agonist Rosiglitazone for 24h before isolating B cells by negative selection. These were co-cultured for 48h with purified iNKT cells from a cell line expanded over 7 days ±anti-CD1d antibody. Cells were stained for CD3 and iTCR, then intracellularly for IFN-γ, IL-4 and IL-10 before analysing by flow cytometry. Percentage change in expression of (A) IFN-γ, (B) IL-4 and (C) IL-10 relative to unstimulated (DMSO) control. Data were analysed by student's t test whereby each treatment was compared to anti-CD1d. *p<0.05

CHAPTER VIII: Discussion

Chapter 8: Discussion

8.1 Conclusions

I report a number of novel findings in relation to the role of iNKT cells in the development of atherosclerosis in SLE patients.

- SLE-P patients had an increased iNKT cell frequency compared to SLE-NP patients and a unique iNKT cell phenotype characterised by differential expression of CD4, CD69, CD161 and PD-1, and increased IL-4 production compared to those without plaque and SLE patients who have suffered cardiovascular events.
- Serum metabolomics analysis identified dyslidaemia in SLE patients compared to healthy donors and significant alterations in VLDL expression and lipid content between SLE-P compared to SLE-NP patients.
- The unique SLE-P iNKT cell phenotype was not associated with differences in CD1d expression or the location of CD1d to lipid raft domains but was associated with more rapid interactions (conjugate formation) between monocytes and iNKT cells and increased signalling via the iTCR.
- Phospholipids isolated from monocytes but not B cells from SLE-P patients were able to induce the SLE-P iNKT cell phenotype in healthy donor cells during *in vitro* culture.
- iNKT cells from SLE-P patients promoted M2-like macrophage polarisation, associated with anti-inflammatory responses in plaque.

I hypothesise that altered serum VLDL contributes to the differential iNKT cell phenotype and cytokine responses observed between SLE-NP and SLE-P patients. The increased IL-4 production by iNKT cells from SLE-P patients consequentially results in polarisation of M2 “anti-inflammatory” macrophages, which may play an athero-protective role in patients with preclinical atherosclerosis. This protective response is probably overwhelmed in more advanced plaques where iNKT cells appear anergic. A diagram outlining the proposed mechanism of iNKT cell activation and responses during different stages of plaque development in SLE patients is shown in Figure 8.1.

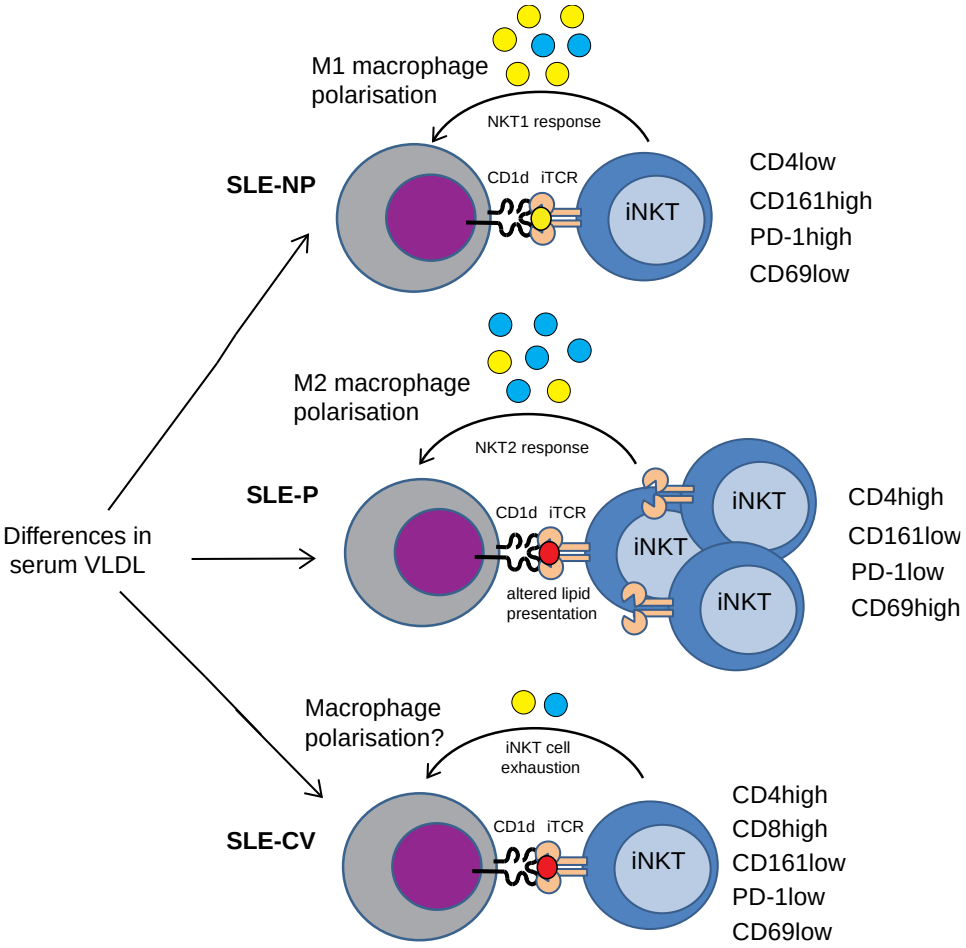

Figure 8.1 Differences in serum VLDL drive an altered iNKT cell phenotype and an anti-inflammatory macrophage response in SLE patients with preclinical plaque

In SLE-NP patients, serum lipids induce altered presentation of endogenous lipids to generate an iNKT cell phenotype characterised by low CD4 expression and CD69 expression, and high CD161 and PD-1 expression associated with high IFN-γ and low IL-4 production. This drives an inflammatory M1 macrophage phenotype. In SLE-P patients, altered presentation of phospholipids drives iNKT cell expansion with high CD4 and CD69 and low CD161 and PD-1 expression. This is associated with increased IL-4 production with drives an M2 macrophage phenotype. In SLE-CV patients, I hypothesise that increased iNKT cell accumulation within the plaque, or M1 macrophage polatisation could promote a proinflammatory iNKT cell response characterised by high CD4 and CD8 expression, with low CD161, PD-1 and CD69. The lack of iNKT cell IL-4 could represent a subsequent failure to induce M2 macrophage differentiation.

8.2 iNKT cell frequency and atherosclerosis in SLE patients

The importance of iNKT cells in the pathogenesis of both atherosclerosis and SLE is supported by my results describing a distinct iNKT cell phenotype in SLE patients with and without preclinical plaque. This included differences in iNKT cell frequency, CD4 and CD8 subsets, activation markers, cytokines and responsiveness to α-GalCer in SLE-NP and SLE-P patients compared to healthy donors (Table 8.1).

The increased iNKT cell frequency in SLE-P compared to SLE-NP patients suggested that iNKT cell expansion could occur as a protective response during early plaque development. Very little is known about iNKT cell function in human health and disease; in particular their ability to orchestrate both pro-inflammatory and immunoregulatory responses and how this balance is maintained. The finding of decreased iNKT cells in SLE-NP patients compared to healthy donors is in support of previous studies showing several iNKT cell defects in SLE, including reduced frequency [233, 436, 439]. Reduced iNKT cell frequency has been described in some animal models of atherosclerosis, where it is thought to be due to increased migration to inflamed tissues although these models typically resemble advanced atherosclerosis [219, 458]. In one of the few models of preclinical plaque, Major et al. observed that iNKT cell numbers were elevated in the spleen and liver of $ApoE^{-/-}$ mice (compared to plaque sites) during early lesion formation, a finding which was reversed during later stages of atherosclerosis [461].

While few human studies have investigated iNKT cell frequency and function in asymptomatic atherosclerosis patients, several studies have observed increased iNKT cell numbers in atherosclerotic lesions, supporting a role for iNKT cells in plaque development [457, 458, 506]. However, in a study by Kyriakakis et al. fewer iNKT cells were detected in the circulation of individuals with asymptomatic plaque compared to healthy donors, which although was true in this study was not found to be significant. Kyriakakis et al. also reported that patients with symptomatic atherosclerosis had lower iNKT cell frequencies compared to asymptomatic individuals, which is also in line with my data showing that SLE patients who had suffered a cardiovascular event had significantly reduced iNKT cell frequencies compared to patients with preclinical plaque [458]. Importantly there were some differences between my study and Kyriakakis et al. which make comparison difficult, in particular the study population were from a previously healthy population with no previous history of autoimmune disease, and had differences in age and gender compared to my SLE patient groups. Overall, whilst my data suggest that iNKT cells are decreased in SLE patients compared to healthy individuals as a

consequence of the disease, differences in iNKT cell frequency during the various stages of atherosclerosis may be indicative of atherosclerosis progression with higher iNKT cell frequencies associated with preclinical plaque. One likely explanation for this would be due to differences in the localisation of iNKT cells within the tissues, whereby one may expect decreased iNKT cells in the peripheral blood of SLE-CV patients due to increased localisation into the plaque, which may not be as apparent during the earlier stages [458].

Strikingly I found a significant positive correlation between iNKT cell number and plaque calcification (grey scale median) which suggested an association between iNKT cells and more stable plaque. This is supported by Rohm et al. who examined T cell markers in stable and unstable plaque specimens [507]. Fewer Tregs but increased T helper and CTLs were detected in the unstable compared to stable plaque specimens [507]. T cells within the plaque were also found to be more activated with increased expression of CD25 and CD69 [507].

I also investigated other plaque related factors including age, patient disease activity and medication, which could influence iNKT cell phenotype. iNKT cell frequency was negatively correlated with age in the healthy controls as described previously [489, 508], but this was lost in SLE patients. Ageing can also influence iNKT cell subset distribution and function with older individuals showing an increase in CD4 positivity [509] and reduced proliferative capacity in response to α-GalCer [508], with a shift from NKT1 to NKT2 type response [509]. Interestingly this could suggest that the iNKT cell phenotype of older individuals is similar to that of SLE patients, and raises the question of whether immunological ageing could be a consequence of SLE. This could be further addressed by assessing conventional T cells for characteristics associated with immunological ageing such as shortened telomere length, and defective responsiveness to antigen challenge i.e. increased Tregs and decreased memory T cell populations as observed in the skin [510]. It would be interesting to correlate such measures with iNKT cell function in SLE patients compared to older individuals.

As reported by Bosma et al. [233] no significant differences in iNKT cell frequency between disease active and inactive patients when categorised according to BILAG at the time of the scan were observed. Similarly, patient medication had little effect on iNKT cell frequency, also reflecting data from Bosma et al. where the relationship between iNKT cell frequency in disease active vs. inactive patients remained the same when medication was taken into account [233]. However, an effect of medication on iNKT cells cannot be completely ruled out. Indeed, Zhao et al observed that treatment with

cyclophosphamide or mycophenolate mofetil resulted in an increase in iNKT cell numbers [511]. Furthermore, in a study on patients with early RA, iNKT cell numbers from naïve-treated patients were found to correlate with the inflammatory markers CRP and ESR [512]. This correlation was lost in patients taking immunosuppressants indicating that DMARD and steroid treatment can influence iNKT cells.

		SLE-NP	SLE-P	SLE-CV
iNKT cell identification and subsets	iNKT cell frequency	↓	-	↓
	CD4	-	↑	-
	CD8	-	-	↑↑
iNKT cell surface markers	CCR6	↑	↑	ND
	CD25	↑	↑	ND
	CD69	-	↑↑	-
	CD161	-	↓↓	↓↓
	PD-1	↑	-	↓
iNKT cell intracellular cytokines and proliferation	Proliferation (Ki67 expression)	↓	↑	ND
	IL-4	↓	↑	↓
	IFNγ	↑	↑	↓

Table 8.1 Surface marker expression relative to healthy individuals

Summary to show differences in iNKT cell frequency, surface marker expression, cytokines and proliferation in response to 7 day stimulation with α-GalCer in SLE-NP, SLE-P and SLE-CV patients relative to healthy donors.

SLE-NP – SLE patients without plaque; SLE-P – SLE patients with plaque; SLE-CV – SLE patients who have suffered a cardiovascular event.

↑ denotes increased expression compared to healthy donors

↓ denotes decreased expression compared to healthy donors

- denotes no change in expression compared to healthy donors

ND denotes not determined

8.3 iNKT cell phenotype and atherosclerosis in SLE patients

I found that $CD4^+$ and $CD4^+CD8^+$ iNKT cell subsets were elevated in SLE-P patients and $CD8^+$ iNKT cells were increased in the SLE-CV patients compared to healthy controls mainly at the expense of the $CD4^-CD8^-$ iNKT cell population. $CD4^+$ iNKT cells have previously been shown to be more atherogenic compared to double negative iNKT cells in mice [465]. Adoptive transfer of $CD4^+$ NKT cells into T and B cell-deficient mice in a study by Li et al. confirmed that NKT cells directly contribute to atherogenesis [225]. They found that the mechanisms employed by $CD4^+$ NKT cells in atherosclerosis were perforin and granzyme-B dependent, and contributed to multiple characteristics of atherosclerosis including necrotic core formation and increased VCAM-1 expression [225]. In contrast, very little is known about $CD8^+$ iNKT cells, although it thought that these cells may play a similar role to $CD8^+$ T cells though their ability to mediate cytotoxic functions [513]. In addition, these cells produce IFN-γ but not IL-4 upon antigenic stimulation in mice [514], which supports my hypothesis of a pathogenic NKT1 response in SLE-CV patients where $CD8^+$ iNKT cells are present.

My hypothesis of differences in the distribution of iNKT cells in SLE patients is supported by the finding of increased $CD8^+$ iNKT cells in SLE-CV patients. Since one would not expect any differences in iNKT cell subset development between SLE-P and SLE-CV patients, it is plausable that $CD4^+$ iNKT cells preferentially localise to the plaque thus leading to an increase in $CD8^+$ compared to $CD4^+$ iNKT cells in the peripheral blood of SLE-CV compared to SLE-P patients, which would be in support of the proatherogenic role for $CD4^+$ iNKT cells which has previously been described [465]. This could be further investigated by analysis of plaque specimens for $CD4^+$ and $CD8^+$ iNKT cells during different stages of atherosclerosis in mouse models of SLE.

Furthermore, I also discovered changes in iNKT cell phenotype. CCR6 and CD25 expression were elevated on iNKT cells from all SLE patients compared to healthy controls, regardless of whether they had plaque. Increased CCR6 expression has been described previously on iNKT cells from SLE patients with active disease [232] and is implicated in the homing of lymphocytes to sites such as the intima, where atherosclerotic plaque forms [515]. This is achieved through binding to its receptor CCL20, which is also expressed on human atherosclerotic plaques [516].

CD25 (IL-2R) contributes to iNKT cell expansion and is thought to play a regulatory role. Similar to Tregs, CD25 is predominantly expressed on $CD4^+$ iNKT cells [347], meaning

its expression on iNKT cells from SLE patients could simply be a result of increased $CD4^+$ iNKT cell numbers. In addition IL-2 enhances iNKT cell expansion, which could suggest that iNKT cells from SLE patients have increased functional potential [358]. One study found that constitutive expression of CD25 by iNKT cells in early life meant that they were primed to respond to lower doses of antigenic stimulation [517].

Expression of CD69, an early activation marker, was raised on iNKT cells from SLE-P patients but not SLE-NP or SLE-CV patients compared to healthy donors. However, the role of CD69 in atherosclerosis remains unknown; whilst CD69 knockout mice are protected from atherosclerosis [518], enhanced expression of CD69 has been observed within unstable carotid plaque specimens in humans [507]. Similar conflicting observations have been seen in SLE where CD69 expression is associated with increased IFN-γ production by NK cells [519], whilst CD69 expression on $CD8^+$ T cells was found to induce apoptosis of other immune cell types [520].

Strikingly, CD161 expression was decreased on iNKT cells from SLE-P patients compared to SLE-NP patients and healthy donors. CD161 is present on both NK cells and Th17 cells, and functions as an inhibitory receptor for cytotoxicity by interacting with its ligand LLT1 [521]. Since there was no difference in IL-17 intracellular cytokine production between SLE-NP and SLE-P patients, it is possible that CD161 could be acting through a negative feedback mechanism to downregulate cytotoxicity in these patients.

Furthermore, CD161 expression has been shown to relate to many functional aspects of iNKT cells. Interestingly, high CD161 expression on iNKT cells was associated with anergy as well as failure to proliferate, secrete cytokines and induce cytolytic activity [522], whereas in conventional T cells, high CD161 expression was found to be associated with IFN-γ production [222]. Both these studies reflect my observation for SLE-NP patients where high CD161 expression on iNKT cells was associated with increased IFN-γ production and poor proliferative responsiveness to α-GalCer. In contrast, the absence of CD161 on iNKT cells was associated with increased IL-4 production in animal models [223], which supports my data from SLE-P patients where CD161 expression correlated with intracellular IL-4.

CD161 may also act by altering the activation threshold of iNKT cells, based on my finding that CD161 expression is positively correlated with activation markers CD25, CD69 and PD-1 in SLE-P patients. This theory has previously been proposed in relation to MAIT cells whereby loss of CD161 during HIV infection was found to be associated with increased levels of MAIT cell activation [224]. Changes to the activation threshold have also been identified in plaque-derived iNKT cells, albeit assessed by responsiveness to α-GalCer, and not CD161 expression [458]. Interestingly, low expression of Ly49 in mice (homologous to CD161 in humans) is associated with atherosclerosis in ApoE deficient mice [465]. This supports my preliminary data showing that CD161 expression is decreased to a greater extent in SLE patients who had suffered a cardiovascular event.

The finding of increased PD-1 in SLE-NP compared to healthy controls and SLE-P patients is supported by studies where PD-1 was shown to play an important role in the inhibition of T cell functions and induction of anergy in iNKT cells through recruitment of intracellular Src homology region 2 domain-containing phosphatase SHP-1 and SHP-2, which inhibits downstream signalling pathways [523, 524]. PD-1 expression may also be linked to cytokine polarisation, since the PD-1 pathway can substantially increase the amount of IFN-γ produced in response to *Mycobacterium tuberculosis* infection [523]. This is particularly relevant to iNKT cells from SLE-NP patients which I have shown predominantly produce IFN-γ. Importantly, blocking PD-1 was found to protect NKT cells from apoptosis in annexin V^+ IFN-γ^+ cells but not annexin V^+ IL-4^+ cells [523], suggesting that the reduction in PD-1 seen in SLE-P patients may be associated with increased survival and maintenance of the iNKT cell population.

Importantly, I have shown that the differences in iNKT cell phenotype observed are mostly iNKT cell-specific; although trends were observed in conventional T cells, these were to a much lesser extent and not significant. Whilst in support of my hypothesis that iNKT cells play a role in the development of atherosclerosis in SLE patients, it does raise the question on the contribution of iNKT cells to the overall immune response due to the fact that they are smaller in number compared to conventional T cells.

Despite my findings, the phenotypic characterisation of iNKT cells in SLE patients is somewhat confusing due to the large degree of heterogeneity. Whilst I observed positive correlations between CD161 and several activation markers in SLE-P patients, their relevance remains under scrutiny since SLE-P patients have lower CD161 expression and higher CD69 expression compared to SLE-NP patients. Functional characterisation of CD25+, CD69+ and CD161+ iNKT cells has not previously been carried out, and

remains a challenge in SLE due to the lower frequency of iNKT cells compared to healthy individuals. This would provide greater insight into how CD161 may affect iNKT cell function. In light of this I decided to focus on iNKT cell function rather than phenotype, and how this may influence atherosclerosis progression.

8.4 iNKT cell responsiveness and cytokine production in SLE patients with plaque

My data has clearly shown differences in iNKT cell responsiveness between healthy, SLE-NP and SLE-P patients, whereby iNKT cells from SLE-NP patients failed to expand in response to α-GalCer. This is partially supported by data from Bosma et al, where iNKT cells from SLE patients were less responsive to α-GalCer [233]. Little is known however about the effect of α-GalCer on iNKT cells from atherosclerosis patients. Although Kyriakakis et al. showed that iNKT cells isolated from the plaque itself are more sensitive to α-GalCer compared to those from peripheral blood, they did not investigate iNKT cell expansion for patients with preclinical plaque [458]. I have therefore shown for the first time that SLE patients with preclinical plaque display altered responsiveness to α-GalCer, which is supported by the finding of increased proliferation (as measured by Ki67 expression) in iNKT cells from SLE-P patients after 7 days. Furthermore, elevated ICOS but not CD40L expression was observed on iNKT cells from SLE-P patients following culture with α-GalCer. It is tempting to speculate that this finding indicates the increased iNKT cell activation in SLE-P patients is due to increased interactions with monocytes, rather than B cells which would also result in elevated CD40L expression as well [388].

Whilst IFN-γ production by iNKT cells was increased in all SLE patients compared to healthy controls, the observation of a significant increase in IL-4 and IL-10 production by iNKT cells in SLE-P patients compared to healthy donors suggests that iNKT cells may play a protective role in SLE patients with preclinical plaque, which is lost in SLE-CV patients. This role for IL-4 in early atherosclerotic lesions is supported by Llorente-Cortés et al. who observed significantly increased *IL-4* gene expression in PBMC from patients with subclinical femoral artery atherosclerosis [525]. Furthermore, decreased expression of transcription factor *T-bet* and increased *GATA-3* in iNKT cells from SLE-P patients compared to SLE-NP patients could indicate that iNKT cells from SLE-P patients were NKT2 cells, a functional iNKT cell subset expressing high CD4, low T-bet and constitutive IL-4 production, with anti-inflammatory properties [265]. Importantly

differences in CD4 and CD8 subsets, in particular the $CD4^+$ iNKT cell subset could explain the increase in IL-4 secretion in SLE-P patients since $CD4^+$ iNKT cells have been shown to be associated with Th2 cytokine secretion in healthy donors and cancer patients [265, 526], which supports my data on SLE-P patients.

My observation of increased IL-10 production by iNKT cells from SLE-P patients is supported by van Puijvelde and colleagues who described increased IL-10 production by iNKT cells during the initiation of atherosclerosis in mice, whilst Lehuen et al. have showed that IL-10 production by iNKT cells contributes to diabetes [468, 527]. A protective role for iNKT cell-derived IL-10 has also been shown in models of obesity, where IL-10 was able to induce a Treg response [251].

However, there is no clear consensus on whether iNKT cells play a protective role in the initial stages of atherosclerosis since most evidence is contradictory. For example, IL-4 knockout mice have been shown to have reduced atherosclerotic lesion size suggesting a pathogenic role for IL-4 in atherosclerosis [528], whilst CD1d knockout mouse models have concluded that iNKT cells contribute to the formation of the fatty streak in preclinical plaques [467]. One the contrary more recent studies have indeed demonstrated that iNKT cells can play an atheroprotective role *in vivo* during the initial stages of plaque development [468], which reflects their function under normal homeostatic conditions [243]. Due to the importance of iNKT cells in immune regulation, I propose that the IL-4 and IL-10 response observed could be an attempt at the resolution of atherosclerosis by the iNKT cells themselves. This is supported by the fact that my work is from an autoimmune perspective where the presence of iNKT cells is thought to be beneficial [409].

Furthermore, analysis of SLE-CV patients who had previously suffered a cardiovascular event confirmed that this NKT2 response is specific to preclinical plaque. Specifically, SLE-CV patients had reduced IL-4 and IFN-γ iNKT cell production similar to $ApoE^{-/-}$ mice with advanced plaques [459], supporting my hypothesis that in the later stages of atherosclerosis iNKT cells become anergic and lose the ability to produce cytokines. However, due to low numbers of SLE-CV patients available I was unable to control for the length of time since the cardiovascular event, which has been shown to influence plaque stability and the immune environment [529, 530].

Finally, it is unknown whether iNKT cells within the plaque have a different phenotype and function compared to those within the blood. This is certainly the case in adipose

tissue from mice, where iNKT cells express lower CD4 and NK1.1, as well as a reduction in IFN-γ and IL-10 [199, 229], and this was attributed to altered gene expression; in particular a reduction in *PLZF* and an increase in both *T-bet* and *GATA-3*. Moreover, parabiotic animal models confirmed that iNKT cells from adipose tissue are tissue resident [251], although it is unknown whether this could be the case for iNKT cells from atherosclerotic plaque.

8.5 SLE patients with plaque have an altered serum metabolic profile

Metabolomics analysis suggested a strong role for differences in serum lipids at driving altered iNKT cell responses, with differences in healthy donors compared to SLE patients, as well as between SLE-NP and SLE-P patients. Differences in small HDL were observed between healthy and SLE patients, but not between SLE-NP and SLE-P patients. The presence of small dense HDL in SLE patients is correlated with complement activation in terms of C3 and C4 titres, as well as the presence of subclinical atherosclerosis in one recent study [531]. Also, reduced circulating HDL levels have been associated with autoimmune diseases in animal models. For example, SR-BI deficient mice exhibit larger HDL particles with increased cholesterol content which are less effective at controlling immune responses [532]. As a result SR-BI null mice show increased B and T lymphocyte proliferation, as well as production of autoantibodies and deposition of immune complexes in the kidney similar to SLE [533]. This finding suggests that in addition to measuring the plasma levels of HDL to assess cardiovascular risk, the functional status of HDL should also be taken into account; a factor which is starting to receive more emphasis. Importantly, abnormal HDL is enriched in triglycerides and depleted in cholesteryl esters, which results in impaired anti-oxidative and anti-inflammatory capacity [534, 535], as well as reduced ability to induce cholesterol efflux [536].

Evidence suggests that HDL may also influence membrane lipids of cells, since HDL and apoA-I can promote cholesterol efflux from cells via ATP-binding cassette transporters ABCA1 and ABCG1 [537]. This is supported by the finding that mice with a combined ABCA1 sand ABCG1 deficiency have very low HDL levels, as well as severe inflammation and atherosclerosis [538]. A study by Rohatgi et al. showed that cholesterol efflux capacity was inversely related to the incidence of cardiovascular events in a cohort of 2924 individuals, and had a stronger influence on cardiovascular disease compared

to the HDL cholesterol level and particle concentration [539]. Such differences could therefore contribute to the altered lipid raft expression on T cells from SLE patients as previously reported [380], as well as differences in lipid rafts on B cells reported in this study. It is unknown whether altered HDL is a cause or consequence of altered immune responses in autoimmune diseases. However, it is clear that autoimmunity can be sustained through defects in circulating lipids, which contribute to impaired lipid metabolism and altered membrane lipid rafts, resulting in hyperactivation of T and B lymphocytes [540].

Many of the differences between SLE-NP and SLE-P patients were related to lipids, which I hypothesised had an anti-inflammatory role in SLE-P patients. For example, the observation of increased omega 6 fatty acids in SLE-P is supported by animal models where omega-6 fatty acids could prevent atherosclerotic plaque development [541]. Similarly, the increased 22:6 docosahexaenoic acid (DHA) seen in SLE-P patients supports its role as an anti-inflammatory marker due to the inverse correlation with markers of inflammation including IL-6 and CRP and association with lower cardiovascular disease incidence [542]. Interestingly, DHA supplementation has been shown to be atheroprotective in mice fed a high fat diet, through its ability to reduce cholesterol levels [543]. Likewise, the increase in monounsaturated fatty acids (MUFA) in SLE-P compared to SLE-NP patients is consistent with their protective role in cardiovascular disease [544], whilst a reduction in cholesterol esters in SLE-P patients is indicative of increased cholesterol catabolism and a reduction in foam cell formation, and thus may be protective against atherosclerosis [160].

The most striking findings between SLE-NP and SLE-P patients were the differences in VLDL (but not IDL, LDL or HDL) concentration as well as difference in its composition; total cholesterol was increased in all SLE patients compared to healthy donors, whilst free cholesterol and phospholipids were increased in SLE-P patients compared to SLE-NP patients. These findings are supported by Chung et al. who observed a difference in the size of particles, whereby VLDL from SLE patients was larger compared to healthy controls [545]. They found that the size of the particles may be a better indicator of atherosclerosis in SLE, due to the association with inflammatory markers such as CRP. However, they observed no relationship between size of LDL and the development of atherosclerosis, again supported by my findings. This was also the case in a study by Olusi et al. which showed that patients with SLE had higher plasma concentrations of VLDL, triglycerides, LDL and ApoB, with lower HDL concentrations [546].

Whilst it is clear that differences in lipoprotein subclasses can predict subclinical atherosclerosis in the general population [547], there is little information on lipoprotein subclasses in SLE patients. Chung et al. found no association between lipoprotein subclasses and coronary atherosclerosis [545], although this unexpected result could have been due to differences in disease activity or the drugs used to treat patients – an important conclusion, since little is known about the effect of corticosteroids on lipoprotein subclasses [545].

One possible mechanism through which VLDL may be increased in SLE patients with preclinical atherosclerosis is through the adipokine resistin, which stimulates VLDL, apoB and lipid secretion by hepatocytes and is associated with cardiovascular disease [548]. Resistin can also influence expression of MTP, which is required for the processing of lipids for CD1d-mediated presentation [548]. This is particularly relevant to SLE patients where increased resistin levels correlate with coronary artery calcification levels [423]. Other molecules linked with VLDL levels in cardiovascular disease include irisin-1 and omentin-1, which are associated with lipoprotein metabolism and could prove important diagnostic biomarkers [549]. There is evidence to suggest that the increased phospholipid content of VLDL observed in SLE-P patients could be protective against atherosclerosis [550]. During lipid transport phospholipids are transferred from VLDL and LDL to HDL mediated by Apo-AI. The finding of increased phospholipids in chylomicrons from SLE-P patients suggests that lipid transport may be defective in SLE-P patients [551].

Interestingly a case-control study by Hua et al. showed that SLE patients with a history of cardiovascular disease (cases) had a significantly greater concentration of VLDL particles compared to SLE patients without cardiovascular disease (controls) [115]. Cases also showed significantly increased small dense LDL, with increased small HDL cholesterol in controls. The finding that VLDL differentiates between cases and controls is supportive of my data, despite the fact that this study involved patients with early atherosclerosis. It would therefore be interesting to carry out metabolomics analysis of serum from the SLE-CV patients in this study.

In addition to lipids, metabolomics analysis identified several other factors in the serum that were associated with SLE and atherosclerosis. These included reduced glutamine levels in SLE patient serum compared to healthy individuals, which is supported in the literature, where is thought to play a role in cerebral disease activity [552]. The observation of increased acetate in SLE patients is indicative of alterations in the endogenous metabolism of glucose, fatty acids and amino acids in SLE patients

compared to healthy individuals [553]. However, the finding of lower concentrations of glycine in SLE-P compared to SLE-NP patient serum was not supportive of an anti-atherogenic response in SLE-P patients due to reports of anti-inflammatory, immunomodulatory and cytoprotective functions for glycine, which protect against oxidative stress and protein glycation in the vasculature [554, 555]. However, whether such differences in biomarkers could influence immune cell function in SLE patients would require further investigation.

In support of the differences in serum metabolites between SLE-NP and SLE-P patients and their relationship with iNKT cell frequency and IL-4 in SLE-P patients, I have shown that serum from SLE-NP and SLE-P patients can recapitulate the iNKT cell expansion and cytokine profile of SLE-NP and SLE-P iNKT cells in healthy PBMCs. This effect was found to be CD1d-mediated, since it was blocked by culturing cells in the presence of anti-CD1d antibody. Similarly, the finding that culture of PBMCs from healthy donors with serum from SLE-NP and SLE-P patients could reduce B cell CD1d expression *in vitro* with a trend towards increased lipid raft expression suggests that factors in the serum can influence CD1d-mediated lipid presentation. Preliminary data suggest that this may occur through alterations in lipid biosynthesis, although this would require further investigation.

No significant differences were seen in serum cytokines levels between SLE-NP and SLE-P patients and this may rule them out as having an influence on iNKT cell function in atherosclerosis. This is supported by findings in the general population where Chironi et al. showed little difference in serum cytokines when healthy individuals were stratified according to the reported frequency of cardiovascular risk factors [556]. Despite differences in iNKT cell-derived IL-4, my finding that it was undetectable in patient serum could suggest that IL-4 participates in a more localised immune response within the plaque itself as indicated by Kyriakakis et al [458]. Importantly, the observation of no difference in IL-12, a cytokine known to directly activate iNKT cells [286], suggests that the differences in iNKT cell activation between SLE-NP and SLE-P patients are due to altered lipids being presented, since I was also able to rule out interactions between CD1d and lipid rafts.

To better understand the differences in serum lipids between healthy donors, SLE-NP and SLE-P patients results would need validating with more healthy donors, as well as taking samples from different time points due to the influence of SLE disease activity, as well as factors like diet and exercise on lipoprotein size, composition and concentration

and other serum metabolites. It also remains to be seen whether lipids from SLE-P patients are more similar to healthy donors or SLE-NP patients, which could be achieved by further statistical analysis.

To determine whether differences in either the concentration or composition of serum VLDL contributes is responsible for the iNKT cell phenotype observed in SLE-P patients, I would carry out VLDL isolation from the serum of healthy, SLE-NP and SLE-P patients. I would then culture VLDL with PBMCs from healthy donors in an attempt to recapitulate the iNKT cell expansion and NKT2 response observed in SLE-P compared to SLE-NP patients.

8.6 Are CD1d and lipid rafts dysfunctional in SLE patients with atherosclerosis?

Analysis of antigen presenting cell phenotype confirms previous work showing that CD1d expression is reduced on B cells (but not monocytes) from SLE patients, with no difference according to whether they had plaque or not [232, 233]. Reasons for this remain unknown although there are several possibilities including decreased activation by PPARγ and vitamin D [383, 387] as well as increased TLR9 stimulation on B cells, which regulates CD1d expression via miR155 and Ets1 [557]. My results were also in line with data from RA patients where no differences in CD1d expression are observed in monocytes [512], which suggests that CD1d-mediated presentation by B cells (and not monocytes) is dysfunctional in autoimmunity.

The altered expression and relationship between CD1d and lipid rafts (CTB) in B cells could therefore be SLE specific rather than associated with the presence of atherosclerosis. This is supported by previous reports showing that membrane lipids are defective in B cells from SLE patients with implications on immune cell function [558]. The position of lipid rafts relative to CD1d was disrupted but only in B cells from SLE patients compared to healthy individuals, a finding which could have functional implications on the recognition of lipid antigen by iNKT cells. C20:2 lipid analogues were shown to induce a Th2 response in the absence of CD1d-lipid raft co-localisation, meaning that one may have expected greater co-localisation for plaque-negative patients [296, 559]. However, this experiment involved murine CD1d and an antigen which may not be physiologically relevant to humans. Therefore, I would need to repeat these experiments using specific lipids identified from patient serum or more relevant lipid antigens. This would enhance our understanding of CD1d and lipid raft co-localisation and its effect on iNKT cell function, as well as whether it could be manipulated by different lipid antigens. However, it remains to be seen whether the lipid being presented or the position of CD1d is more important in determining iNKT cell effector responses.

Reduced B cell CD1d expression in SLE patients has previously been associated with increased internalisation and recycling to the plasma membrane [233]. However I did not identify differences in lysosome or endosome numbers in SLE B cells (only endosome numbers were increased in monocytes from SLE-NP patients) and no differences in CD1d-endosome or lysosome co-localisation were detected. Yet I cannot rule out differences in recycling since other factors such as the pH of endosomal compartments

have been found to regulate the loading of Th1-biasing vs Th2-biasing forms of α-GalCer onto CD1d [377], whilst differences in the various chaperones which regulate CD1d recycling could play a role in the rate of CD1d recycling [279]. It is also possible that differences in other antigen presenting cells may be contributing to altered iNKT cell activation since I observed a trend towards increased lipid rafts in pDCs from SLE patients. This supports data from Menon et al. showing that pDCs are defective in SLE patients [560], but whether this is due to their effect on iNKT cells remains to be determined.

8.7 iNKT cell signalling

My results showing that B cells and monocytes interact differently with iNKT cells provides an insight into how lipid antigen presentation by different antigen presenting cells could differentially influence iNKT cell function. No previous reports have investigated the dynamics of iNKT cell-APC interactions and it will be interesting to investigate the underlying mechanisms further using endogenous lipids rather than α-GalCer as the stimulating lipid. Changes in conjugate formation were associated with altered iNKT cell intracellular signalling. Despite forming fewer and more transient conjugates with iNKT cells B cells induced stronger phosphorylation of iTCRζ and ERK1/2. This observation warrants further study as to the reasons behind this, and its consequence.

In vitro, blockade of ERK has been shown to significantly decrease numbers of iNKT cells in mice with a concurrent increase in CD69 expression and IFN-γ secretion, in a similar fashion to iNKT cells in the SLE-NP patients [561, 562]. However, there were some differences in vivo; namely a reduction in CD69 expression and IFN-γ secretion [561], thus highlighting that the differences observed in ERK signalling in this study may not be representative of the signalling *in vivo*. They also indicate a requirement of other pathways including NFκB and p38 MAPK signalling.

Therapeutically, several molecules have been developed which interfere with ERK signalling, thus altering iNKT cell activation and function. These include DPPE-PEG_{350}, which has been shown to attenuate allergen-induced airways hypersensitivity by binding to CD1d and inhibiting ERK phosphorylation [563]. This antagonist has also been shown to reduce atherosclerosis in mice in an iNKT cell-dependent manner and could play a role here [564].

Lipids could therefore play more of a role in the formation of conjugates between CD1d and iNKT cells as well as the subsequent signal strength, so it would be interesting to repeat this experiment with cellular lipids isolated from B cells or monocytes from healthy, SLE-NP and SLE-P patients instead of α-GalCer – in particular, fraction 5 (neutral phospholipids, PC, PE and sphingomyelin), which I found could induce iNKT cell expansion in SLE-P patients. This was blocked by anti-CD1d antibody suggesting lipid antigen presentation to iNKT cells was responsible for this effect. Whilst fraction 5 from SLE-P monocytes appeared to induce IL-4 and IL-13 in the culture supernatants in cultures with healthy PBMCs and was blocked with anti-CD1d, intracellular cytokine staining would be useful in confirming cytokines to be iNKT cell-derived.

8.8 Monocytes and iNKT cell activation

Importantly, my results have showed a role for differences in monocytic lipids between healthy donors, SLE-NP and SLE-P patients contributing to altered iNKT cell activation, a result which warrants further study. In this study, phospholipids from SLE-P monocytes induced significant iNKT cell expansion compared to phospholipids from SLE-NP patients. This was associated with increased IL-4 and IL-13, and decreased IFN-γ concentrations suggesting that differences in phospholipids in SLE-P patients contributed to the iNKT cell expansion and NKT2 response observed. Phospholipids have been identified previously to activate iNKT cells [314]. For example LPC is upregulated during inflammation and serves as danger signals to activate iNKT cells [243, 565]. In addition to indicating differences in lipid metabolism between healthy donors, SLE-NP and SLE-P patients, these results also suggest that monocytes may play more of a role compared to B cells in the development of atherosclerosis. However, it remains to be determined whether monocytes were indeed responsible for the presentation of endogenous lipids in these experiments.

This potential role for monocytes in atherosclerosis is supported by the finding of increased expression of scavenger receptors LOX-1, LDLR and CD36 on monocytes compared to B cells. Comparison of patient groups revealed a trend towards decreased CD36 expression on monocytes from SLE-P patients, which is supported by Syväranta et al, who found significantly reduced expression by qPCR in aortic valve stenosis (AVS) patients [566]. The reduction in CD36 expression could be seen as either downregulation due to activation or alternatively as an immunoregulatory mechanism in

an attempt to resolve inflammation within the plaque. As a result, further work is needed to look at responses associated with CD36 activation in SLE patients, and its known influence on CD86 and HLA-DR expression, which may contribute to atherosclerosis development [567]. The increase in LOX-1 expression seen in AVS was observed on monocytes from SLE patients who had suffered a cardiovascular event but not in SLE-P patients suggesting that increased LOX-1 expression is characteristic of later stages in atherosclerosis [566].

I observed increased numbers of intermediate $CD14^{++}CD16^{+}$ monocytes in the SLE-NP patients and SLE patients who had cardiovascular disease compared to healthy controls and SLE-P patients. The importance of $CD14^{++}CD16^{+}$ monocytes during later stages of atherosclerosis is highlighted by a study on haemodialysis patients where individuals with advanced carotid plaques had higher counts of classical and intermediate monocytes compared to individuals without advanced plaques [568]. Moreover, in obese patients with subclinical atherosclerosis $CD16^{+}$ monocyte frequency is similar to the general population, but is decreased following bariatric surgery where intermediate monocyte frequency was associated with decreased intima media thickness [168]. Importantly, a study by Mikolajczyk et al. showed that increased $CD14^{++}CD16^{+}$ monocyte frequency was associated with IMT in SLE patients but not healthy individuals [569].

8.9 M1 and M2 macrophage polarisation

In recent years, the balance between pro-inflammatory M1 and anti-atherogenic M2 macrophages has been implicated in atherosclerosis progression, with M2 macrophages postulated to play a role during its early stages [190]. My results showing increased $CD14^{+}CD206^{+}CX3CR1^{+}$ (M2-like) monocytes in SLE patients with preclinical atherosclerosis is in direct contrast to the study by Fadini et al. where patients with asymptomatic lesions had increased numbers of $CD14^{+}CD68^{+}CCR2^{+}$ (M1-like) monocytes and decreased numbers of $CD14^{+}CD206^{+}CX3CR1^{+}$ (M2-like) monocytes [497]. However, Fadini et al. studied patients with Familial and Non-familial hyper-cholesterolaemia, which are characterised by a very severe form of dyslipidaemia with significantly elevated LDL and total cholesterol but normal HDL and triglyceride levels; in this study SLE patients were all within the standard normal ranges used in clinical practice and only triglycerides were found to be significantly different between SLE-NP and SLE-P patients. Furthermore, the cohort in the study by Fadini et al. had no previous history of autoimmune disease, and therefore didn't have the same underlying immune

cell dysfunction observed in SLE patients. Importantly, the M1:M2 ratio observed for the healthy donors in my study was similar to that of Fadini et al. despite some differences in the size of M1 and M2 monocyte-macrophage populations, which could be explained by the finding that the donors in my study were much younger. However, it is important to note that no other groups have adopted this monocyte-macrophage gating strategy yet and despite being a recent publication, no functional data available on the roles of the M1 and M2 subsets, apart from their altered distribution in pre-diabetic and hypercholesteroleamic patients [497, 570]. The relevance of these markers to monocyte function therefore warrants further investigation.

The finding of increased numbers of $CD14^{+}CD206^{+}CX3CR1^{+}$ "M2-like" monocytes and a decreased M1: M2 monocyte ratio in SLE patients with preclinical plaque is in line with previous data supporting a role for M2 macrophages in early plaque development [571], and could therefore point to a protective anti-atherogenic immune response.

Using the mannose receptor (CD206) as an M2 marker, M2 cells have been identified in areas surrounding the plaque while M1 cells were located in the lipid core [572, 573]. Whilst recent studies have shown that symptomatic carotid lesions exhibit increased lipid and inflammatory cell infiltration, with a greater proportion of M1-like pro-inflammatory macrophages over M2-like anti-inflammatory macrophages [574], both macrophage subsets have also been reported to be present in early lesions (fatty streaks) as well as more advanced lesions [500]. Furthermore, Lee et al. studied plaque in patients with acute myocardial infarction compared to stable angina and revealed differences in macrophage phenotype [575], supporting disease specific variations in macrophage/monocyte polarisation meaning that macrophage polarisation may vary according to the type of plaque or cardiovascular event.

In contrast, Oksala et al. found M2 macrophages to be associated with more unstable plaque [576], however this shown to be related to the expression of the cell adhesion molecule kindlin-3 (FERMT3). Another explanation would be the possibility that alternative M2 macrophage subsets (M2a, M2b, M2c and M2d) may be responsible for differential effects within the plaque since M2a are described as being involved in wound healing, M2b in immunoregulation, M2c in efferocytosis and M2d in tumour growth [577]. It is difficult to conclude precisely which macrophage/monocyte subsets predominate at different stages of plaque development; alterations in the plaque microenvironment are

likely to play an important role in influencing macrophage (and most likely monocyte) polarisation and therefore influence plaque progression and regression.

Despite the fairly extensive work on monocyte/macrophage subsets *in vitro* and in animal models of atherosclerosis, evidence of their patho-physiological relevance in a clinical setting remains scarce and in some cases conflicting [171, 188]. Whilst my work has looked at the *ex vivo* phenotype on monocytes, the results presented here are in line with previous studies on human plaques that demonstrate the presence of both M1 and M2 macrophages during developing atherosclerosis with a role for M2 macrophages during early stages [500]. More extensive work such as plaque biopsies would therefore be needed to investigate the roles of M1 and M2 macrophages in SLE patients with atherosclerosis.

Importantly, SLE-P serum was able to induce M2-like macrophage polarisation but only in the presence of iNKT cells. This finding corroborates previous observations in experimental autoimmune encephalomyelitis, where iNKT cell deficient mice have fewer M2 macrophages and worse disease [433]. Similarly, treatment with α-GalCer in a mouse model of obesity significantly increased both iNKT cell numbers and M2 macrophage polarisation, and reduced M1 macrophage-associated iNOS expression [251]. I observed increased expression of the CD206 and CD200R genes in THP-1 macrophages upon co-culture with iNKT cells preconditioned using SLE-P serum. Such genes are known to be upregulated in response to IL-4 [501, 503], suggesting that iNKT cells promote M2 macrophage differentiation through IL-4 production.

Whilst I have uncovered a potential role for M2 macrophages in preclinical atherosclerosis within the context of autoimmunity, I hypothesise that during the later stages of atherosclerosis this protective response is probably overwhelmed as can be seen through the predominance of M1 macrophages in more advanced rupture prone lesions [188, 189, 575], shifting the balance from tissue repair in favour of chronic inflammation. This is likely associated with the increase in iNKT cell frequency and a shift from IL-4 to IFN-γ production observed in animal models of atherosclerosis [459-461] and could be driven by alterations in other cell types, such as vascular smooth muscle cells, which have been shown to induce iNKT cell proliferation and the characteristic Th1 response observed in atherosclerotic lesions [578].

8.10 iNKT cells and interactions with Tregs

In addition to IL-4, M2 macrophage polarisation can also occur through IL-10; the role of which remains to be determined in our patients. Previous work by Lynch et al. demonstrated a role for iNKT cells in M2 macrophage polarisation, mediated by both IL-4 and IL-10 in murine adipose tissue [251]. They also observed that iNKT cells could transactivate Tregs to induce M2 macrophage via IL-10, suggesting a role for Tregs in iNKT cell-mediated macrophage polarisation [251]. Indeed, increased levels of IL-10 have previously been observed in SLE patient serum [579], which could be due to increased M2 macrophage polarisation as a result of immune complex formation and TLR stimulation [580].

Whilst IL-10 can also contribute to M2 macrophage polarisation [581], the little difference in IL-10 secretion observed between SLE-NP and SLE-P patients suggests that IL-10 is not required for the M2 macrophage polarisation observed in the SLE-P patients. This is supported by Brochériou et al. where the addition of IL-10 to M-CSF cultures induced comparable M2 macrophage gene expression compared to cultures with M-CSF alone [500]. Brochériou et al. also show that M2 macrophage polarisation by GM-CSF is associated with decreased CD36 expression in monocytes [500]; this supports the previously described trend towards decreased CD36 expression in monocytes from SLE-P patients, which although not significant may have been associated with increased M2 macrophage polarisation.

Previous work has indicated that a lack of iNKT cells in adipose tissue induces a proinflammatory environment, with increased polarisation towards M1-like macrophages [199, 229], and as such suggests that the presence of iNKT cells enhances M2 macrophage polarisation. This is supported by the finding that treatment with α-GalCer can increase the frequency of $CD206^+$ macrophages, whilst decreasing $CD11c^+$ M1 macrophages, in models of both obesity, as well as EAE [433]. This increase in M2 macrophages was found to be IL-4-dependent in EAE, but IL-10-dependent in obesity; the iNKT cells being the source of each cytokine in both case [251].

Since α-GalCer treatment has also been associated with an increase in Treg frequency in the adipose tissue but not the spleen of mice, it appears that this iNKT cell regulatory environment is tissue–specific, whilst indicating that iNKT cells can control Tregs and promote immunoregulation [251]. Adipose tissue Treg show a higher expression of IL-10 in the resting state [230], and increased expression of lectin-like inhibitory receptor KLRG1, indicating a strong immunoregulatory function [251]. The role of Tregs in this

model would therefore warrant further investigation as it may be that iNKT cell-Treg interactions are confined to the plaque site in SLE patients with preclinical atherosclerosis.

8.11 Lipid biosynthesis and iNKT cell responses in SLE patients with atherosclerosis

The finding of differences in the ability of endogenous monocytic lipids from SLE-NP compared to SLE-P patients to activate iNKT cells suggested potential differences in lipid biosynthesis in APCs from healthy donors, SLE-NP and SLE-P patients. Furthermore, differences in cholesterol expression on B cells and monocytes from healthy and SLE-NP patients could indicate potential differences in lipid metabolism and lipid raft expression, similar to differences previously described in T cells from SLE patients [380]. To confirm this I would need to be increase sample numbers for membrane order and ABCA1 expression, and repeat HPLC analysis of B cell and monocyte GSL species. An alternative approach would be to carry out qPCR on genes involved in lipid metabolism in APCs from healthy donors, SLE-NP and SLE-P patients.

Whilst it is hard to come to any major conclusions on whether PPARγ and LXR agonist can influence CD1d and lipid raft expression due to lack of sample numbers and donor variability, trends in the data suggest that PPARγ and LXR differentially regulate lipid raft expression in monocytes and B cells. Previously published literature shows that PPARγ regulates CD1d gene expression in dendritic cells, but more work is needed to confirm whether this is true for B cells and monocytes in humans [383]. Furthermore, I observed that such differences in lipid metabolism in APCs could have a knock-on effect on iNKT cell function. This was most striking for iNKT cell frequency where PPARγ stimulation of B cells resulted in iNKT cell expansion, in contrast to LXR stimulation which resulted in a decrease in iNKT cell frequency. This is in support of data from patients with obesity where defects in PPARγ function were associated with altered CD1d expression and a decrease in iNKT cell frequency [384]. Unexpectedly, LXR-stimulated B cells were found to promote a Th1 response in iNKT cells, characterised by increased intracellular IFN-γ and decreased IL-4. Despite not supporting the well-described anti-inflammatory role for LXR [374, 380], this finding suggests that differences in lipid rafts (as a consequence of lipid biosynthesis) can influence iNKT cell cytokine production in support of data by Im et al [377].

Differences in lipid metabolism in APCs from SLE-P patients could also contribute to the M2 polarisation observed in SLE patients with preclinical plaque. PPARγ is induced by oxLDL and is present in M1-like macrophage foam cells in atherosclerosis, where it exerts pro-atherosclerotic functions such as driving phagocytosis of oxLDL via CD36 [582]. This is exemplified by the PPARγ agonist rosiglitazone, which was withdrawn from the market following trials where it increased the risk of MI in diabetes patients [583, 584]. However, statin-induced PPARγ activation has been shown to induce M2 macrophage polarisation, whilst having marked improvements on insulin sensitivity [585, 586].

LXR stimulation appears to protect against atherosclerosis by inducing cholesterol efflux from cells [587] and by inducing upregulation of the *MERTK* gene which encodes the Proto-oncogene tyrosine-protein kinase MER [588]. MerTK in turn prevents the uptake of lipoproteins and exerts anti-apoptotic effects on macrophages [589]. Importantly, the presence of IL-4 may contribute to atherosclerosis development in certain scenarios, since IL-4 can augment PPARγ expression in the presence of oxLDL, whilst downregulating LXR expression [573, 590]; the resultant defect in cholesterol efflux contributes to foam cell formation. Additionally, IL-4 decreases both *MERTK* and *CD163* expression resulting in poor clearance of apoptotic macrophages [588, 591], thus promoting both SLE- and atherosclerosis-associated inflammation. Overall, these data suggest that altered lipid metabolism in B cells can influence CD1d-mediated iNKT cell activation in healthy donors; although it is unknown whether this plays a role in SLE patients.

8.12 Limitations

There are several limitations to this current study, which confines its clinical potential to SLE patients who have not previously suffered a cardiovascular event. Most important is the absence of ultrasound scan data for healthy individuals in order to act as a direct comparison to SLE patients who have not previously suffered a cardiovascular event (SLE-NP and SLE-P groups).

Whilst comparison of SLE-CV with SLE-P patients has proved useful in understanding monocyte and iNKT cell phenotypes in SLE patients within a pre-clinical cardiovascular setting compared to those who had suffered a cardiovascular event, it is extremely difficult to control for factors such as length of time since event as well as type of event

(stroke or MI) due to low numbers of patients. Once again ultrasound scan data from these patients, as well as atherosclerosis patients with no previous history of autoimmune disease, would be useful for comparison to SLE-NP and SLE-P patients. Longitudinal analysis of these patients by performing follow up scans and through reassessment of their cardiovascular disease history would prove extremely useful in being able to monitor how iNKT cells may protect against or promote atherosclerosis at different stages of the disease in SLE patients. It would also be necessary to repeat these experiments with PBMCs from other patient groups in order to confirm whether a similar iNKT cell protective response is present in healthy donors with preclinical atherosclerosis.

8.13 Therapeutic implications

From this study there is much evidence to suggest that lipoprotein size, composition and concentration is a much better indicator of cardiovascular disease risk in SLE patients compared to the standard clinical measures used to diagnose dyslipidemia. SLE patient's serum lipids should therefore be routinely monitored, and a lower threshold should be used when diagnosing dyslipidaemia in these patients. This is particularly evident since SLE patients with preclinical atherosclerosis displayed significantly higher concentrations of triglycerides and greater cholesterol: HDL ratio compared to SLE-NP patients, despite being within normal range for healthy adults. This could lead to earlier intervention with statins, and consequentially improved prognosis for SLE patients.

There is much controversy however surrounding serum lipid measurements as a predictor of atherosclerosis, termed "the lipid paradox", where patients with lowest LDL levels have an increased cardiovascular risk compared to those with moderate LDL levels [592]. The cardiovascular risk profile of RA patients as determined by LDL levels is not significantly different to the general population [592], a finding which points to the lipid composition as perhaps playing more of a role than otherwise thought.

Additionally, I would advocate a better system to identify patients at high risk of cardiovascular disease by taking into account SLE-specific factors. The use of an adjusted cardiovascular risk validation score has recently been investigated in RA, where patients are similarly at increased risk of cardiovascular disease [593]. Variables

identified for inclusion in an RA-specific cardiovascular risk model were RA disease activity and disease duration, which enabled the correct prediction of cardiovascular risk in 17% more RA patients [593].

Interactions between antigen presenting cells and iNKT cells could also represent important therapeutic targets for atherosclerosis in SLE patients. The use of α-GalCer as a vaccine adjuvant has recently been explored in cancer treatment [594], although whether expansion of iNKT cells in SLE patients could prove beneficial remains to be determined. This would depend on the stage of atherosclerosis as the challenge would be to harness the protective iNKT cell function in SLE patients with preclinical plaque. Alternatively, more specific targeting of serum lipids in SLE patients, such as VLDL could prove beneficial in re-directing the iNKT cell response towards a NKT2-type response would also be useful; however this would require a greater understanding of the different lipoproteins present in patient serum as well as their effect on not only iNKT cell activation, but other immune cell types in the plaque.

Finally, a recent publication from the group has suggested targeting of lipid biosynthesis in order to improve SLE disease activity since treatment with NB-DNJ was found to restore lipid raft function in SLE patients [380]. However, its effect on CD1d and lipid raft expression in antigen presenting cells as well as whether it could induce presentation of different endogenous lipid antigens to iNKT cells remains to be investigated.

8.14 Summary

In summary, I show evidence of a potentially atheroprotective iNKT cell response in SLE patients with preclinical atherosclerosis characterised by IL-4 production, which promoted macrophage polarisation towards an anti-inflammatory M2 phenotype. I also provide evidence that this protective iNKT cell response is specific to preclinical atherosclerosis in SLE patients due to impaired cytokine production and surface marker expression in SLE patients who have suffered a cardiovascular event. Whilst I have shown that there are differences in serum lipids (in particular VLDL size and composition) between SLE-NP and SLE-P patients the causal relationship between altered serum metabolites and iNKT cell function remains to be determined. Other factors which may be driving altered iNKT cell function in SLE patients with preclinical plaque include altered cellular lipid metabolism in monocytes, and differences in CD1d-lipid raft co-localisation in B cells. Subject to further research, I would advocate targeting either

serum lipids or lipid biosynthesis in antigen presenting cells in order to enhance the atheroprotective NKT2-type response in SLE patients.

Accepted abstracts, talks and poster presentations

Posters

- 7th International symposium on CD1 and NKT cells – Tours, France (2013)
- British Society for Immunology – Liverpool, UK (2013)
- Division of Medicine Research Retreat – London, UK (2014)
- UCL Cardiovascular Science Symposium – London, UK (2016), for which I won a prize for the best use of the “3rs”.

Talks

- Institute of Child Health Symposium – London, UK (2014)
- Royal Free Division of Medicine Postgraduate Research Day – London, UK (2014)
- British Society for Immunology – Brighton, UK (2014)
- UCL Rheumatology clinicians meeting – London, UK (2015)

References

1. Johnson, A.E., et al., *The prevalence and incidence of systemic lupus erythematosus in Birmingham, England.* Arthritis & Rheumatism, 1995. **38**(4): p. 551-558.
2. Murphy, G. and D. Isenberg, *Effect of gender on clinical presentation in systemic lupus erythematosus.* Rheumatology, 2013. **52**(12): p. 2108-2115.
3. Lu, L.-J., et al., *Review: Male systemic lupus erythematosus: a review of sex disparities in this disease.* Lupus, 2010. **19**(2): p. 119-129.
4. UROWITZ, M.B., et al., *Changing Patterns in Mortality and Disease Outcomes for Patients with Systemic Lupus Erythematosus.* The Journal of Rheumatology, 2008. **35**(11): p. 2152-2158.
5. Rus V, M.E., Hochberg MC, Wallace DJ, Hahn BH, *The epidemiology of systemic lupus erythematosus.* , in *Dubois' Lupus Erythematosus*, H.B. Wallace DJ, Editor. 2002: Lippincott Williams and Wilkins.
6. Nikpour, M., et al., *Frequency and determinants of flare and persistently active disease in systemic lupus erythematosus.* Arthritis Care & Research, 2009. **61**(9): p. 1152-1158.
7. Bernatsky, S., et al., *Mortality in systemic lupus erythematosus.* Arthritis & Rheumatism, 2006. **54**(8): p. 2550-2557.
8. Moss, K.E., et al., *Outcome of a cohort of 300 patients with systemic lupus erythematosus attending a dedicated clinic for over two decades.* Annals of the Rheumatic Diseases, 2002. **61**(5): p. 409-413.
9. Skaggs, B.J., B.H. Hahn, and M. McMahon, *Accelerated atherosclerosis in patients with SLE[mdash]mechanisms and management.* Nat Rev Rheumatol, 2012. **8**(4): p. 214-223.
10. Tan EM, C.A., Fries JF, Masi AT, McShane DJ, Rothfield NF, Schaller JG, Talal N, Winchester RJ, *The 1982 revised criteria for the classification of systemic lupus erythematosus.* Arthritis Rheum, 1982. **25**(11): p. 1271-7.
11. Harrison, M.J.M., MS *; Ravdin, Lisa D. PhD, *Cognitive dysfunction in neuropsychiatric systemic lupus erythematosus.* Current Opinion in Rheumatology, 2002. **14**(5): p. 510-14.
12. Leonhardt, E.T.G., *Family studies in lupus erythematosus.* Clinical and Experimental Immunology, 1967. **2**(Suppl): p. 743-759.
13. Block, S.R., *A brief history of twins.* Lupus, 2006. **15**(2): p. 61-64.
14. Mohan, C. and C. Putterman, *Genetics and pathogenesis of systemic lupus erythematosus and lupus nephritis.* Nat Rev Nephrol, 2015. **11**(6): p. 329-341.
15. Namjou, B., et al., *High-density genotyping of STAT4 reveals multiple haplotypic associations with systemic lupus erythematosus in different racial groups.* Arthritis & Rheumatism, 2009. **60**(4): p. 1085-1095.
16. Arechiga, A.F., et al., *Cutting Edge: The PTPN22 Allelic Variant Associated with Autoimmunity Impairs B Cell Signaling.* The Journal of Immunology, 2009. **182**(6): p. 3343-3347.
17. Lu, R., et al., *Genetic associations of LYN with systemic lupus erythematosus.* Genes Immun, 2009. **10**(5): p. 397-403.
18. Liu, P., et al., *<italic>IL-10</italic> Gene Polymorphisms and Susceptibility to Systemic Lupus Erythematosus: A Meta-Analysis.* PLoS ONE, 2013. **8**(7): p. e69547.
19. LÖFGREN, S.E., et al., *Promoter Insertion/Deletion in the IRF5 Gene Is Highly Associated with Susceptibility to Systemic Lupus Erythematosus in Distinct Populations, But Exerts a Modest Effect on Gene Expression in Peripheral Blood Mononuclear Cells.* The Journal of Rheumatology, 2010. **37**(3): p. 574-578.

20. Salloum, R., et al., *Genetic variation at the IRF7/PHRF1 locus is associated with autoantibody profile and serum interferon-α activity in lupus patients.* Arthritis & Rheumatism, 2010. **62**(2): p. 553-561.
21. Baccala, R., et al., *Essential requirement for IRF8 and SLC15A4 implicates plasmacytoid dendritic cells in the pathogenesis of lupus.* Proceedings of the National Academy of Sciences, 2013. **110**(8): p. 2940-2945.
22. Shen, N., et al., *Sex-specific association of X-linked Toll-like receptor 7 (TLR7) with male systemic lupus erythematosus.* Proceedings of the National Academy of Sciences, 2010. **107**(36): p. 15838-15843.
23. Zhang, J., et al., *Association study of TLR-9 polymorphisms and systemic lupus erythematosus in Northern Chinese Han population.* Gene, 2014. **533**(1): p. 385-388.
24. Zhou, X.-j., et al., *Genetic association of PRDM1-ATG5 intergenic region and autophagy with systemic lupus erythematosus in a Chinese population.* Annals of the Rheumatic Diseases, 2011. **70**(7): p. 1330-1337.
25. Shin, H.D., et al., *Common DNase I polymorphism associated with autoantibody production among systemic lupus erythematosus patients.* Human Molecular Genetics, 2004. **13**(20): p. 2343-2350.
26. Roubinian, J.R., et al., *Effect of castration and sex hormone treatment on survival, anti-nucleic acid antibodies, and glomerulonephritis in NZB/NZW F1 mice.* The Journal of Experimental Medicine, 1978. **147**(6): p. 1568-1583.
27. Heard, E., *Recent advances in X-chromosome inactivation.* Current Opinion in Cell Biology, 2004. **16**(3): p. 247-255.
28. Sawalha, A.H., J.B. Harley, and R.H. Scofield, *Autoimmunity and Klinefelter's syndrome: When men have two X chromosomes.* Journal of Autoimmunity, 2009. **33**(1): p. 31-34.
29. Manson JJ, I.D., *The pathogenesis of systemic lupus erythematosus.* Neth J Med, 2003. **61**(11): p. 343-6.
30. Miller, F.W., et al., *Epidemiology of environmental exposures and human autoimmune diseases: Findings from a National Institute of Environmental Health Sciences Expert Panel Workshop.* Journal of Autoimmunity, 2012. **39**(4): p. 259-271.
31. Ekblom-Kullberg, S., et al., *Smoking and the risk of systemic lupus erythematosus.* Clinical Rheumatology, 2013. **32**(8): p. 1219-1222.
32. Parks, C.G., et al., *Association of Epstein-Barr virus with systemic lupus erythematosus: Effect modification by race, age, and cytotoxic T lymphocyte-associated antigen 4 genotype.* Arthritis and Rheumatism, 2005. **52**(4): p. 1148-1159.
33. Yung, R.L., et al., *Mechanism of drug-induced lupus. I. Cloned Th2 cells modified with DNA methylation inhibitors in vitro cause autoimmunity in vivo.* The Journal of Immunology, 1995. **154**(6): p. 3025-35.
34. Cornacchia, E., et al., *Hydralazine and procainamide inhibit T cell DNA methylation and induce autoreactivity.* The Journal of Immunology, 1988. **140**(7): p. 2197-200.
35. Kamen, D.L., *Environmental Influences on Systemic Lupus Erythematosus Expression.* Rheumatic Disease Clinics of North America, 2014. **40**(3): p. 401-412.
36. Hochberg, M.C., *Updating the American College of Rheumatology revised criteria for the classification of systemic lupus erythematosus.* Arthritis Rheum, 1997. **40**(9): p. 1725.
37. Feld, J. and D. Isenberg, *Why and how should we measure disease activity and damage in lupus?* La Presse Médicale, 2014. **43**(6, Part 2): p. e151-e156.
38. Isenberg, D.A., 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, 2005. **44**(7): p. 902-906.
39. Yee, C.-S., et al., *Numerical scoring for the BILAG-2004 index.* Rheumatology, 2010. **49**(9): p. 1665-1669.

40. MORROW, W.J.W., et al., *Useful Laboratory Measurements in the Management of Systemic Lupus Erythematosus.* QJM, 1982. **51**(2): p. 125-138.
41. Yildirim-Toruner, C. and B. Diamond, *Current and novel therapeutics in the treatment of systemic lupus erythematosus.* Journal of Allergy and Clinical Immunology, 2011. **127**(2): p. 303-312.
42. The, A.T.G., *Treatment of Lupus Nephritis With Abatacept: The Abatacept and Cyclophosphamide Combination Efficacy and Safety Study.* Arthritis & Rheumatology, 2014. **66**(11): p. 3096-3104.
43. Stohl, W. and D.M. Hilbert, *The discovery and development of belimumab: the anti-BLyS-lupus connection.* Nat Biotech, 2012. **30**(1): p. 69-77.
44. Merrill, J.T., 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 & Rheumatism, 2010. **62**(1): p. 222-233.
45. Navarra, S.V., et al., *Efficacy and safety of belimumab in patients with active systemic lupus erythematosus: a randomised, placebo-controlled, phase 3 trial.* The Lancet. **377**(9767): p. 721-731.
46. Merrill, J.T., J.P. Buyon, and T. Utset, *A 2014 update on the management of patients with systemic lupus erythematosus.* Seminars in Arthritis and Rheumatism, 2014. **44**(2): p. e1-e2.
47. Fattah, Z. and D.A. Isenberg, *Recent developments in the treatment of patients with systemic lupus erythematosus: focusing on biologic therapies.* Expert Opinion on Biological Therapy, 2014. **14**(3): p. 311-326.
48. Anolik, J., *B cell biology: implications for treatment of systemic lupus erythematosus.* Lupus, 2013. **22**(4): p. 342-349.
49. Lu, T.Y.T., et al., *A retrospective seven-year analysis of the use of B cell depletion therapy in systemic lupus erythematosus at university college london hospital: The first fifty patients.* Arthritis Care & Research, 2009. **61**(4): p. 482-487.
50. Reddy, V., et al., *B-cell depletion in SLE: clinical and trial experience with rituximab and ocrelizumaband implications for study design.* Arthritis Research & Therapy, 2013. **15**(1): p. 1-16.
51. Urowitz, M.B., et al., *The bimodal mortality pattern of systemic lupus erythematosus.* The American Journal of Medicine, 1976. **60**(2): p. 221-225.
52. Lockshin, M.D., J.E. Salmon, and M.J. Roman, *Atherosclerosis and lupus: A work in progress.* Arthritis & Rheumatism, 2001. **44**(10): p. 2215-2217.
53. Manzi, S., et al., *Age-specific Incidence Rates of Myocardial Infarction and Angina in Women with Systemic Lupus Erythematosus: Comparison with the Framingham Study.* American Journal of Epidemiology, 1997. **145**(5): p. 408-415.
54. KANNEL, W.B., et al., *AN INVESTIGATION OF CORONARY HEART DISEASE IN FAMILIES: THE FRAMINGHAM OFFSPRING STUDY.* American Journal of Epidemiology, 1979. **110**(3): p. 281-290.
55. Roman, M.J., et al., *Prevalence and Correlates of Accelerated Atherosclerosis in Systemic Lupus Erythematosus.* New England Journal of Medicine, 2003. **349**(25): p. 2399-2406.
56. Roman, M.J., et al., *Rate and determinants of progression of atherosclerosis in systemic lupus erythematosus.* Arthritis & Rheumatism, 2007. **56**(10): p. 3412-3419.
57. Rua-Figueroa, I., et al., *Factors involved in the progress of preclinical atherosclerosis associated with systemic lupus erythematosus: a 2-year longitudinal study.* Annals of the Rheumatic Diseases, 2010. **69**(6): p. 1136-1139.
58. Kiani, A.N., et al., *Predictors of progression in atherosclerosis over 2 years in systemic lupus erythematosus.* Rheumatology, 2011. **50**(11): p. 2071-2079.

59. Thompson, T., et al., *Progression of carotid intima-media thickness and plaque in women with systemic lupus erythematosus.* Arthritis & Rheumatism, 2008. **58**(3): p. 835-842.
60. HAQUE, S., et al., *Risk Factors for Clinical Coronary Heart Disease in Systemic Lupus Erythematosus: The Lupus and Atherosclerosis Evaluation of Risk (LASER) Study.* The Journal of Rheumatology, 2010. **37**(2): p. 322-329.
61. Lluis-Ganella, C., et al., *Assessment of the value of a genetic risk score in improving the estimation of coronary risk.* Atherosclerosis, 2012. **222**(2): p. 456-463.
62. Alagona Jr, P. and T.A. Ahmad, *Cardiovascular Disease Risk Assessment and Prevention: Current Guidelines and Limitations.* Medical Clinics of North America, 2015. **99**(4): p. 711-731.
63. Long, M.T. and C.S. Fox, *The Framingham Heart Study [mdash] 67 years of discovery in metabolic disease.* Nat Rev Endocrinol, 2016. **12**(3): p. 177-183.
64. Mak, A. and N.Y. Kow, *Imbalance between Endothelial Damage and Repair: A Gateway to Cardiovascular Disease in Systemic Lupus Erythematosus.* BioMed Research International, 2014. **2014**: p. 11.
65. Parker, B., et al., *An analysis of the metabolic syndrome phenotype in systemic lupus erythematosus.* Lupus, 2011. **20**(14): p. 1459-1465.
66. Sabio, J., et al., *Metabolic syndrome in patients with systemic lupus erythematosus from Southern Spain.* Lupus, 2008. **17**(9): p. 849-859.
67. García-Carrasco, M., et al., *Lack of subclinical myocardial ischaemia in Mexican patients with systemic lupus erythematosus without traditional risk factors for coronary artery disease.* Lupus, 2007. **16**(4): p. 298-301.
68. Zonana-Nacach A, S.-S.E., Jiménez-Balderas FJ, Camargo-Coronel A., *Prevalence and factors associated with metabolic syndrome in patients with rheumatoid arthritis and systemic lupus erythematosus.* Journal of Clinical Rheumatology, 2008. **14**(2): p. 74-77.
69. Asanuma, Y., et al., *Premature Coronary-Artery Atherosclerosis in Systemic Lupus Erythematosus.* New England Journal of Medicine, 2003. **349**(25): p. 2407-2415.
70. Esdaile, J.M., et al., *Traditional Framingham risk factors fail to fully account for accelerated atherosclerosis in systemic lupus erythematosus.* Arthritis & Rheumatism, 2001. **44**(10): p. 2331-2337.
71. Westerweel, P.E., et al., *Premature atherosclerotic cardiovascular disease in systemic lupus erythematosus.* Arthritis & Rheumatism, 2007. **56**(5): p. 1384-1396.
72. Leonard, D., et al., *Coronary Heart Disease in Systemic Lupus Erythematosus Is Associated With Interferon Regulatory Factor-8 Gene Variants.* Circulation: Cardiovascular Genetics, 2013. **6**(3): p. 255-263.
73. Bahrehmand, F., et al., *Matrix metalloproteinase-2 functional promoter polymorphism G1575A is associated with elevated circulatory MMP-2 levels and increased risk of cardiovascular disease in systemic lupus erythematosus patients.* Lupus, 2012. **21**(6): p. 616-624.
74. Bicakcigil, M., et al., *Role of fibrinolytic parameters and plasminogen activator inhibitor 1 (PAI-1) promoter polymorphism on premature atherosclerosis in SLE patients.* Lupus, 2011. **20**(10): p. 1063-1071.
75. TROELSEN, L.N., et al., *Genetically Determined Serum Levels of Mannose-Binding Lectin Correlate Negatively with Common Carotid Intima-Media Thickness in Systemic Lupus Erythematosus.* The Journal of Rheumatology, 2010. **37**(9): p. 1815-1821.
76. Huang, W.-N., T.K. Tso, and H.-Y. Huang, *Enhanced oxidative status but not corresponding elevated antioxidative status by anticardiolipin antibody and disease activity in patients with systemic lupus erythematosus.* Rheumatology International, 2006. **27**(5): p. 453-458.

77. Narshi, C., I. Giles, and A. Rahman, *The endothelium: an interface between autoimmunity and atherosclerosis in systemic lupus erythematosus?* Lupus, 2011. **20**(1): p. 5-13.
78. Madamanchi, N.R., A. Vendrov, and M.S. Runge, *Oxidative Stress and Vascular Disease.* Arteriosclerosis, Thrombosis, and Vascular Biology, 2005. **25**(1): p. 29-38.
79. Mengya, Z., et al., *Th17/Treg imbalance induced by increased incidence of atherosclerosis in patients with Systemic Lupus Erythematosus (SLE).* Clinical Rheumatology, 2013. **32**(7): p. 1045-1052.
80. Ruiz-Limon, P., et al., *Atherosclerosis and cardiovascular disease in systemic lupus erythematosus: effects of in vivo statin treatment.* Annals of the Rheumatic Diseases, 2014.
81. Kirou, K.A., et al., *Activation of the interferon-α pathway identifies a subgroup of systemic lupus erythematosus patients with distinct serologic features and active disease.* Arthritis & Rheumatism, 2005. **52**(5): p. 1491-1503.
82. Yamaoka, J., et al., *Cytotoxicity of IFN-γ and TNF-α for Vascular Endothelial Cell Is Mediated by Nitric Oxide.* Biochemical and Biophysical Research Communications, 2002. **291**(4): p. 780-786.
83. Denny, M.F., et al., *Interferon-α promotes abnormal vasculogenesis in lupus: a potential pathway for premature atherosclerosis*. Vol. 110. 2007. 2907-2915.
84. Giles, I. and A. Rahman, *How to manage patients with systemic lupus erythematosus who are also antiphospholipid antibody positive.* Best Practice & Research Clinical Rheumatology, 2009. **23**(4): p. 525-537.
85. Wu, R., et al., *Antibodies Against Cardiolipin and Oxidatively Modified LDL in 50-Year-Old Men Predict Myocardial Infarction.* Arteriosclerosis, Thrombosis, and Vascular Biology, 1997. **17**(11): p. 3159-3163.
86. Vaarala, O., et al., *Anti-Cardiolipin Antibodies and Risk of Myocardial Infarction in a Prospective Cohort of Middle-Aged Men.* Circulation, 1995. **91**(1): p. 23-27.
87. Meroni, P.L., et al., *Endothelial cell activation by antiphospholipid antibodies.* Clinical Immunology, 2004. **112**(2): p. 169-174.
88. Ames, P.R.J., et al., *Premature atherosclerosis in primary antiphospholipid syndrome: preliminary data.* Annals of the Rheumatic Diseases, 2005. **64**(2): p. 315-317.
89. O'Neill, S.G., et al., *Antibodies to apolipoprotein A-I, high-density lipoprotein, and C-reactive protein are associated with disease activity in patients with systemic lupus erythematosus.* Arthritis & Rheumatism, 2010. **62**(3): p. 845-854.
90. Croca, S., et al., *IgG anti-apolipoprotein A-1 antibodies in patients with systemic lupus erythematosus are associated with disease activity and corticosteroid therapy: an observational study.* Arthritis Research & Therapy, 2015. **17**(1): p. 1-10.
91. Fesmire, J., M. Wolfson-Reichlin, and M. Reichlin, *Efeitos de autoanticorpos antilipoproteína lipase, antilipoproteína de baixa densidade e antilipoproteína de baixa densidade oxidada sobre o metabolismo lipídico e aterosclerose no lúpus eritematoso sistêmico.* Revista Brasileira de Reumatologia, 2010. **50**: p. 539-551.
92. Hansson, G.K. and A. Hermansson, *The immune system in atherosclerosis.* Nat Immunol, 2011. **12**(3): p. 204-212.
93. Hrycek, A. and P. Cieślik, *Annexin A5 and anti-annexin antibodies in patients with systemic lupus erythematosus.* Rheumatology International, 2012. **32**(5): p. 1335-1342.
94. Rand, J.H., et al., *Antiphospholipid Antibodies Accelerate Plasma Coagulation by Inhibiting Annexin-V Binding to Phospholipids: A "Lupus Procoagulant" Phenomenon*. Vol. 92. 1998. 1652-1660.
95. Garcia-Romo, G.S., et al., *Netting Neutrophils Are Major Inducers of Type I IFN Production in Pediatric Systemic Lupus Erythematosus.* Science Translational Medicine, 2011. **3**(73): p. 73ra20.

96. Lande, R., et al., *Neutrophils Activate Plasmacytoid Dendritic Cells by Releasing Self-DNA–Peptide Complexes in Systemic Lupus Erythematosus.* Science Translational Medicine, 2011. **3**(73): p. 73ra19.
97. Von Feldt, J.M., et al., *Homocysteine levels and disease duration independently correlate with coronary artery calcification in patients with systemic lupus erythematosus.* Arthritis & Rheumatism, 2006. **54**(7): p. 2220-2227.
98. Potter, K., et al., *Homocysteine or Renal Impairment: Which Is the Real Cardiovascular Risk Factor?* Arteriosclerosis, Thrombosis, and Vascular Biology, 2008. **28**(6): p. 1158-1164.
99. Wall, R.T., et al., *Homocysteine-induced endothelial cell injury in vitro: A model for the study of vascular injury.* Thrombosis Research. **18**(1–2): p. 113-121.
100. Upchurch, G.R., et al., *Homocyst(e)ine Decreases Bioavailable Nitric Oxide by a Mechanism Involving Glutathione Peroxidase.* Journal of Biological Chemistry, 1997. **272**(27): p. 17012-17017.
101. Malinow, M.R., et al., *Carotid artery intimal-medial wall thickening and plasma homocyst(e)ine in asymptomatic adults. The Atherosclerosis Risk in Communities Study.* Circulation, 1993. **87**(4): p. 1107-13.
102. Taleb, S., et al., *Defective Leptin/Leptin Receptor Signaling Improves Regulatory T Cell Immune Response and Protects Mice From Atherosclerosis.* Arteriosclerosis, Thrombosis, and Vascular Biology, 2007. **27**(12): p. 2691-2698.
103. Al, M., et al., *Adipokines as novel biomarkers in paediatric systemic lupus erythematosus.* Rheumatology, 2009. **48**(5): p. 497-501.
104. Garcia-Gonzalez, A., et al., *Serum leptin levels in women with systemic lupus erythematosus.* Rheumatology International, 2002. **22**(4): p. 138-141.
105. Reynolds, H.R., et al., *Association of plasma soluble E-selectin and adiponectin with carotid plaque in patients with systemic lupus erythematosus.* Atherosclerosis, 2010. **210**(2): p. 569-574.
106. Pereira Silva, J.A.D., et al., *C-reactive protein levels in systemic lupus erythematosus: a classification criterion?* Arthritis & Rheumatism, 1980. **23**(6): p. 770-771.
107. Toloza, S.M.A., et al., *Systemic lupus erythematosus in a multiethnic US cohort (LUMINA): XXIII. Baseline predictors of vascular events.* Arthritis & Rheumatism, 2004. **50**(12): p. 3947-3957.
108. Pons-Estel, G.J., et al., *Predictors of cardiovascular damage in patients with systemic lupus erythematosus: data from LUMINA (LXVIII), a multiethnic US cohort.* Rheumatology, 2009. **48**(7): p. 817-822.
109. Bessant, R., et al., *Prevalence of conventional and lupus-specific risk factors for cardiovascular disease in patients with systemic lupus erythematosus: A case–control study.* Arthritis Care & Research, 2006. **55**(6): p. 892-899.
110. Tanay, A., et al., *Vascular Elasticity of Systemic Lupus Erythematosus Patients Is Associated with Steroids and Hydroxychloroquine Treatment.* Annals of the New York Academy of Sciences, 2007. **1108**(1): p. 24-34.
111. Mak, A., D.A. Isenberg, and C.-S. Lau, *Global trends, potential mechanisms and early detection of organ damage in SLE.* Nat Rev Rheumatol, 2013. **9**(5): p. 301-310.
112. Croca, S.C. and A. Rahman, *Imaging Assessment of Cardiovascular Disease in Systemic Lupus Erythematosus.* Clinical and Developmental Immunology, 2012. **2012**: p. 7.
113. Barsalou, J., et al., *Impact of Disease Duration on Vascular Surrogates of Early Atherosclerosis in Childhood-Onset Systemic Lupus Erythematosus.* Arthritis & Rheumatology, 2016. **68**(1): p. 237-246.
114. Tselios, K., et al., *Dyslipidemia in systemic lupus erythematosus: just another comorbidity?* Seminars in Arthritis and Rheumatism, 2016.

115. Hua, X., et al., *Dyslipidaemia and lipoprotein pattern in systemic lupus erythematosus (SLE) and SLE-related cardiovascular disease.* Scandinavian Journal of Rheumatology, 2009. **38**(3): p. 184-189.
116. Pego-Reigosa, J.M., et al., *Long-term improvement of lipid profile in patients with refractory systemic lupus erythematosus treated with B-cell depletion therapy: a retrospective observational study.* Rheumatology, 2010. **49**(4): p. 691-696.
117. Forrester, J.S. and P. Libby, *The Inflammation Hypothesis and Its Potential Relevance to Statin Therapy.* The American Journal of Cardiology, 2007. **99**(5): p. 732-738.
118. Aikawa, M., et al., *An HMG-CoA Reductase Inhibitor, Cerivastatin, Suppresses Growth of Macrophages Expressing Matrix Metalloproteinases and Tissue Factor In Vivo and In Vitro.* Circulation, 2001. **103**(2): p. 276-283.
119. Wæhre, T., et al., *Increased Expression of Interleukin-1 in Coronary Artery Disease With Downregulatory Effects of HMG-CoA Reductase Inhibitors.* Circulation, 2004. **109**(16): p. 1966-1972.
120. López-Pedrera, C., et al., *Global effects of fluvastatin on the prothrombotic status of patients with antiphospholipid syndrome.* Annals of the Rheumatic Diseases, 2011. **70**(4): p. 675-682.
121. Mok, C.C., et al., *Effects of rosuvastatin on vascular biomarkers and carotid atherosclerosis in lupus: A randomized, double-blind, placebo-controlled trial.* Arthritis Care & Research, 2011. **63**(6): p. 875-883.
122. Fatemi, A., et al., *Atorvastatin effect on systemic lupus erythematosus disease activity: a double-blind randomized clinical trial.* Clinical Rheumatology, 2014: p. 1-6.
123. Petri, M.A., et al., *Lupus Atherosclerosis Prevention Study (LAPS).* Annals of the Rheumatic Diseases, 2011. **70**(5): p. 760-765.
124. Schanberg, L.E., et al., *Use of atorvastatin in systemic lupus erythematosus in children and adolescents.* Arthritis & Rheumatism, 2012. **64**(1): p. 285-296.
125. Elkon, K.B. and D.M. Santer, *Complement, interferon and lupus.* Current Opinion in Immunology, 2012. **24**(6): p. 665-670.
126. Majai, G., et al., *Decreased apopto-phagocytic gene expression in the macrophages of systemic lupus erythematosus patients.* Lupus, 2013.
127. Borba, E.F., et al., *Mechanisms of Dyslipoproteinemias in Systemic Lupus Erythematosus.* Clinical and Developmental Immunology, 2006. **13**(2-4): p. 203-208.
128. Munoz, L.E., et al., *The role of defective clearance of apoptotic cells in systemic autoimmunity.* Nat Rev Rheumatol, 2010. **6**(5): p. 280-289.
129. Ravichandran, Kodi S., *Beginnings of a Good Apoptotic Meal: The Find-Me and Eat-Me Signaling Pathways.* Immunity, 2011. **35**(4): p. 445-455.
130. Tas, S.W., et al., *Macrophages from patients with SLE and rheumatoid arthritis have defective adhesion in vitro, while only SLE macrophages have impaired uptake of apoptotic cells.* Annals of the Rheumatic Diseases, 2006. **65**(2): p. 216-221.
131. Leadbetter, E.A., et al., *Chromatin-IgG complexes activate B cells by dual engagement of IgM and Toll-like receptors.* Nature, 2002. **416**(6881): p. 603-607.
132. Crow, M.K., M. Olferiev, and K.A. Kirou, *Targeting of type I interferon in systemic autoimmune diseases.* Translational Research, 2015. **165**(2): p. 296-305.
133. Fransen, J., et al., *The role of dendritic cells in the pathogenesis of systemic lupus erythematosus.* Arthritis Research & Therapy, 2010. **12**(2): p. 207.
134. Peng, S.L., *Altered T and B lymphocyte signaling pathways in lupus.* Autoimmunity Reviews, 2009. **8**(3): p. 179-183.
135. Singh, R.P., et al., *Th17 cells in inflammation and autoimmunity.* Autoimmunity Reviews, 2014. **13**(12): p. 1174-1181.

136. Valencia, X., et al., *Deficient CD4+CD25high T Regulatory Cell Function in Patients with Active Systemic Lupus Erythematosus.* The Journal of Immunology, 2007. **178**(4): p. 2579-2588.
137. Chu, V.T., et al., *Systemic activation of the immune system induces aberrant BAFF and APRIL expression in B cells in patients with systemic lupus erythematosus.* Arthritis & Rheumatism, 2009. **60**(7): p. 2083-2093.
138. Manea, M., et al., *Increased expression of CD154 and FAS in SLE patients' lymphocytes.* Rheumatology International, 2009. **30**(2): p. 181-185.
139. Tabas, I., K.J. Williams, and J. Borén, *Subendothelial Lipoprotein Retention as the Initiating Process in Atherosclerosis: Update and Therapeutic Implications.* Circulation, 2007. **116**(16): p. 1832-1844.
140. Skalen, K., et al., *Subendothelial retention of atherogenic lipoproteins in early atherosclerosis.* Nature, 2002. **417**(6890): p. 750-754.
141. Vikramadithyan, R.K., et al., *Atherosclerosis in perlecan heterozygous mice.* Journal of Lipid Research, 2004. **45**(10): p. 1806-1812.
142. Subramanian, M. and I. Tabas, *Dendritic cells in atherosclerosis.* Seminars in Immunopathology, 2014. **36**(1): p. 93-102.
143. Moore, Kathryn J. and I. Tabas, *Macrophages in the Pathogenesis of Atherosclerosis.* Cell, 2011. **145**(3): p. 341-355.
144. Silvestre-Roig, C., et al., *Atherosclerotic Plaque Destabilization: Mechanisms, Models, and Therapeutic Strategies.* Circulation Research, 2014. **114**(1): p. 214-226.
145. Clarke, M.C.H., et al., *Apoptosis of vascular smooth muscle cells induces features of plaque vulnerability in atherosclerosis.* Nat Med, 2006. **12**(9): p. 1075-1080.
146. Williams, H., et al., *MMP-7 mediates cleavage of N-cadherin and promotes smooth muscle cell apoptosis.* Cardiovascular Research, 2010. **87**(1): p. 137-146.
147. den Dekker, W.K., et al., *Mast Cells Induce Vascular Smooth Muscle Cell Apoptosis via a Toll-Like Receptor 4 Activation Pathway.* Arteriosclerosis, Thrombosis, and Vascular Biology, 2012. **32**(8): p. 1960-1969.
148. Soehnlein, O. and C. Weber, *Myeloid cells in atherosclerosis: initiators and decision shapers.* Seminars in Immunopathology, 2009. **31**(1): p. 35-47.
149. Izma Abdul Zani, S.L.S., Nadeem A. Mughal, David Russell, Shervanthi Homer-Vanniasinkam, Stephen B. Wheatcroft and Sreenivasan Ponnambalam, *Scavenger Receptor Structure and Function in Health and Disease.* Cells, 2015. **4**(2): p. 178-201.
150. Zani, I., et al., *Scavenger Receptor Structure and Function in Health and Disease.* Cells, 2015. **4**(2): p. 178.
151. Silverstein, R.L. and M. Febbraio, *CD36, a Scavenger Receptor Involved in Immunity, Metabolism, Angiogenesis, and Behavior*. Vol. 2. 2009. re3-re3.
152. Park, Y.M., *CD36, a scavenger receptor implicated in atherosclerosis.* Exp Mol Med, 2014. **46**: p. e99.
153. Inoue, M., et al., *Astaxanthin functions differently as a selective peroxisome proliferator-activated receptor γ modulator in adipocytes and macrophages.* Biochemical Pharmacology, 2012. **84**(5): p. 692-700.
154. Gao, D., et al., *Palmitate promotes monocyte atherogenicity via de novo ceramide synthesis.* Free Radical Biology and Medicine, 2012. **53**(4): p. 796-806.
155. Ren, Y., et al., *CD36 gene transfer confers capacity for phagocytosis of cells undergoing apoptosis.* The Journal of Experimental Medicine, 1995. **181**(5): p. 1857-1862.
156. Podrez, E.A., et al., *A Novel Family of Atherogenic Oxidized Phospholipids Promotes Macrophage Foam Cell Formation via the Scavenger Receptor CD36 and Is Enriched in Atherosclerotic Lesions.* Journal of Biological Chemistry, 2002. **277**(41): p. 38517-38523.

157. Febbraio, M., D.P. Hajjar, and R.L. Silverstein, *CD36: a class B scavenger receptor involved in angiogenesis, atherosclerosis, inflammation, and lipid metabolism.* The Journal of Clinical Investigation, 2001. **108**(6): p. 785-791.
158. Sawamura, T., I. Wakabayashi, and T. Okamura, *LOX-1 in atherosclerotic disease.* Clinica Chimica Acta, 2015. **440**: p. 157-163.
159. Kume, N., et al., *Inducible Expression of LOX-1, a Novel Receptor for Oxidized LDL, in Macrophages and Vascular Smooth Muscle Cells.* Annals of the New York Academy of Sciences, 2000. **902**(1): p. 323-327.
160. Chistiakov, D.A., Y.V. Bobryshev, and A.N. Orekhov, *Macrophage-mediated cholesterol handling in atherosclerosis.* Journal of Cellular and Molecular Medicine, 2016. **20**(1): p. 17-28.
161. Kataoka, H., et al., *Expression of Lectinlike Oxidized Low-Density Lipoprotein Receptor-1 in Human Atherosclerotic Lesions.* Circulation, 1999. **99**(24): p. 3110-3117.
162. Zhang, L., et al., *Feedback Regulation of Cholesterol Uptake by the LXR–IDOL–LDLR Axis.* Arteriosclerosis, Thrombosis, and Vascular Biology, 2012. **32**(11): p. 2541-2546.
163. Chen, X.-w., et al., *Anti-class a scavenger receptor autoantibodies from systemic lupus erythematosus patients impair phagocytic clearance of apoptotic cells by macrophages in vitro.* Arthritis Research & Therapy, 2011. **13**(1): p. R9.
164. Reiss, A.B., et al., *A BRIEF COMMUNICATION: Enhanced CD36 Scavenger Receptor Expression in THP-1 Human Monocytes in the Presence of Lupus Plasma: Linking Autoimmunity and Atherosclerosis.* Experimental Biology and Medicine, 2009. **234**(3): p. 354-360.
165. Li, J., et al., *Interferon-α priming promotes lipid uptake and macrophage-derived foam cell formation: A novel link between interferon-α and atherosclerosis in lupus.* Arthritis & Rheumatism, 2011. **63**(2): p. 492-502.
166. Mitchell, A.J., B. Roediger, and W. Weninger, *Monocyte homeostasis and the plasticity of inflammatory monocytes.* Cellular Immunology, 2014. **291**(1–2): p. 22-31.
167. Nahrendorf, M., et al., *The healing myocardium sequentially mobilizes two monocyte subsets with divergent and complementary functions.* The Journal of Experimental Medicine, 2007. **204**(12): p. 3037-3047.
168. Poitou, C., et al., *CD14dimCD16+ and CD14+CD16+ Monocytes in Obesity and During Weight Loss: Relationships With Fat Mass and Subclinical Atherosclerosis.* Arteriosclerosis, Thrombosis, and Vascular Biology, 2011. **31**(10): p. 2322-2330.
169. Timmerman, K.L., et al., *Exercise training-induced lowering of inflammatory (CD14+CD16+) monocytes: a role in the anti-inflammatory influence of exercise?* Journal of Leukocyte Biology, 2008. **84**(5): p. 1271-1278.
170. Schlitt, A., et al., *CD14+CD16+ monocytes in coronary artery disease and their relationship to serum TNF-α levels.* Thrombosis and Haemostasis, 2004. **92**(8): p. 419-424.
171. Zawada, A.M., et al., *Monocyte heterogeneity in human cardiovascular disease.* Immunobiology, 2012. **217**(12): p. 1273-84.
172. Burbano, C., G. Vasquez, and M. Rojas, *Modulatory Effects of CD14+CD16++ Monocytes on CD14++CD16− Monocytes: A Possible Explanation of Monocyte Alterations in Systemic Lupus Erythematosus.* Arthritis & Rheumatology, 2014. **66**(12): p. 3371-3381.
173. Sümegi, A., et al., *Glucocorticosteroid therapy decreases CD14-expression and CD14-mediated LPS-binding and activation of monocytes in patients suffering from systemic lupus erythematosus.* Clinical Immunology, 2005. **117**(3): p. 271-279.
174. Cairns, A., A. Crockard, and A. Bell, *The CD14+ CD16+ monocyte subset in rheumatoid arthritis and systemic lupus erythematosus.* Rheumatology International, 2002. **21**(5): p. 189-192.

175. Mukherjee, R., et al., *Non-Classical monocytes display inflammatory features: Validation in Sepsis and Systemic Lupus Erythematous.* Scientific Reports, 2015. **5**: p. 13886.
176. Ghattas, A., et al., *Monocytes in Coronary Artery Disease and Atherosclerosis: Where Are We Now?* Journal of the American College of Cardiology, 2013. **62**(17): p. 1541-1551.
177. Lee, P.Y., et al., *A novel type i IFN-producing cell subset in murine lupus.* Journal of Immunology, 2008. **180**(7): p. 5101-5108.
178. Feng, D., 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 and Rheumatism, 2010. **62**(2): p. 562-573.
179. Korman, B., et al., *Inflammatory expression profiles in monocyte-to-macrophage differentiation in patients with systemic lupus erythematosus and relationship with atherosclerosis.* Arthritis Research & Therapy, 2014. **16**(4): p. R147.
180. Wingrove, J.A., et al., *Correlation of Peripheral-Blood Gene Expression With the Extent of Coronary Artery Stenosis.* Circulation: Cardiovascular Genetics, 2008. **1**(1): p. 31-38.
181. Huang, C.-C., et al., *Activated TLR Signaling in Atherosclerosis among Women with Lower Framingham Risk Score: The Multi-Ethnic Study of Atherosclerosis.* PLoS ONE, 2011. **6**(6): p. e21067.
182. Li, X., et al., *Reevaluation of the Role of VEGF-B Suggests a Restricted Role in the Revascularization of the Ischemic Myocardium.* Arteriosclerosis, Thrombosis, and Vascular Biology, 2008. **28**(9): p. 1614-1620.
183. Blanco, P., et al., *Induction of Dendritic Cell Differentiation by IFN-α in Systemic Lupus Erythematosus.* Science, 2001. **294**(5546): p. 1540-1543.
184. Herrmann, M., R.E. Voll, and J.R. Kalden, *Etiopathogenesis of systemic lupus erythematosus.* Immunology Today, 2000. **21**(9): p. 424-426.
185. Naumov, Y.N., et al., *Activation of CD1d-restricted T cells protects NOD mice from developing diabetes by regulating dendritic cell subsets.* Proceedings of the National Academy of Sciences, 2001. **98**(24): p. 13838-13843.
186. Peled, M. and E.A. Fisher, *Dynamic Aspects of Macrophage Polarization during Atherosclerosis Progression and Regression.* Frontiers in Immunology, 2014. **5**.
187. Mills, C.D., et al., *M-1/M-2 Macrophages and the Th1/Th2 Paradigm.* The Journal of Immunology, 2000. **164**(12): p. 6166-6173.
188. Chistiakov, D.A., et al., *Macrophage phenotypic plasticity in atherosclerosis: The associated features and the peculiarities of the expression of inflammatory genes.* International Journal of Cardiology, 2015. **184**: p. 436-445.
189. Cho, K.Y., et al., *The Phenotype of Infiltrating Macrophages Influences Arteriosclerotic Plaque Vulnerability in the Carotid Artery.* Journal of Stroke and Cerebrovascular Diseases, 2013. **22**(7): p. 910-918.
190. Khallou-Laschet, J., et al., *Macrophage Plasticity in Experimental Atherosclerosis.* PLoS ONE, 2010. **5**(1): p. e8852.
191. Stöger, J.L., et al., *Distribution of macrophage polarization markers in human atherosclerosis.* Atherosclerosis, 2012. **225**(2): p. 461-468.
192. Li, F., et al., *Macrophage Polarization Modulates Development of Systemic Lupus Erythematosus.* Cellular Physiology and Biochemistry, 2015. **37**(4): p. 1279-1288.
193. Iwata, Y., et al., *Aberrant Macrophages Mediate Defective Kidney Repair That Triggers Nephritis in Lupus-Susceptible Mice.* The Journal of Immunology, 2012. **188**(9): p. 4568-4580.
194. Santana-de Anda, K., et al., *Plasmacytoid dendritic cells: Key players in viral infections and autoimmune diseases.* Seminars in Arthritis and Rheumatism, 2013. **43**(1): p. 131-136.

195. Martín-Gayo, E., et al., *Plasmacytoid dendritic cells resident in human thymus drive natural Treg cell development*. Vol. 115. 2010. 5366-5375.
196. Ala-Korpela, M., A. Kangas, and P. Soininen, *Quantitative high-throughput metabolomics: a new era in epidemiology and genetics.* Genome Medicine, 2012. **4**(4): p. 36.
197. Zhou, L.J. and T.F. Tedder, *CD14+ blood monocytes can differentiate into functionally mature CD83+ dendritic cells.* Proceedings of the National Academy of Sciences of the United States of America, 1996. **93**(6): p. 2588-2592.
198. Wilhelm, A.J., et al., *Dysregulated CD4+ T cells from SLE-susceptible mice are sufficient to accelerate atherosclerosis in LDLr−/− mice.* Annals of the Rheumatic Diseases, 2015. **74**(4): p. 778-785.
199. Lynch, L., et al., *Adipose Tissue Invariant NKT Cells Protect against Diet-Induced Obesity and Metabolic Disorder through Regulatory Cytokine Production.* Immunity, 2012. **37**(3): p. 574-587.
200. Williams, L.M., et al., *Interleukin-10 suppression of myeloid cell activation — a continuing puzzle.* Immunology, 2004. **113**(3): p. 281-292.
201. Pei, B., et al., *Invariant NKT Cells Require Autophagy To Coordinate Proliferation and Survival Signals during Differentiation.* The Journal of Immunology, 2015.
202. Griffin, M., et al., *Carotid and femoral arterial wall changes and the prevalence of clinical cardiovascular disease.* Vascular Medicine, 2009. **14**(3): p. 227-232.
203. Liao, J., C. Chang, and Q. Lu, *Cell-based therapies for systemic lupus erythematosus.* Autoimmunity Reviews, (0).
204. Wigren, M., J. Nilsson, and D. Kolbus, *Lymphocytes in atherosclerosis.* Clinica Chimica Acta, 2012. **413**(19–20): p. 1562-1568.
205. Major, A.S., S. Fazio, and M.F. Linton, *B-Lymphocyte Deficiency Increases Atherosclerosis in LDL Receptor–Null Mice.* Arteriosclerosis, Thrombosis, and Vascular Biology, 2002. **22**(11): p. 1892-1898.
206. Doran, A.C., et al., *B-Cell Aortic Homing and Atheroprotection Depend on Id3.* Circulation Research, 2012. **110**(1): p. e1-e12.
207. Kyaw, T., et al., *Depletion of B2 but Not B1a B Cells in BAFF Receptor-Deficient ApoE^{−/−} Mice Attenuates Atherosclerosis by Potently Ameliorating Arterial Inflammation.* PLoS ONE, 2012. **7**(1): p. e29371.
208. Chou, M.-Y., et al., *Oxidation-specific epitopes are dominant targets of innate natural antibodies in mice and humans.* The Journal of Clinical Investigation, 2009. **119**(5): p. 1335-1349.
209. Perry, H.M., T.P. Bender, and C.A. McNamara, *B cell subsets in atherosclerosis.* Frontiers in Immunology, 2012. **3**.
210. Sabetai, M.M., et al., *Reproducibility of Computer-Quantified Carotid Plaque Echogenicity: Can We Overcome the Subjectivity?* Stroke, 2000. **31**(9): p. 2189-2196.
211. Jonasson, L., et al., *Regional accumulations of T cells, macrophages, and smooth muscle cells in the human atherosclerotic plaque.* Arteriosclerosis, Thrombosis, and Vascular Biology, 1986. **6**(2): p. 131-8.
212. Jonasson, L., et al., *Expression of class II transplantation antigen on vascular smooth muscle cells in human atherosclerosis.* The Journal of Clinical Investigation, 1985. **76**(1): p. 125-131.
213. Hansson, G.K., et al., *Innate and Adaptive Immunity in the Pathogenesis of Atherosclerosis.* Circulation Research, 2002. **91**(4): p. 281-291.
214. Li, N., *CD4+ T cells in atherosclerosis: Regulation by platelets.* Thrombosis and Haemostasis, 2013. **109**(6): p. 980-990.

215. Zhou, X., et al., *Adoptive Transfer of CD4+ T Cells Reactive to Modified Low-Density Lipoprotein Aggravates Atherosclerosis.* Arteriosclerosis, Thrombosis, and Vascular Biology, 2006. **26**(4): p. 864-870.
216. Tegos, T.J., et al., *Comparability of the ultrasonic tissue characteristics of carotid plaques.* Journal of Ultrasound in Medicine, 2000. **19**(6): p. 399-407.
217. Griffin M, N.A., Kyriacou E., *Normalisation of ultrasonic images of atherosclerotic plaques and reproducibility of grey scale median using dedicated software.* International Angiology, 2007. **4**(372-7).
218. Makris, G.C., et al., *Three-dimensional ultrasound imaging for the evaluation of carotid atherosclerosis.* Atherosclerosis, 2011. **219**(2): p. 377-383.
219. Liu, L.-L., et al., *Lower prevalence of circulating invariant natural killer T (iNKT) cells in patients with acute myocardial infarction undergoing primary coronary stenting.* International Immunopharmacology, 2011. **11**(4): p. 480-484.
220. Fontenot, J.D., M.A. Gavin, and A.Y. Rudensky, *Foxp3 programs the development and function of CD4+CD25+ regulatory T cells.* Nat Immunol, 2003. **4**(4): p. 330-336.
221. Allan, S.E., et al., *Activation-induced FOXP3 in human T effector cells does not suppress proliferation or cytokine production.* International Immunology, 2007. **19**(4): p. 345-354.
222. Aldemir, H., et al., *Cutting Edge: Lectin-Like Transcript 1 Is a Ligand for the CD161 Receptor.* The Journal of Immunology, 2005. **175**(12): p. 7791-7795.
223. Chen, Y.-T. and J.T. Kung, *CD1d-Independent Developmental Acquisition of Prompt IL-4 Gene Inducibility in Thymus CD161(NK1)−CD44lowCD4+CD8− T Cells Is Associated with Complementarity Determining Region 3-Diverse and Biased Vβ2/Vβ7/Vβ8/Vα3.2 T Cell Receptor Usage.* The Journal of Immunology, 2005. **175**(10): p. 6537-6550.
224. Eberhard, J.M., et al., *CD161⁺ MAIT Cells Are Severely Reduced in Peripheral Blood and Lymph Nodes of HIV-Infected Individuals Independently of Disease Progression.* PLoS ONE, 2014. **9**(11): p. e111323.
225. Li, Y., et al., *CD4+ Natural Killer T Cells Potently Augment Aortic Root Atherosclerosis by Perforin- and Granzyme B-Dependent Cytotoxicity.* Circulation Research, 2015. **116**(2): p. 245-254.
226. Major, A.S., S. Joyce, and L. Van Kaer, *Lipid metabolism, atherogenesis and CD1-restricted antigen presentation.* Trends in Molecular Medicine, 2006. **12**(6): p. 270-278.
227. de Boer, O.J., et al., *Low Numbers of FOXP3 Positive Regulatory T Cells Are Present in all Developmental Stages of Human Atherosclerotic Lesions.* PLoS ONE, 2007. **2**(8): p. e779.
228. Veillard, N.R., et al., *Differential Expression Patterns of Proinflammatory and Antiinflammatory Mediators During Atherogenesis in Mice.* Arteriosclerosis, Thrombosis, and Vascular Biology, 2004. **24**(12): p. 2339-2344.
229. Ji, Y., et al., *Activation of Natural Killer T Cells Promotes M2 Macrophage Polarization in Adipose Tissue and Improves Systemic Glucose Tolerance via Interleukin-4 (IL-4)/STAT6 Protein Signaling Axis in Obesity.* Journal of Biological Chemistry, 2012. **287**(17): p. 13561-13571.
230. Feuerer, M., et al., *Lean, but not obese, fat is enriched for a unique population of regulatory T cells that affect metabolic parameters.* Nat Med, 2009. **15**(8): p. 930-939.
231. Godfrey, D.I., et al., *NKT cells: what's in a name?* Nat Rev Immunol, 2004. **4**(3): p. 231-237.
232. Hofmann, S.C., et al., *Invariant natural killer T cells are enriched at the site of cutaneous inflammation in lupus erythematosus.* Journal of Dermatological Science, 2013. **71**(1): p. 22-28.
233. Bosma, A., et al., *Lipid-Antigen Presentation by CD1d+ B Cells Is Essential for the Maintenance of Invariant Natural Killer T Cells.* Immunity, 2012. **36**(3): p. 477-490.

234. Major, A.S., et al., *The role of invariant natural killer T cells in lupus and atherogenesis.* Immunologic research, 2006. **34**(1): p. 49-66.
235. Budd, R.C., et al., *Developmentally regulated expression of T cell receptor beta chain variable domains in immature thymocytes.* The Journal of Experimental Medicine, 1987. **166**(2): p. 577-582.
236. Fowlkes, B.J., et al., *A novel population of T-cell receptor [alpha][beta]-bearing thymocytes which predominantly expresses a single V[beta] gene family.* Nature, 1987. **329**(6136): p. 251-254.
237. Ceredig, R., F. Lynch, and P. Newman, *Phenotypic properties, interleukin 2 production, and developmental origin of a "mature" subpopulation of Lyt-2- L3T4- mouse thymocytes.* Proceedings of the National Academy of Sciences, 1987. **84**(23): p. 8578-8582.
238. Makino, Y., et al., *Predominant expression of invariant V alpha 14+ TCR alpha chain in NK1.1+ T cell populations.* International immunology, 1995. **7**(7): p. 1157-1161.
239. Brossay, L., et al., *Mouse CD1-Autoreactive T Cells Have Diverse Patterns of Reactivity to CD1+ Targets.* The Journal of Immunology, 1998. **160**(8): p. 3681-3688.
240. Berzins, S.P., M.J. Smyth, and A.G. Baxter, *Presumed guilty: natural killer T cell defects and human disease.* Nat Rev Immunol, 2011. **11**(2): p. 131-142.
241. Godfrey, D.I., S. Stankovic, and A.G. Baxter, *Raising the NKT cell family.* Nat Immunol, 2010. **11**(3): p. 197-206.
242. Kim, E.Y., et al., *The transcriptional programs of iNKT cells.* Seminars in Immunology, 2015. **27**(1): p. 26-32.
243. Brennan, P.J., M. Brigl, and M.B. Brenner, *Invariant natural killer T cells: an innate activation scheme linked to diverse effector functions.* Nat Rev Immunol, 2013. **13**(2): p. 101-117.
244. Constantinides, M.G. and A. Bendelac, *Transcriptional regulation of the NKT cell lineage.* Current Opinion in Immunology, 2013. **25**(2): p. 161-167.
245. Godfrey, D.I. and S.P. Berzins, *Control points in NKT-cell development.* Nat Rev Immunol, 2007. **7**(7): p. 505-518.
246. Griewank, K., et al., *Homotypic Interactions Mediated by Slamf1 and Slamf6 Receptors Control NKT Cell Lineage Development.* Immunity, 2007. **27**(5): p. 751-762.
247. Hu, T., et al., *The Ras/MAPK Pathway Is Required for Generation of iNKT Cells.* PLoS ONE, 2011. **6**(5): p. e19890.
248. Lazarevic, V., et al., *The gene encoding early growth response 2, a target of the transcription factor NFAT, is required for the development and maturation of natural killer T cells.* Nat Immunol, 2009. **10**(3): p. 306-313.
249. Seiler, M.P., et al., *Elevated and sustained expression of the transcription factors Egr1 and Egr2 controls NKT lineage differentiation in response to TCR signaling.* Nat Immunol, 2012. **13**(3): p. 264-271.
250. Savage, A.K., M.G. Constantinides, and A. Bendelac, *Promyelocytic Leukemia Zinc Finger Turns on the Effector T Cell Program without Requirement for Agonist TCR Signaling.* The Journal of Immunology, 2011. **186**(10): p. 5801-5806.
251. Lynch, L., et al., *Regulatory iNKT cells lack expression of the transcription factor PLZF and control the homeostasis of Treg cells and macrophages in adipose tissue.* Nat Immunol, 2015. **16**(1): p. 85-95.
252. Engel, I. and M. Kronenberg, *Making memory at birth: understanding the differentiation of natural killer T cells.* Current Opinion in Immunology, 2012. **24**(2): p. 184-190.
253. Constantinides, M.G., et al., *A Naive-Like Population of Human CD1d-Restricted T Cells Expressing Intermediate Levels of Promyelocytic Leukemia Zinc Finger.* The Journal of Immunology, 2011. **187**(1): p. 309-315.

254. Savage, A.K., et al., *The Transcription Factor PLZF Directs the Effector Program of the NKT Cell Lineage.* Immunity, 2008. **29**(3): p. 391-403.
255. Alonzo, E.S. and D.B. Sant'Angelo, *Development of PLZF-expressing innate T cells.* Current Opinion in Immunology, 2011. **23**(2): p. 220-227.
256. Das, R., D.B. Sant'Angelo, and K.E. Nichols, *Transcriptional control of invariant NKT cell development.* Immunological Reviews, 2010. **238**(1): p. 195-215.
257. Finlay, D.K., et al., *Temporal Differences in the Dependency on Phosphoinositide-Dependent Kinase 1 Distinguish the Development of Invariant Vα14 NKT Cells and Conventional T Cells.* The Journal of Immunology, 2010. **185**(10): p. 5973-5982.
258. Mycko, M.P., et al., *Selective Requirement for c-Myc at an Early Stage of Vα14i NKT Cell Development.* The Journal of Immunology, 2009. **182**(8): p. 4641-4648.
259. Gadue, P., N. Morton, and P.L. Stein, *The Src Family Tyrosine Kinase Fyn Regulates Natural Killer T Cell Development.* The Journal of Experimental Medicine, 1999. **190**(8): p. 1189-1196.
260. Eberl, G., B. Lowin-Kropf, and H.R. MacDonald, *Cutting Edge: NKT Cell Development Is Selectively Impaired in Fyn- Deficient Mice.* The Journal of Immunology, 1999. **163**(8): p. 4091-4094.
261. Dose, M., et al., *Intrathymic proliferation wave essential for Vα14+ natural killer T cell development depends on c-Myc.* Proceedings of the National Academy of Sciences, 2009. **106**(21): p. 8641-8646.
262. Zhang, L., et al., *Mammalian Target of Rapamycin Complex 1 Orchestrates Invariant NKT Cell Differentiation and Effector Function.* The Journal of Immunology, 2014. **193**(4): p. 1759-1765.
263. Henao-Mejia, J., et al., *The MicroRNA miR-181 Is a Critical Cellular Metabolic Rheostat Essential for NKT Cell Ontogenesis and Lymphocyte Development and Homeostasis.* Immunity, 2013. **38**(5): p. 984-997.
264. Shin, J., et al., *Mechanistic target of rapamycin complex 1 is critical for invariant natural killer T-cell development and effector function.* Proceedings of the National Academy of Sciences, 2014. **111**(8): p. E776-E783.
265. Chandra, S. and M. Kronenberg, *Chapter Three - Activation and Function of iNKT and MAIT Cells*, in *Advances in Immunology*, W.A. Frederick, Editor. 2015, Academic Press. p. 145-201.
266. Napolitano, A., et al., *Functional Education of Invariant NKT Cells by Dendritic Cell Tuning of SHP-1.* The Journal of Immunology, 2013. **190**(7): p. 3299-3308.
267. Eidson, M., et al., *Altered Development of NKT Cells, γδ T Cells, CD8 T Cells and NK Cells in a PLZF Deficient Patient.* PLoS ONE, 2011. **6**(9): p. e24441.
268. Girardi, E. and D.M. Zajonc, *Molecular basis of lipid antigen presentation by CD1d and recognition by natural killer T cells.* Immunological Reviews, 2012. **250**(1): p. 167-179.
269. Exley, M., et al., *CD1d structure and regulation on human thymocytes, peripheral blood T cells,B cells and monocytes.* Immunology, 2000. **100**(1): p. 37-47.
270. Canchis , B., Landau , Yang , Balk , and Blumberg R S, *Tissue distribution of the non-polymorphic major histocompatibility complex class I-like molecule, CD1d.* Immunology, 1993. **80**(4): p. 561-5.
271. Borg, N.A., et al., *CD1d-lipid-antigen recognition by the semi-invariant NKT T-cell receptor.* Nature, 2007. **448**(7149): p. 44-49.
272. Zeng, Z.-H., et al., *Crystal Structure of Mouse CD1: An MHC-Like Fold with a Large Hydrophobic Binding Groove.* Science, 1997. **277**(5324): p. 339-345.
273. Kawano, T., et al., *CD1d-Restricted and TCR-Mediated Activation of Vα14 NKT Cells by Glycosylceramides.* Science, 1997. **278**(5343): p. 1626-1629.
274. Kaer, L.V., *[alpha]-Galactosylceramide therapy for autoimmune diseases: prospects and obstacles.* Nat Rev Immunol, 2005. **5**(1): p. 31-42.

275. Elzen, P.v.d., et al., *Apolipoprotein-mediated pathways of lipid antigen presentation.* Nature, 2005. **437**(7060): p. 906-910.
276. Freigang, S., et al., *Scavenger receptors target glycolipids for natural killer T cell activation.* The Journal of Clinical Investigation, 2012. **122**(11): p. 3943-3954.
277. Lang, G.A., et al., *BCR targeting of biotin-α-galactosylceramide leads to enhanced presentation on CD1d and requires transport of BCR to CD1d-containing endocytic compartments.* International Immunology, 2005. **17**(7): p. 899-908.
278. Barral, P., et al., *B cell receptor-mediated uptake of CD1d-restricted antigen augments antibody responses by recruiting invariant NKT cell help in vivo.* Proceedings of the National Academy of Sciences, 2008. **105**(24): p. 8345-8350.
279. Brigl, M. and M.B. Brenner, *CD1: Antigen Presentation and T Cell Function.* Annual Review of Immunology, 2004. **22**(1): p. 817-890.
280. Huang, J.-R., et al., *α-Galactosylceramide but Not Phenyl-Glycolipids Induced NKT Cell Anergy and IL-33–Mediated Myeloid-Derived Suppressor Cell Accumulation via Upregulation of egr2/3.* The Journal of Immunology, 2014. **192**(4): p. 1972-1981.
281. Stirnemann, K., et al., *Sustained activation and tumor targeting of NKT cells using a CD1d–anti-HER2–scFv fusion protein induce antitumor effects in mice.* The Journal of Clinical Investigation, 2008. **118**(3): p. 994-1005.
282. Gonzalez-Aseguinolaza, G., et al., *Natural Killer T Cell Ligand α-Galactosylceramide Enhances Protective Immunity Induced by Malaria Vaccines.* The Journal of Experimental Medicine, 2002. **195**(5): p. 617-624.
283. Carreño, L.J., Shalu S. Kharkwal, and S.A. Porcelli, *Optimizing NKT cell ligands as vaccine adjuvants.* Immunotherapy, 2014. **6**(3): p. 309-320.
284. Fujii, S.-i., et al., *Activation of Natural Killer T Cells by α-Galactosylceramide Rapidly Induces the Full Maturation of Dendritic Cells In Vivo and Thereby Acts as an Adjuvant for Combined CD4 and CD8 T Cell Immunity to a Coadministered Protein.* The Journal of Experimental Medicine, 2003. **198**(2): p. 267-279.
285. Schmieg, J., et al., *Superior Protection against Malaria and Melanoma Metastases by a C-glycoside Analogue of the Natural Killer T Cell Ligand α-Galactosylceramide.* The Journal of Experimental Medicine, 2003. **198**(11): p. 1631-1641.
286. Brigl, M., et al., *Mechanism of CD1d-restricted natural killer T cell activation during microbial infection.* Nat Immunol, 2003. **4**(12): p. 1230-1237.
287. Tyznik, A.J., et al., *Cutting Edge: The Mechanism of Invariant NKT Cell Responses to Viral Danger Signals.* The Journal of Immunology, 2008. **181**(7): p. 4452-4456.
288. Tyznik, A.J., et al., *Distinct Requirements for Activation of NKT and NK Cells during Viral Infection.* The Journal of Immunology, 2014. **192**(8): p. 3676-3685.
289. Holzapfel, K.L., et al., *Antigen-Dependent versus -Independent Activation of Invariant NKT Cells during Infection.* The Journal of Immunology, 2014. **192**(12): p. 5490-5498.
290. Vahl, J.C., et al., *NKT Cell-TCR Expression Activates Conventional T Cells in Vivo, but Is Largely Dispensable for Mature NKT Cell Biology.* PLoS Biology, 2013. **11**(6): p. e1001589.
291. Exley, M., et al., *CD161 (NKR-P1A) Costimulation of CD1d-dependent Activation of Human T Cells Expressing Invariant Vα24JαQ T Cell Receptor α Chains.* The Journal of Experimental Medicine, 1998. **188**(5): p. 867-876.
292. Joshi, S.K. and M.L. Lang, *Fine tuning a well-oiled machine: Influence of NK1.1 and NKG2D on NKT cell development and function.* International Immunopharmacology, 2013. **17**(2): p. 260-266.
293. Kuylenstierna, C., et al., *NKG2D performs two functions in invariant NKT cells: Direct TCR-independent activation of NK-like cytolysis and co-stimulation of activation by CD1d.* European Journal of Immunology, 2011. **41**(7): p. 1913-1923.

294. Rossjohn, J., et al., *Recognition of CD1d-restricted antigens by natural killer T cells.* Nat Rev Immunol, 2012. **12**(12): p. 845-857.
295. Cerundolo, V., et al., *Harnessing invariant NKT cells in vaccination strategies.* Nat Rev Immunol, 2009. **9**(1): p. 28-38.
296. Venkataswamy, M.M. and S.A. Porcelli, *Lipid and glycolipid antigens of CD1d-restricted natural killer T cells.* Seminars in Immunology, 2010. **22**(2): p. 68-78.
297. Miyamoto, K., S. Miyake, and T. Yamamura, *A synthetic glycolipid prevents autoimmune encephalomyelitis by inducing TH2 bias of natural killer T cells.* Nature, 2001. **413**(6855): p. 531-534.
298. Aspeslagh, S., et al., *Galactose-modified iNKT cell agonists stabilized by an induced fit of CD1d prevent tumour metastasis.* EMBO J, 2011. **30**(11): p. 2294-2305.
299. Yu, K.O.A., et al., *Modulation of CD1d-restricted NKT cell responses by using N-acyl variants of α-galactosylceramides.* Proceedings of the National Academy of Sciences of the United States of America, 2005. **102**(9): p. 3383-3388.
300. Andoh, Y., et al., *Natural killer T cells are required for lipopolysaccharide-mediated enhancement of atherosclerosis in apolipoprotein E-deficient mice.* Immunobiology, 2013. **218**(4): p. 561-569.
301. Kawahara, K., et al., *Structural analysis of two glycosphingolipids from the lipopolysaccharide-lacking bacterium Sphingomonas capsulata.* European Journal of Biochemistry, 2000. **267**(6): p. 1837-1846.
302. Kawahara, K., et al., *Occurrence of an α-galacturonosyl-ceramide in the dioxin-degrading bacterium Sphingomonas wittichii*. Vol. 214. 2002. 289-294.
303. Wu, D., et al., *Design of natural killer T cell activators: Structure and function of a microbial glycosphingolipid bound to mouse CD1d.* Proceedings of the National Academy of Sciences of the United States of America, 2006. **103**(11): p. 3972-3977.
304. Beaudoin, L., et al., *Plasmacytoid dendritic cells license regulatory T cells, upon iNKT-cell stimulation, to prevent autoimmune diabetes.* European Journal of Immunology, 2014. **44**(5): p. 1454-1466.
305. Albacker, L.A., et al., *Invariant natural killer T cells recognize a fungal glycosphingolipid that can induce airway hyperreactivity.* Nat Med, 2013. **19**(10): p. 1297-1304.
306. Kinjo, Y., et al., *Natural killer T cells recognize diacylglycerol antigens from pathogenic bacteria.* Nat Immunol, 2006. **7**(9): p. 978-986.
307. Wang, J., et al., *Lipid binding orientation within CD1d affects recognition of Borrelia burgorferi antigens by NKT cells.* Proceedings of the National Academy of Sciences, 2010. **107**(4): p. 1535-1540.
308. Ito, Y., et al., *<italic>Helicobacter pylori</italic> Cholesteryl α-Glucosides Contribute to Its Pathogenicity and Immune Response by Natural Killer T Cells.* PLoS ONE, 2013. **8**(12): p. e78191.
309. Lotter, H., et al., *Natural Killer T Cells Activated by a Lipopeptidophosphoglycan from <italic>Entamoeba histolytica</italic> Are Critically Important To Control Amebic Liver Abscess.* PLoS Pathog, 2009. **5**(5): p. e1000434.
310. Zhou, D., et al., *Lysosomal Glycosphingolipid Recognition by NKT Cells.* Science, 2004. **306**(5702): p. 1786-1789.
311. Gadola, S.D., et al., *Vα24-JαQ-Independent, CD1d-Restricted Recognition of α-Galactosylceramide by Human CD4+ and CD8αβ+ T Lymphocytes.* The Journal of Immunology, 2002. **168**(11): p. 5514-5520.
312. Schümann, J., et al., *Differential alteration of lipid antigen presentation to NKT cells due to imbalances in lipid metabolism.* European Journal of Immunology, 2007. **37**(6): p. 1431-1441.

313. Sanderson, J.P., et al., *CD1d protein structure determines species-selective antigenicity of isoglobotrihexosylceramide (iGb3) to invariant NKT cells.* European Journal of Immunology, 2013. **43**(3): p. 815-825.
314. Fox, L.M., et al., *Recognition of Lyso-Phospholipids by Human Natural Killer T Lymphocytes.* PLoS Biol, 2009. **7**(10): p. e1000228.
315. Cox, D., et al., *Determination of Cellular Lipids Bound to Human CD1d Molecules.* PLoS ONE, 2009. **4**(5): p. e5325.
316. Panagiotis Kougias, H.C., Peter H. Lin, Alan B. Lumsden, Qizhi Yao, Changyi Chen, *Lysophosphatidylcholine and secretory phospholipase A2 in vascular disease: mediators of endothelial dysfunction and atherosclerosis.* Med Sci Monit, 2006. **12**(1): p. 5-16.
317. López-Sagaseta, J., et al., *The Molecular Basis for Recognition of CD1d/α-Galactosylceramide by a Human Non-Vα24 T Cell Receptor.* PLoS Biol, 2012. **10**(10): p. e1001412.
318. Brennan, P.J., et al., *Invariant natural killer T cells recognize lipid self antigen induced by microbial danger signals.* Nat Immunol, 2011. **12**(12): p. 1202-1211.
319. Oku, H., et al., *Tumor specific cytotoxicity of β-glucosylceramide: structure–cytotoxicity relationship and anti-tumor activity in vivo.* Cancer Chemotherapy and Pharmacology, 2009. **64**(3): p. 485-496.
320. Long, J., et al., *Improvement of HBsAg Gene-Modified Dendritic Cell-Based Vaccine Efficacy by Optimizing Immunization Method or the Application of β-glucosylceramide.* Immunological Investigations, 2013. **42**(2): p. 137-155.
321. Madi El Haj, A.B.Y.a., Gadi Lalazar, Yaron Ilan, *Potential role of NKT regulatory cell ligands for the treatment of immune mediated colitis.* World J Gastroenterol 2007. **13**(44): p. 5799-5804.
322. Brennan, P.J., et al., *Activation of iNKT cells by a distinct constituent of the endogenous glucosylceramide fraction.* Proceedings of the National Academy of Sciences, 2014. **111**(37): p. 13433-13438.
323. Webb, T.J., et al., *Molecular Identification of GD3 as a Suppressor of the Innate Immune Response in Ovarian Cancer.* Cancer Research, 2012. **72**(15): p. 3744-3752.
324. Mallevaey, T. and T. Selvanantham, *Strategy of lipid recognition by invariant natural killer T cells: 'one for all and all for one'.* Immunology, 2012. **136**(3): p. 273-282.
325. Sullivan, B.A., et al., *Mechanisms for Glycolipid Antigen-Driven Cytokine Polarization by Vα14i NKT Cells.* The Journal of Immunology, 2010. **184**(1): p. 141-153.
326. Zeissig, S., et al., *Hepatitis B virus-induced lipid alterations contribute to natural killer T cell-dependent protective immunity.* Nat Med, 2012. **18**(7): p. 1060-1068.
327. Facciotti, F., et al., *Peroxisome-derived lipids are self antigens that stimulate invariant natural killer T cells in the thymus.* Nat Immunol, 2012. **13**(5): p. 474-480.
328. Gumperz, J.E., et al., *Murine CD1d-Restricted T Cell Recognition of Cellular Lipids.* Immunity, 2000. **12**(2): p. 211-221.
329. Lantz, O. and A. Bendelac, *An invariant T cell receptor alpha chain is used by a unique subset of major histocompatibility complex class I-specific CD4+ and CD4-8- T cells in mice and humans.* The Journal of Experimental Medicine, 1994. **180**(3): p. 1097-1106.
330. Matsuda, J.L., et al., *Natural killer T cells reactive to a single glycolipid exhibit a highly diverse T cell receptor β repertoire and small clone size.* Proceedings of the National Academy of Sciences, 2001. **98**(22): p. 12636-12641.
331. Exley, M., et al., *Requirements for CD1d Recognition by Human Invariant Vα24+ CD4−CD8− T Cells.* The Journal of Experimental Medicine, 1997. **186**(1): p. 109-120.
332. van Schaik, B., et al., *Discovery of Invariant T Cells by Next-Generation Sequencing of the Human TCR α-Chain Repertoire.* The Journal of Immunology, 2014. **193**(10): p. 5338-5344.

333. Patel, O., et al., *Vβ2 natural killer T cell antigen receptor-mediated recognition of CD1d-glycolipid antigen.* Proceedings of the National Academy of Sciences, 2011. **108**(47): p. 19007-19012.
334. Joyce, S., E. Girardi, and D.M. Zajonc, *NKT Cell Ligand Recognition Logic: Molecular Basis for a Synaptic Duet and Transmission of Inflammatory Effectors.* The Journal of Immunology, 2011. **187**(3): p. 1081-1089.
335. Mallevaey, T., et al., *A Molecular Basis for NKT Cell Recognition of CD1d-Self-Antigen.* Immunity, 2011. **34**(3): p. 315-326.
336. Mallevaey, T., et al., *T Cell Receptor CDR2β and CDR3β Loops Collaborate Functionally to Shape the iNKT Cell Repertoire.* Immunity, 2009. **31**(1): p. 60-71.
337. Matulis, G., et al., *Innate-Like Control of Human iNKT Cell Autoreactivity via the Hypervariable CDR3β Loop.* PLoS Biol, 2010. **8**(6): p. e1000402.
338. Pellicci, D.G., et al., *Recognition of [beta]-linked self glycolipids mediated by natural killer T cell antigen receptors.* Nat Immunol, 2011. **12**(9): p. 827-833.
339. Florence, W.C., et al., *Adaptability of the semi-invariant natural killer T-cell receptor towards structurally diverse CD1d-restricted ligands*. Vol. 28. 2009. 3579-3590.
340. Koch, M., et al., *The crystal structure of human CD1d with and without [alpha]-galactosylceramide.* Nat Immunol, 2005. **6**(8): p. 819-826.
341. Zajonc, D.M., et al., *Structure and function of a potent agonist for the semi-invariant natural killer T cell receptor.* Nat Immunol, 2005. **6**(8): p. 810-818.
342. Zajonc, D.M., et al., *Crystal Structures of Mouse CD1d-iGb3 Complex and its Cognate Vα14 T Cell Receptor Suggest a Model for Dual Recognition of Foreign and Self Glycolipids.* Journal of Molecular Biology, 2008. **377**(4): p. 1104-1116.
343. Yu, E.D., et al., *Cutting Edge: Structural Basis for the Recognition of β-Linked Glycolipid Antigens by Invariant NKT Cells.* The Journal of Immunology, 2011. **187**(5): p. 2079-2083.
344. Corse, E., R.A. Gottschalk, and J.P. Allison, *Strength of TCR–Peptide/MHC Interactions and In Vivo T Cell Responses.* The Journal of Immunology, 2011. **186**(9): p. 5039-5045.
345. Wun, Kwok S., et al., *A Molecular Basis for the Exquisite CD1d-Restricted Antigen Specificity and Functional Responses of Natural Killer T Cells.* Immunity, 2011. **34**(3): p. 327-339.
346. Stetson, D.B., et al., *Constitutive Cytokine mRNAs Mark Natural Killer (NK) and NK T Cells Poised for Rapid Effector Function.* The Journal of Experimental Medicine, 2003. **198**(7): p. 1069-1076.
347. Lee, P.T., et al., *Distinct Functional Lineages of Human Vα24 Natural Killer T Cells.* The Journal of Experimental Medicine, 2002. **195**(5): p. 637-641.
348. Crowe, N.Y., et al., *Differential antitumor immunity mediated by NKT cell subsets in vivo.* The Journal of Experimental Medicine, 2005. **202**(9): p. 1279-1288.
349. Osada, T., et al., *Ex vivo expanded human CD4+ regulatory NKT cells suppress expansion of tumor antigen-specific CTLs.* International Immunology, 2005. **17**(9): p. 1143-1155.
350. Takahashi, T., et al., *Cutting Edge: Analysis of Human Vα24+CD8+ NK T Cells Activated by α-Galactosylceramide-Pulsed Monocyte-Derived Dendritic Cells.* The Journal of Immunology, 2002. **168**(7): p. 3140-3144.
351. Doisne, J.-M., et al., *Skin and Peripheral Lymph Node Invariant NKT Cells Are Mainly Retinoic Acid Receptor-Related Orphan Receptor γt+ and Respond Preferentially under Inflammatory Conditions.* The Journal of Immunology, 2009. **183**(3): p. 2142-2149.
352. Lee, Y.J., et al., *Steady-state production of IL-4 modulates immunity in mouse strains and is determined by lineage diversity of iNKT cells.* Nat Immunol, 2013. **14**(11): p. 1146-1154.

353. Michel, M.-L., et al., *Critical role of ROR-γt in a new thymic pathway leading to IL-17-producing invariant NKT cell differentiation.* Proceedings of the National Academy of Sciences, 2008. **105**(50): p. 19845-19850.
354. Watarai, H., et al., *Development and Function of Invariant Natural Killer T Cells Producing T_H2- and T_H17-Cytokines.* PLoS Biol, 2012. **10**(2): p. e1001255.
355. Gleimer, M., H. Von Boehmer, and T. Kreslavsky, *PLZF controls the expression of a limited number of genes essential for NKT cell function.* Frontiers in Immunology, 2012. **3**.
356. Sag, D., et al., *IL-10–producing NKT10 cells are a distinct regulatory invariant NKT cell subset.* The Journal of Clinical Investigation, 2014. **124**(9): p. 3725-3740.
357. Sag, D., et al., *IL-10–producing NKT10 cells are a distinct regulatory invariant NKT cell subset.* The Journal of Clinical Investigation, 2014. **124**(9): p. 0-0.
358. Rampuria, P. and M.L. Lang, *CD1d-dependent expansion of NKT follicular helper cells in vivo and in vitro is a product of cellular proliferation and differentiation.* International Immunology, 2015. **27**(5): p. 253-263.
359. Lee, You J., et al., *Tissue-Specific Distribution of iNKT Cells Impacts Their Cytokine Response.* Immunity, 2015. **43**(3): p. 566-578.
360. Venken, K., et al., *Bacterial CD1d–Restricted Glycolipids Induce IL-10 Production by Human Regulatory T Cells upon Cross-Talk with Invariant NKT Cells.* The Journal of Immunology, 2013. **191**(5): p. 2174-2183.
361. Arora, P., et al., *A Single Subset of Dendritic Cells Controls the Cytokine Bias of Natural Killer T Cell Responses to Diverse Glycolipid Antigens.* Immunity, 2014. **40**(1): p. 105-116.
362. DeLyria, E.S., et al., *Sublingual injection of microparticles containing glycolipid ligands for NKT cells and subunit vaccines induces antibody responses in oral cavity.* Carbohydrate Research, 2015. **405**: p. 87-92.
363. Blair, P.A., et al., *CD19+CD24hiCD38hi B Cells Exhibit Regulatory Capacity in Healthy Individuals but Are Functionally Impaired in Systemic Lupus Erythematosus Patients.* Immunity, 2010. **32**(1): p. 129-140.
364. Ikonen, E., *Cellular cholesterol trafficking and compartmentalization.* Nat Rev Mol Cell Biol, 2008. **9**(2): p. 125-138.
365. Jury, E.C., F. Flores-Borja, and P.S. Kabouridis, *Lipid rafts in T cell signalling and disease.* Seminars in Cell & Developmental Biology, 2007. **18**(5): p. 608-615.
366. Robinson, J.M. and M.J. Karnovsky, *Evaluation of the polyene antibiotic filipin as a cytochemical probe for membrane cholesterol.* Journal of Histochemistry & Cytochemistry, 1980. **28**(2): p. 161-8.
367. Jury, E.C., et al., *Altered lipid raft–associated signaling and ganglioside expression in T lymphocytes from patients with systemic lupus erythematosus.* The Journal of Clinical Investigation, 2004. **113**(8): p. 1176-1187.
368. Flores-Borja F, K.P., Jury EC, Isenberg DA, Mageed RA., *Decreased Lyn expression and translocation to lipid raft signaling domains in B lymphocytes from patients with systemic lupus erythematosus.* Arthritis Rheum., 2005. **52**(12): p. 3955-65.
369. Jury, E.C., et al., *Increased ubiquitination and reduced expression of LCK in T lymphocytes from patients with systemic lupus erythematosus.* Arthritis & Rheumatism, 2003. **48**(5): p. 1343-1354.
370. Moulton, V. and G. Tsokos, *Abnormalities of T cell signaling in systemic lupus erythematosus.* Arthritis Research & Therapy, 2011. **13**(2): p. 207.
371. Simons, K. and D. Toomre, *Lipid rafts and signal transduction.* Nat Rev Mol Cell Biol, 2000. **1**(1): p. 31-39.

372. Janes, P.W., et al., *The role of lipid rafts in T cell antigen receptor (TCR) signalling.* Seminars in Immunology, 2000. **12**(1): p. 23-34.
373. Jury, E.C., et al., *Atorvastatin Restores Lck Expression and Lipid Raft-Associated Signaling in T Cells from Patients with Systemic Lupus Erythematosus.* The Journal of Immunology, 2006. **177**(10): p. 7416-7422.
374. Waddington, Kirsty E., Elizabeth C. Jury, and I. Pineda-Torra, *Liver X receptors in immune cell function in humans.* Biochemical Society Transactions, 2015. **43**(4): p. 752-757.
375. Kabouridis, P.S. and E.C. Jury, *Lipid rafts and T-lymphocyte function: Implications for autoimmunity.* FEBS Letters, 2008. **582**(27): p. 3711-3718.
376. Lang, G.A., et al., *Presentation of α-galactosylceramide by murine CD1d to natural killer T cells is facilitated by plasma membrane glycolipid rafts.* Immunology, 2004. **112**(3): p. 386-396.
377. Im, J.S., et al., *Kinetics and Cellular Site of Glycolipid Loading Control the Outcome of Natural Killer T Cell Activation.* Immunity, 2009. **30**(6): p. 888-898.
378. Lalazar, G., et al., *β-Glycoglycosphingolipid-Induced Alterations of the STAT Signaling Pathways Are Dependent on CD1d and the Lipid Raft Protein Flotillin-2.* The American Journal of Pathology, 2009. **174**(4): p. 1390-1399.
379. Speak, A.O., et al., *Invariant natural killer T cells are not affected by lysosomal storage in patients with Niemann-Pick disease type C.* European Journal of Immunology, 2012. **42**(7): p. 1886-1892.
380. McDonald, G., et al., *Normalizing glycosphingolipids restores function in CD4+ T cells from lupus patients.* The Journal of Clinical Investigation, 2014. **124**(2): p. 712-724.
381. Kathryn, J.M., et al., *The role of PPAR-γ in macrophage differentiation and cholesterol uptake.* Nature Medicine, 2001. **7**(1): p. 41-47.
382. Nakken, B., et al., *Peroxisome Proliferator-Activated Receptor γ-Regulated Cathepsin D Is Required for Lipid Antigen Presentation by Dendritic Cells.* The Journal of Immunology, 2011. **187**(1): p. 240-247.
383. Szatmari, I., et al., *PPARγ controls CD1d expression by turning on retinoic acid synthesis in developing human dendritic cells.* The Journal of Experimental Medicine, 2006. **203**(10): p. 2351-2362.
384. Huh, J.Y., et al., *A Novel Function of Adipocytes in Lipid Antigen Presentation to iNKT Cells.* Molecular and Cellular Biology, 2013. **33**(2): p. 328-339.
385. Giulia, C., et al., *PPAR-α and PPAR-γ activators induce cholesterol removal from human macrophage foam cells through stimulation of the ABCA1 pathway.* Nature Medicine, 2001. **7**(1): p. 53-58.
386. David, M.M. and P.E. Justin, *Exploring the full spectrum of macrophage activation.* Nature Reviews Immunology, 2008. **8**(12): p. 958-969.
387. Yu, S. and M.T. Cantorna, *The vitamin D receptor is required for iNKT cell development.* Proceedings of the National Academy of Sciences, 2008. **105**(13): p. 5207-5212.
388. van den Heuvel, M.J., et al., *NKT cell costimulation: experimental progress and therapeutic promise.* Trends in Molecular Medicine, 2011. **17**(2): p. 65-77.
389. Williams, J.A., et al., *Regulation of Thymic NKT Cell Development by the B7-CD28 Costimulatory Pathway.* The Journal of Immunology, 2008. **181**(2): p. 907-917.
390. Chung, Y., et al., *A Critical Role of Costimulation during Intrathymic Development of Invariant NK T Cells.* The Journal of Immunology, 2008. **180**(4): p. 2276-2283.
391. Wang, J., et al., *Cutting Edge: CD28 Engagement Releases Antigen-Activated Invariant NKT Cells from the Inhibitory Effects of PD-1.* The Journal of Immunology, 2009. **182**(11): p. 6644-6647.

392. Uldrich, A.P., et al., *NKT Cell Stimulation with Glycolipid Antigen In Vivo: Costimulation-Dependent Expansion, Bim-Dependent Contraction, and Hyporesponsiveness to Further Antigenic Challenge.* The Journal of Immunology, 2005. **175**(5): p. 3092-3101.
393. Akbari, O., et al., *ICOS/ICOSL Interaction Is Required for CD4+ Invariant NKT Cell Function and Homeostatic Survival.* The Journal of Immunology, 2008. **180**(8): p. 5448-5456.
394. Kaneda, H., et al., *ICOS costimulates invariant NKT cell activation.* Biochemical and Biophysical Research Communications, 2005. **327**(1): p. 201-207.
395. Tomura, M., et al., *A Novel Function of Vα14+CD4+NKT Cells: Stimulation of IL-12 Production by Antigen-Presenting Cells in the Innate Immune System.* The Journal of Immunology, 1999. **163**(1): p. 93-101.
396. Tonti, E., et al., *NKT-cell help to B lymphocytes can occur independently of cognate interaction*. Vol. 113. 2009. 370-376.
397. Kitamura, H., et al., *The Natural Killer T (NKT) Cell Ligand α-Galactosylceramide Demonstrates Its Immunopotentiating Effect by Inducing Interleukin (IL)-12 Production by Dendritic Cells and IL-12 Receptor Expression on NKT Cells.* The Journal of Experimental Medicine, 1999. **189**(7): p. 1121-1128.
398. Pál, E., et al., *Costimulation-Dependent Modulation of Experimental Autoimmune Encephalomyelitis by Ligand Stimulation of Vα14 NK T Cells.* The Journal of Immunology, 2001. **166**(1): p. 662-668.
399. Raifer, H., et al., *Unlike αβ T cells, γδ T cells, LTi cells and NKT cells do not require IRF4 for the production of IL-17A and IL-22.* European Journal of Immunology, 2012. **42**(12): p. 3189-3201.
400. Wei, J., K. Yang, and H. Chi, *Cutting Edge: Discrete Functions of mTOR Signaling in Invariant NKT Cell Development and NKT17 Fate Decision.* The Journal of Immunology, 2014. **193**(9): p. 4297-4301.
401. Prevot, N., et al., *Mammalian Target of Rapamycin Complex 2 Regulates Invariant NKT Cell Development and Function Independent of Promyelocytic Leukemia Zinc-Finger.* The Journal of Immunology, 2015. **194**(1): p. 223-230.
402. Stuart, J.K., et al., *Negative modulation of invariant natural killer T cell responses to glycolipid antigens by p38 MAP kinase.* International Immunopharmacology, 2010. **10**(9): p. 1068-1076.
403. Tian, J., et al., *The Differential Roles of mTOR, ERK, and JNK Pathways in Invariant Natural Killer T-cell Function and Survival.* Inflammation, 2014. **37**(6): p. 2013-2019.
404. Veenbergen, S., et al., *A pivotal role for antigen-presenting cells overexpressing SOCS3 in controlling invariant NKT cell responses during collagen-induced arthritis.* Annals of the Rheumatic Diseases, 2011. **70**(12): p. 2167-2175.
405. Singh, N., et al., *Cutting Edge: Activation of NK T Cells by CD1d and α-Galactosylceramide Directs Conventional T Cells to the Acquisition of a Th2 Phenotype.* The Journal of Immunology, 1999. **163**(5): p. 2373-2377.
406. Lisbonne, M., et al., *Cutting Edge: Invariant Vα14 NKT Cells Are Required for Allergen-Induced Airway Inflammation and Hyperreactivity in an Experimental Asthma Model.* The Journal of Immunology, 2003. **171**(4): p. 1637-1641.
407. Galli, G., et al., *CD1d-restricted Help To B Cells By Human Invariant Natural Killer T Lymphocytes.* The Journal of Experimental Medicine, 2003. **197**(8): p. 1051-1057.
408. Chaudhry, M.S. and A. Karadimitris, *Role and Regulation of CD1d in Normal and Pathological B Cells.* The Journal of Immunology, 2014. **193**(10): p. 4761-4768.
409. Mars, L.T., et al., *Therapeutic manipulation of iNKT cells in autoimmunity: modes of action and potential risks.* Trends in Immunology, 2004. **25**(9): p. 471-476.

410. Lehuen, A., et al., *Overexpression of Natural Killer T Cells Protects Vα14-Jα281 Transgenic Nonobese Diabetic Mice against Diabetes.* The Journal of Experimental Medicine, 1998. **188**(10): p. 1831-1839.
411. Beaudoin, L., et al., *NKT Cells Inhibit the Onset of Diabetes by Impairing the Development of Pathogenic T Cells Specific for Pancreatic β Cells.* Immunity, 2002. **17**(6): p. 725-736.
412. Chen, Y.-G., et al., *Activated NKT Cells Inhibit Autoimmune Diabetes through Tolerogenic Recruitment of Dendritic Cells to Pancreatic Lymph Nodes.* The Journal of Immunology, 2005. **174**(3): p. 1196-1204.
413. Martin-Murphy, B.V., et al., *Mice Lacking Natural Killer T Cells Are More Susceptible to Metabolic Alterations following High Fat Diet Feeding.* PLoS ONE, 2014. **9**(1): p. e80949.
414. Monteiro, M., et al., *Identification of Regulatory Foxp3+ Invariant NKT Cells Induced by TGF-β.* The Journal of Immunology, 2010. **185**(4): p. 2157-2163.
415. Engelmann, P., et al., *Characterization of human invariant natural killer T cells expressing FoxP3.* International Immunology, 2011. **23**(8): p. 473-484.
416. Huijts, C.M., et al., *mTOR Inhibition Per Se Induces Nuclear Localization of FOXP3 and Conversion of Invariant NKT (iNKT) Cells into Immunosuppressive Regulatory iNKT Cells.* The Journal of Immunology, 2015. **195**(5): p. 2038-2045.
417. Baselga, J., et al., *Everolimus in Postmenopausal Hormone-Receptor–Positive Advanced Breast Cancer.* New England Journal of Medicine, 2012. **366**(6): p. 520-529.
418. Molina, A., M. Ginsberg, and R. Motzer, *Long-term response with everolimus for metastatic renal cell carcinoma refractory to sunitinib.* Medical Oncology, 2011. **28**(4): p. 1527-1529.
419. Strauss, L., et al., *Differential Responses of Human Regulatory T Cells (Treg) and Effector T Cells to Rapamycin.* PLoS ONE, 2009. **4**(6): p. e5994.
420. Diana, J., et al., *Viral infection prevents diabetes by inducing regulatory T cells through NKT cell–plasmacytoid dendritic cell interplay.* The Journal of Experimental Medicine, 2011. **208**(4): p. 729-745.
421. Schneidawind, D., et al., *CD4+ invariant natural killer T cells protect from murine GVHD lethality through expansion of donor CD4+CD25+FoxP3+ regulatory T cells.* Blood, 2014. **124**(22): p. 3320-3328.
422. Pillai, A.B., et al., *Host natural killer T cells induce an interleukin-4–dependent expansion of donor CD4+CD25+Foxp3+ T regulatory cells that protects against graft-versus-host disease.* Blood, 2009. **113**(18): p. 4458-4467.
423. BAKER, J.F., et al., *Resistin Levels in Lupus and Associations with Disease-specific Measures, Insulin Resistance, and Coronary Calcification.* The Journal of Rheumatology, 2011. **38**(11): p. 2369-2375.
424. Chen, W., et al., *Conversion of Peripheral CD4+CD25− Naive T Cells to CD4+CD25+ Regulatory T Cells by TGF-β Induction of Transcription Factor Foxp3.* The Journal of Experimental Medicine, 2003. **198**(12): p. 1875-1886.
425. Sawitzki, B., et al., *IFN-γ production by alloantigen-reactive regulatory T cells is important for their regulatory function in vivo.* The Journal of Experimental Medicine, 2005. **201**(12): p. 1925-1935.
426. Hua, J., et al., *The Interaction between Regulatory T Cells and NKT Cells in the Liver: A CD1d Bridge Links Innate and Adaptive Immunity.* PLoS ONE, 2011. **6**(11): p. e27038.
427. Azuma, T., et al., *Human CD4+ CD25+ Regulatory T Cells Suppress NKT Cell Functions.* Cancer Research, 2003. **63**(15): p. 4516-4520.
428. Bailey, J.C., et al., *Inhibition of CD1d-mediated antigen presentation by the transforming growth factor-β/Smad signalling pathway.* Immunology, 2014. **143**(4): p. 679-691.

429. Parekh, V.V., et al., *Activated Invariant NKT Cells Control Central Nervous System Autoimmunity in a Mechanism That Involves Myeloid-Derived Suppressor Cells.* The Journal of Immunology, 2013. **190**(5): p. 1948-1960.
430. De Santo, C., et al., *Invariant NKT cells reduce the immunosuppressive activity of influenza A virus–induced myeloid-derived suppressor cells in mice and humans.* The Journal of Clinical Investigation, 2008. **118**(12): p. 4036-4048.
431. Kmieciak, M., et al., *Activated NKT Cells and NK Cells Render T Cells Resistant to Myeloid-Derived Suppressor Cells and Result in an Effective Adoptive Cellular Therapy against Breast Cancer in the FVBN202 Transgenic Mouse.* The Journal of Immunology, 2011. **187**(2): p. 708-717.
432. Liu, W., et al., *Cross-Regulation of T Regulatory—Cell Response after Coxsackievirus B3 Infection by NKT and γδ T Cells in the Mouse.* The American Journal of Pathology, 2013. **183**(2): p. 441-449.
433. Denney, L., et al., *Activation of Invariant NKT Cells in Early Phase of Experimental Autoimmune Encephalomyelitis Results in Differentiation of Ly6Chi Inflammatory Monocyte to M2 Macrophages and Improved Outcome.* The Journal of Immunology, 2012. **189**(2): p. 551-557.
434. Chuang, Y.-P., et al., *Modulatory Function of Invariant Natural Killer T Cells in Systemic Lupus Erythematosus.* Clinical and Developmental Immunology, 2012. **2012**: p. 8.
435. Sumida, T., *TCR AV24 gene expression in double negative T cells in systemic lupus erythematosus [1].* Lupus, 1998. **7**(8): p. 565-568.
436. Cho, Y.-N., et al., *Numerical and functional deficiencies of natural killer T cells in systemic lupus erythematosus: their deficiency related to disease activity.* Rheumatology, 2011. **50**(6): p. 1054-1063.
437. Takahashi, T., et al., *Cutting edge: Analysis of human Vα24 +CD8 + NKT cells activated by α-galactosylceramide-pulsed monocyte-derived dendritic cells.* Journal of Immunology, 2002. **168**(7): p. 3140-3144.
438. Mitsuo, A., et al., *Decreased CD161 +CD8 + T cells in the peripheral blood of patients suffering from rheumatic diseases.* Rheumatology, 2006. **45**(12): p. 1477-1484.
439. Green, M.R.J., et al., *Natural killer T cells in families of patients with systemic lupus erythematosus: Their possible role in regulation of IgG production.* Arthritis and Rheumatism, 2007. **56**(1): p. 303-310.
440. Wither, J., et al., *Reduced proportions of natural killer T cells are present in the relatives of lupus patients and are associated with autoimmunity.* Arthritis Research and Therapy, 2008. **10**(5).
441. Gately, C.M., et al., *Invariant Natural Killer T-cell anergy to endogenous myelin acetyl-glycolipids in multiple sclerosis.* Journal of Neuroimmunology, 2013. **259**(1–2): p. 1-7.
442. Morshed, S.R., et al., *β-galactosylceramide alters invariant natural killer T cell function and is effective treatment for lupus.* Clinical Immunology, 2009. **132**(3): p. 321-333.
443. Ortaldo, J.R., et al., *Dissociation of NKT Stimulation, Cytokine Induction, and NK Activation In Vivo by the Use of Distinct TCR-Binding Ceramides.* The Journal of Immunology, 2004. **172**(2): p. 943-953.
444. Smyth, M.J., et al., *Differential Tumor Surveillance by Natural Killer (Nk) and Nkt Cells.* The Journal of Experimental Medicine, 2000. **191**(4): p. 661-668.
445. Zhou, G., et al., *Identification of systemically expanded activated T cell clones in MRL/lpr and NZB/W F1 lupus model mice.* Clinical & Experimental Immunology, 2004. **136**(3): p. 448-455.
446. Singh, A.K., et al., *The natural killer T cell ligand α-galactosylceramide prevents or promotes pristane-induced lupus in mice.* European Journal of Immunology, 2005. **35**(4): p. 1143-1154.

447. Yang, J.-Q., et al., *CD1d deficiency exacerbates inflammatory dermatitis in MRL-lpr/lpr mice.* European Journal of Immunology, 2004. **34**(6): p. 1723-1732.
448. Forestier, C., et al., *Expansion and Hyperactivity of CD1d-Restricted NKT Cells during the Progression of Systemic Lupus Erythematosus in (New Zealand Black × New Zealand White)F1 Mice.* The Journal of Immunology, 2005. **175**(2): p. 763-770.
449. Yang, J.Q., et al., *Intrinsic hyporesponsiveness of invariant natural killer T cells precedes the onset of lupus.* Clinical & Experimental Immunology, 2013. **173**(1): p. 18-27.
450. Zeng, D., et al., *Activation of natural killer T cells in NZB/W mice induces Th1-type immune responses exacerbating lupus.* The Journal of Clinical Investigation, 2003. **112**(8): p. 1211-1222.
451. Takahashi, T. and S. Strober, *Natural killer T cells and innate immune B cells from lupus-prone NZB/W mice interact to generate IgM and IgG autoantibodies.* European Journal of Immunology, 2008. **38**(1): p. 156-165.
452. Wermeling, F., et al., *Invariant NKT cells limit activation of autoreactive CD1d-positive B cells.* The Journal of Experimental Medicine, 2010. **207**(5): p. 943-952.
453. Yang, J.-Q., et al., *Invariant NKT Cells Inhibit Autoreactive B Cells in a Contact- and CD1d-Dependent Manner.* The Journal of Immunology, 2011. **186**(3): p. 1512-1520.
454. Tan, Andy H.-M., et al., *Aberrant Presentation of Self-Lipids by Autoimmune B Cells Depletes Peripheral iNKT Cells.* Cell Reports, 2014. **9**(1): p. 24-31.
455. Darmoise, A., et al., *Lysosomal α-Galactosidase Controls the Generation of Self Lipid Antigens for Natural Killer T Cells.* Immunity, 2010. **33**(2): p. 216-228.
456. Melián, A., et al., *CD1 Expression in Human Atherosclerosis: A Potential Mechanism for T Cell Activation by Foam Cells.* The American Journal of Pathology, 1999. **155**(3): p. 775-786.
457. Bobryshev, Y.V. and R.S.A. Lord, *Co-accumulation of Dendritic Cells and Natural Killer T Cells within Rupture-prone Regions in Human Atherosclerotic Plaques.* Journal of Histochemistry & Cytochemistry, 2005. **53**(6): p. 781-785.
458. Kyriakakis, E., et al., *Invariant natural killer T cells: Linking inflammation and neovascularization in human atherosclerosis.* European Journal of Immunology, 2010. **40**(11): p. 3268-3279.
459. Nakai, Y., et al., *Natural killer T cells accelerate atherogenesis in mice.* Blood, 2004. **104**(7): p. 2051-2059.
460. Tupin, E., et al., *CD1d-dependent Activation of NKT Cells Aggravates Atherosclerosis.* The Journal of Experimental Medicine, 2004. **199**(3): p. 417-422.
461. Major, A.S., et al., *Quantitative and Qualitative Differences in Proatherogenic NKT Cells in Apolipoprotein E–Deficient Mice.* Arteriosclerosis, Thrombosis, and Vascular Biology, 2004. **24**(12): p. 2351-2357.
462. VanderLaan, P.A., et al., *Characterization of the Natural Killer T-Cell Response in an Adoptive Transfer Model of Atherosclerosis.* The American Journal of Pathology, 2007. **170**(3): p. 1100-1107.
463. Rogers, L., et al., *Deficiency of invariant Vα14 natural killer T cells decreases atherosclerosis in LDL receptor null mice.* Cardiovascular Research, 2008. **78**(1): p. 167-174.
464. Subramanian, S., et al., *Increased levels of invariant natural killer T lymphocytes worsens metabolic abnormalities and atherosclerosis in obese mice.* Journal of Lipid Research, 2013.
465. To, K., et al., *NKT Cell Subsets Mediate Differential Proatherogenic Effects in ApoE−/− Mice.* Arteriosclerosis, Thrombosis, and Vascular Biology, 2009. **29**(5): p. 671-677.
466. Novak, J., et al., *Potential role of invariant natural killer T cells in outcomes of acute myocardial infarction.* International Journal of Cardiology, 2015. **187**: p. 663-665.

467. Aslanian, A.M., H.A. Chapman, and I.F. Charo, *Transient Role for CD1d-Restricted Natural Killer T Cells in the Formation of Atherosclerotic Lesions.* Arteriosclerosis, Thrombosis, and Vascular Biology, 2005. **25**(3): p. 628-632.
468. van Puijvelde, G.H., et al., *Effect of natural killer T cell activation on the initiation of atherosclerosis.* Thrombosis and haemostasis, 2009. **102**(2): p. 223-230.
469. Braun, N.A., R. Covarrubias, and A.S. Major, *Natural Killer T Cells and Atherosclerosis: Form and Function Meet Pathogenesis.* Journal of Innate Immunity, 2010. **2**(4): p. 316-324.
470. Ström, Å., et al., *Involvement of the CD1d–Natural Killer T Cell Pathway in Neointima Formation After Vascular Injury.* Circulation Research, 2007. **101**(8): p. e83-e89.
471. Maldonado, N., et al., *A mechanistic analysis of the role of microcalcifications in atherosclerotic plaque stability: potential implications for plaque rupture.* American Journal of Physiology - Heart and Circulatory Physiology, 2012. **303**(5): p. H619-H628.
472. Belcaro G, B.A., Nicolaides AN., *"Ultrasonic biopsy"--a non-invasive screening technique to evaluate the cardiovascular risk and to follow up the progression and the regression of arteriosclerosis.* Vasa, 1991. **20**(1): p. 40-50.
473. Elatrozy T, N.A., Tegos T, Zarka AZ, Griffin M, Sabetai M., *The effect of B-mode ultrasonic image standardisation on the echodensity of symptomatic and asymptomatic carotid bifurcation plaques.* International Angiology, 1998. **17**(3): p. 179-86.
474. Biasi, G.M., et al., *Computer Analysis of Ultrasonic Plaque Echolucency in Identifying High Risk Carotid Bifurcation Lesions.* European Journal of Vascular and Endovascular Surgery, 1999. **17**(6): p. 476-479.
475. el-Barghouty N, N.A., Bahal V, Geroulakos G, Androulakis A., *The identification of the high risk carotid plaque.* Eur J Vasc Endovasc Surg., 1996. **11**(4): p. 470-8.
476. Lind, L., et al., *The echogenecity of the intima–media complex in the common carotid artery is closely related to the echogenecity in plaques.* Atherosclerosis, 2007. **195**(2): p. 411-414.
477. Salem, M.K., et al., *Identification of Patients with a Histologically Unstable Carotid Plaque Using Ultrasonic Plaque Image Analysis.* European Journal of Vascular and Endovascular Surgery, 2014. **48**(2): p. 118-125.
478. Denzel, C., et al., *Carotid Artery Calcium: Accuracy of a Calcium Score by Computed Tomography—An In vitro Study with Comparison to Sonography and Histology.* European Journal of Vascular and Endovascular Surgery, 2004. **28**(2): p. 214-220.
479. Markussis, V., et al., *Originally published as Volume 2, Issue 8829Detection of premature atherosclerosis by high-resolution ultrasonography in symptom-free hypopituitary adults.* The Lancet, 1992. **340**(8829): p. 1188-1192.
480. Belcaro, G., et al., *Ultrasound Morphology Classification of the Arterial Wall and Cardiovascular Events in a 6-Year Follow-up Study.* Arteriosclerosis, Thrombosis, and Vascular Biology, 1996. **16**(7): p. 851-856.
481. Stary, H.C., et al., *A Definition of Advanced Types of Atherosclerotic Lesions and a Histological Classification of Atherosclerosis: A Report From the Committee on Vascular Lesions of the Council on Arteriosclerosis, American Heart Association.* Arteriosclerosis, Thrombosis, and Vascular Biology, 1995. **15**(9): p. 1512-1531.
482. Reis, E.G., et al., *NK and NKT cell dynamics after rituximab therapy for systemic lupus erythematosus and rheumatoid arthritis.* Rheumatology International, 2009. **29**(4): p. 469-475.
483. Miguel, L., et al., *Primary Human CD4+ T Cells Have Diverse Levels of Membrane Lipid Order That Correlate with Their Function.* The Journal of Immunology, 2011. **186**(6): p. 3505-3516.

484. Carlson, L.A., *Extraction of lipids from human whole serum and lipoproteins and from rat liver tissue with methylene chloride-methanol: a comparison with extraction with chloroform: methanol.* Clinica Chimica Acta, 1985. **149**.
485. Untergasser, A., et al., *Primer3—new capabilities and interfaces.* Nucleic Acids Research, 2012. **40**(15): p. e115.
486. Koressaar, T. and M. Remm, *Enhancements and modifications of primer design program Primer3.* Bioinformatics, 2007. **23**(10): p. 1289-1291.
487. Hoefer, I.E., et al., *Novel methodologies for biomarker discovery in atherosclerosis.* European Heart Journal, 2015. **36**(39): p. 2635-2642.
488. Wigren, M., J. Nilsson, and M.J. Kaplan, *Pathogenic immunity in systemic lupus erythematosus and atherosclerosis: common mechanisms and possible targets for intervention.* Journal of Internal Medicine, 2015. **278**(5): p. 494-506.
489. DelaRosa, O., et al., *Vα24+ NKT cells are decreased in elderly humans.* Experimental Gerontology, 2002. **37**(2–3): p. 213-217.
490. Fergusson, J.R., et al., *CD161 defines a transcriptional and functional phenotype across distinct human T cell lineages.* Cell Rep, 2014. **9**(3): p. 1075-88.
491. Pesenacker, A.M., et al., *CD161 defines the subset of FoxP3+ T cells capable of producing proinflammatory cytokines.* Blood, 2013. **121**(14): p. 2647-58.
492. Annunziato, F., et al., *Main features of human T helper 17 cells.* Ann N Y Acad Sci, 2013. **1284**: p. 66-70.
493. Sztajzel, R., et al., *Correlation of Cerebrovascular Symptoms and Microembolic Signals With the Stratified Gray-Scale Median Analysis and Color Mapping of the Carotid Plaque.* Stroke, 2006. **37**(3): p. 824-829.
494. Nakou, E., et al., *Statin-Induced Immunomodulation Alters Peripheral Invariant Natural Killer T-cell Prevalence in Hyperlipidemic Patients.* Cardiovascular Drugs and Therapy, 2012. **26**(4): p. 293-299.
495. Braun, N.A., et al., *Development of Spontaneous Anergy in Invariant Natural Killer T Cells in a Mouse Model of Dyslipidemia.* Arteriosclerosis, Thrombosis, and Vascular Biology, 2010. **30**(9): p. 1758-1765.
496. Järvisalo, M.J., et al., *Increased Aortic Intima-Media Thickness: A Marker of Preclinical Atherosclerosis in High-Risk Children.* Circulation, 2001. **104**(24): p. 2943-2947.
497. Fadini, G.P., et al., *Pro-inflammatory monocyte-macrophage polarization imbalance in human hypercholesterolemia and atherosclerosis.* Atherosclerosis, 2014. **237**(2): p. 805-808.
498. Martin, S.S., et al., *Dyslipidemia, Coronary Artery Calcium, and Incident Atherosclerotic Cardiovascular Disease: Implications for Statin Therapy From the Multi-Ethnic Study of Atherosclerosis.* Circulation, 2014. **129**(1): p. 77-86.
499. Kettunen, J., et al., *Genome-wide association study identifies multiple loci influencing human serum metabolite levels.* Nat Genet, 2012. **44**(3): p. 269-276.
500. Brochériou, I., et al., *Antagonistic regulation of macrophage phenotype by M-CSF and GM-CSF: Implication in atherosclerosis.* Atherosclerosis, 2011. **214**(2): p. 316-324.
501. Ambarus, C.A., et al., *Systematic validation of specific phenotypic markers for in vitro polarized human macrophages.* Journal of Immunological Methods, 2012. **375**(1–2): p. 196-206.
502. Roma-Lavisse, C., et al., *M1 and M2 macrophage proteolytic and angiogenic profile analysis in atherosclerotic patients reveals a distinctive profile in type 2 diabetes.* Diabetes and Vascular Disease Research, 2015. **12**(4): p. 279-289.
503. Boß, M., et al., *FABP4 inhibition suppresses PPARγ activity and VLDL-induced foam cell formation in IL-4-polarized human macrophages.* Atherosclerosis, 2015. **240**(2): p. 424-430.

504. Barish, G.D., *Peroxisome Proliferator-Activated Receptors and Liver X Receptors in Atherosclerosis and Immunity.* The Journal of Nutrition, 2006. **136**(3): p. 690-694.
505. Parikh, M., et al., *Liver X Receptor: A Cardinal Target for Atherosclerosis and Beyond.* Journal of Atherosclerosis and Thrombosis, 2014. **21**(6): p. 519-531.
506. Chan, W.L., et al., *NKT cell subsets in infection and inflammation.* Immunology Letters, 2003. **85**(2): p. 159-163.
507. Rohm, I., et al., *Decreased Regulatory T Cells in Vulnerable Atherosclerotic Lesions: Imbalance between Pro- and Anti-Inflammatory Cells in Atherosclerosis.* Mediators of Inflammation, 2015. **2015**: p. 13.
508. Peralbo, E., et al., *Decreased frequency and proliferative response of invariant Vα24Vβ11 natural killer T (iNKT) cells in healthy elderly.* Biogerontology, 2006. **7**(5-6): p. 483-492.
509. Jing, Y., et al., *Aging is associated with a rapid decline in frequency, alterations in subset composition, and enhanced Th2 response in CD1d-restricted NKT cells from human peripheral blood.* Experimental Gerontology, 2007. **42**(8): p. 719-732.
510. Vukmanovic-Stejic, M., et al., *Immune responses in the skin in old age.* Current Opinion in Immunology, 2011. **23**(4): p. 525-531.
511. Zhao, L., et al., *Changes in immune cell frequencies after cyclophosphamide or mycophenolate mofetil treatments in patients with systemic lupus erythematosus.* Clinical Rheumatology, 2012. **31**(6): p. 951-959.
512. Mansour, S., et al., *Structural and Functional Changes of the Invariant NKT Clonal Repertoire in Early Rheumatoid Arthritis.* The Journal of Immunology, 2015.
513. Wen, X., et al., *A Subset of CD8αβ+ Invariant NKT Cells in a Humanized Mouse Model.* The Journal of Immunology, 2015. **195**(4): p. 1459-1469.
514. Lee, H., et al., *The presence of CD8+ invariant NKT cells in mice.* Exp Mol Med, 2009. **41**: p. 866-872.
515. Wan, W., et al., *Genetic Deletion of Chemokine Receptor Ccr6 Decreases Atherogenesis in ApoE-Deficient Mice.* Circulation Research, 2011. **109**(4): p. 374-381.
516. Yilmaz, A., et al., *Accumulation of immune cells and high expression of chemokines/chemokine receptors in the upstream shoulder of atherosclerotic carotid plaques.* Experimental and Molecular Pathology, 2007. **82**(3): p. 245-255.
517. Ladd, M., et al., *Natural killer T cells constitutively expressing the interleukin-2 receptor α chain early in life are primed to respond to lower antigenic stimulation.* Immunology, 2010. **131**(2): p. 289-299.
518. Gómez, M., et al., *Atherosclerosis development in apolipoprotein E-null mice deficient for CD69.* Cardiovascular Research, 2009. **81**(1): p. 197-205.
519. Hervier, B., et al., *Phenotype and function of natural killer cells in systemic lupus erythematosus: Excess interferon-γ production in patients with active disease.* Arthritis & Rheumatism, 2011. **63**(6): p. 1698-1706.
520. Rus, V., et al., *Increased expression and release of functional tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) by T cells from lupus patients with active disease.* Clinical Immunology, 2005. **117**(1): p. 48-56.
521. Peralbo, E., C. Alonso, and R. Solana, *Invariant NKT and NKT-like lymphocytes: Two different T cell subsets that are differentially affected by ageing.* Experimental Gerontology, 2007. **42**(8): p. 703-708.
522. Takahashi, T., S. Dejbakhsh-Jones, and S. Strober, *Expression of CD161 (NKR-P1A) Defines Subsets of Human CD4 and CD8 T Cells with Different Functional Activities.* The Journal of Immunology, 2006. **176**(1): p. 211-216.
523. Singh, A., et al., *Programmed death-1 receptor suppresses γ-IFN producing NKT cells in human tuberculosis.* Tuberculosis, 2014. **94**(3): p. 197-206.

524. Chang, W.-S., et al., *Cutting Edge: Programmed Death-1/Programmed Death Ligand 1 Interaction Regulates the Induction and Maintenance of Invariant NKT Cell Anergy.* The Journal of Immunology, 2008. **181**(10): p. 6707-6710.
525. Llorente-Cortés, V., et al., *Signature of subclinical femoral artery atherosclerosis in peripheral blood mononuclear cells.* European Journal of Clinical Investigation, 2014. **44**(6): p. 539-548.
526. Bricard, G., et al., *Enrichment of Human CD4+ Vα24/Vβ11 Invariant NKT Cells in Intrahepatic Malignant Tumors.* The Journal of Immunology, 2009. **182**(8): p. 5140-5151.
527. Ghazarian, L., et al., *Rôle régulateur des lymphocytes NKT dans la prévention du diabète de type 1.* Med Sci (Paris), 2013. **29**(8-9): p. 722-728.
528. Ketelhuth, D.F.J. and G.K. Hansson, *Cellular immunity, low-density lipoprotein and atherosclerosis: Break of tolerance in the artery wall.* Thrombosis and haemostasis, 2011. **106**(11): p. 779-786.
529. Wang, K.-F.a., e; Huang, Po-Hsun a,d,e; Chiang, Chia-Hung a,e; Hsu, Chien-Yi a,e; Leu, Hsin-Bang a,c,d,e; Chen, Jaw-Wen a,b,e,f; Lin, Shing-Jong a,b,d,e, *Usefulness of plasma matrix metalloproteinase-9 level in predicting future coronary revascularization in patients after acute myocardial infarction.* Coronary Artery Disease, 2013. **24**(1): p. 23-8.
530. Baruch, A., et al., *Anti-Inflammatory Strategies for Plaque Stabilization after Acute Coronary Syndromes.* Current Atherosclerosis Reports, 2013. **15**(6): p. 1-11.
531. Parra, S., et al., *Complement system and small HDL particles are associated with subclinical atherosclerosis in SLE patients.* Atherosclerosis, 2012. **225**(1): p. 224-230.
532. Feng, H., et al., *Deficiency of Scavenger Receptor BI Leads to Impaired Lymphocyte Homeostasis and Autoimmune Disorders in Mice.* Arteriosclerosis, Thrombosis, and Vascular Biology, 2011. **31**(11): p. 2543-2551.
533. Rigotti, A., et al., *A targeted mutation in the murine gene encoding the high density lipoprotein (HDL) receptor scavenger receptor class B type I reveals its key role in HDL metabolism.* Proceedings of the National Academy of Sciences, 1997. **94**(23): p. 12610-12615.
534. Kontush, A., et al., *A normotriglyceridemic, low HDL-cholesterol phenotype is characterised by elevated oxidative stress and HDL particles with attenuated antioxidative activity.* Atherosclerosis, 2005. **182**(2): p. 277-285.
535. Calabresi, L., et al., *Elevated Soluble Cellular Adhesion Molecules in Subjects With Low HDL-Cholesterol.* Arteriosclerosis, Thrombosis, and Vascular Biology, 2002. **22**(4): p. 656-661.
536. Brites, F.D., et al., *Alterations in the main steps of reverse cholesterol transport in male patients with primary hypertriglyceridemia and low HDL-cholesterol levels.* Atherosclerosis, 2000. **152**(1): p. 181-192.
537. Yvan-Charvet, L., N. Wang, and A.R. Tall, *Role of HDL, ABCA1, and ABCG1 Transporters in Cholesterol Efflux and Immune Responses.* Arteriosclerosis, Thrombosis, and Vascular Biology, 2010. **30**(2): p. 139-143.
538. Out, R., et al., *Coexistence of Foam Cells and Hypocholesterolemia in Mice Lacking the ABC Transporters A1 and G1.* Circulation Research, 2008. **102**(1): p. 113-120.
539. Rohatgi, A., et al., *HDL Cholesterol Efflux Capacity and Incident Cardiovascular Events.* New England Journal of Medicine, 2014. **371**(25): p. 2383-2393.
540. Catapano, A.L., et al., *HDL in innate and adaptive immunity*. Vol. 103. 2014. 372-383.
541. Machado, R.M., et al., *Omega-6 polyunsaturated fatty acids prevent atherosclerosis development in LDLr-KO mice, in spite of displaying a pro-inflammatory profile similar to trans fatty acids.* Atherosclerosis, 2012. **224**(1): p. 66-74.

542. de Oliveira Otto, M.C., et al., *Circulating and Dietary Omega-3 and Omega-6 Polyunsaturated Fatty Acids and Incidence of CVD in the Multi-Ethnic Study of Atherosclerosis.* Journal of the American Heart Association, 2013. **2**(6).
543. Adan, Y., et al., *Effects of Docosahexaenoic and Eicosapentaenoic Acid on Lipid Metabolism, Eicosanoid Production, Platelet Aggregation and Atherosclerosis in Hypercholesterolemic Rats.* Bioscience, Biotechnology, and Biochemistry, 1999. **63**(1): p. 111-119.
544. Gillingham, L.G., S. Harris-Janz, and P.J.H. Jones, *Dietary Monounsaturated Fatty Acids Are Protective Against Metabolic Syndrome and Cardiovascular Disease Risk Factors.* Lipids, 2011. **46**(3): p. 209-228.
545. Chung, C., et al., *Lipoprotein subclasses and particle size determined by nuclear magnetic resonance spectroscopy in systemic lupus erythematosus.* Clinical Rheumatology, 2008. **27**(10): p. 1227-1233.
546. Olusi, S.O. and S. George, *Prevalence of LDL atherogenic phenotype in patients with systemic lupus erythematosus.* Vascular Health and Risk Management, 2011. **7**: p. 75-80.
547. Mora, S., et al., *LDL particle subclasses, LDL particle size, and carotid atherosclerosis in the Multi-Ethnic Study of Atherosclerosis (MESA).* Atherosclerosis, 2007. **192**(1): p. 211-217.
548. Costandi, J., et al., *Human Resistin Stimulates Hepatic Overproduction of Atherogenic ApoB-Containing Lipoprotein Particles by Enhancing ApoB Stability and Impairing Intracellular Insulin Signaling.* Circulation Research, 2011. **108**(6): p. 727-742.
549. Panagiotou, G., et al., *Circulating irisin, omentin-1, and lipoprotein subparticles in adults at higher cardiovascular risk.* Metabolism, 2014. **63**(10): p. 1265-1271.
550. Zhao, Y., et al., *Lack of Phosphatidylethanolamine N-Methyltransferase Alters Plasma VLDL Phospholipids and Attenuates Atherosclerosis in Mice.* Arteriosclerosis, Thrombosis, and Vascular Biology, 2009. **29**(9): p. 1349-1355.
551. Mahmood Hussain, M., *A proposed model for the assembly of chylomicrons.* Atherosclerosis, 2000. **148**(1): p. 1-15.
552. Cagnoli, P., et al., *Reduced Insular Glutamine and N-Acetylaspartate in Systemic Lupus Erythematosus: A Single-Voxel 1H-MR Spectroscopy Study.* Academic Radiology, 2013. **20**(10): p. 1286-1296.
553. Verbeke, K.A., et al., *Towards microbial fermentation metabolites as markers for health benefits of prebiotics.* Nutrition Research Reviews, 2015. **28**(1): p. 42-66.
554. Carvajal Sandoval G1, M.S.R., Juárez E, RamosMartínez G, Carvajal Juárez ME., *Effect of glycine on hemoglobin glycation in diabetic patients.* Proc West Pharmacol Soc., 1999. **42**(1): p. 31-2.
555. Alvarado-Vásquez, N., et al., *Oral glycine administration attenuates diabetic complications in streptozotocin-induced diabetic rats.* Life Sciences, 2006. **79**(3): p. 225-232.
556. Chironi, G., et al., *Relationship of Circulating Biomarkers of Inflammation and Hemostasis with Preclinical Atherosclerotic Burden in Nonsmoking Hypercholesterolemic Men*.* American Journal of Hypertension, 2006. **19**(10): p. 1025-1031.
557. Liu, F., et al., *TLR9-induced miR-155 and Ets-1 decrease expression of CD1d on B cells in SLE.* European Journal of Immunology, 2015. **45**(7): p. 1934-1945.
558. Dong, G.F., et al., *Effect of Leflunomide on the Abnormal Expression of Lipid Rafts and F-Actin in B Lymphocytes from Patients with Systemic Lupus Erythematosus.* Journal of Immunology Research, 2015. **2015**: p. 8.

559. Im, J.S., et al., *Expression of CD1d Molecules by Human Schwann Cells and Potential Interactions with Immunoregulatory Invariant NK T Cells.* The Journal of Immunology, 2006. **177**(8): p. 5226-5235.
560. Menon, M., et al., *A Regulatory Feedback between Plasmacytoid Dendritic Cells and Regulatory B Cells Is Aberrant in Systemic Lupus Erythematosus.* Immunity, 2016. **44**(3): p. 683-697.
561. Li, L., et al., *Activation of decidual invariant natural killer T cells promotes lipopolysaccharide-induced preterm birth.* Molecular Human Reproduction, 2015. **21**(4): p. 369-381.
562. González-Amaro, R., et al., *Is CD69 an effective brake to control inflammatory diseases?* Trends in Molecular Medicine, 2013. **19**(10): p. 625-632.
563. Lombardi, V., et al., *A CD1d-Dependent Antagonist Inhibits the Activation of Invariant NKT Cells and Prevents Development of Allergen-Induced Airway Hyperreactivity.* The Journal of Immunology, 2010. **184**(4): p. 2107-2115.
564. Li, Y., et al., *A CD1d-dependent lipid antagonist to Natural Killer T (NKT) cells ameliorates atherosclerosis in ApoE−/- mice by reducing lesion necrosis and inflammation.* Cardiovascular Research, 2015.
565. Perrin-Cocon, L., et al., *Modified lipoproteins provide lipids that modulate dendritic cell immune function.* Biochimie, 2013. **95**(1): p. 103-108.
566. Syväranta, S., et al., *Potential pathological roles for oxidized low-density lipoprotein and scavenger receptors SR-AI, CD36, and LOX-1 in aortic valve stenosis.* Atherosclerosis. **235**(2): p. 398-407.
567. Chávez-Sánchez, L., et al., *The role of TLR2, TLR4 and CD36 in macrophage activation and foam cell formation in response to oxLDL in humans.* Human Immunology, 2014. **75**(4): p. 322-329.
568. Ulrich, C., et al., *Monocyte Angiotensin Converting Enzyme Expression May Be Associated with Atherosclerosis Rather than Arteriosclerosis in Hemodialysis Patients.* Clinical Journal of the American Society of Nephrology, 2011. **6**(3): p. 505-511.
569. Mikołajczyk, T.P., et al., *Heterogeneity of peripheral blood monocytes, endothelial dysfunction and subclinical atherosclerosis in patients with systemic lupus erythematosus.* Lupus, 2015.
570. Fadini, G.P., et al., *Monocyte–macrophage polarization balance in pre-diabetic individuals.* Acta Diabetologica, 2013. **50**(6): p. 977-982.
571. Cardilo-Reis, L., et al., *Interleukin-13 protects from atherosclerosis and modulates plaque composition by skewing the macrophage phenotype*. Vol. 4. 2012. 1072-1086.
572. Bouhlel, M.A., et al., *PPARgamma activation primes human monocytes into alternative M2 macrophages with anti-inflammatory properties.* Cell Metab, 2007. **6**(2): p. 137-43.
573. Chinetti-Gbaguidi, G., et al., *Human Atherosclerotic Plaque Alternative Macrophages Display Low Cholesterol Handling but High Phagocytosis Because of Distinct Activities of the PPARγ and LXRα Pathways.* Circulation Research, 2011. **108**(8): p. 985-995.
574. Shaikh, S., et al., *Macrophage subtypes in symptomatic carotid artery and femoral artery plaques.* Eur J Vasc Endovasc Surg, 2012. **44**(5): p. 491-7.
575. Lee, C.W., et al., *Macrophage Heterogeneity of Culprit Coronary Plaques in Patients With Acute Myocardial Infarction or Stable Angina.* American Journal of Clinical Pathology, 2013. **139**(3): p. 317-322.
576. Oksala, N., et al., *Kindlin 3 (FERMT3) is associated with unstable atherosclerotic plaques, anti-inflammatory type II macrophages and upregulation of beta-2 integrins in all major arterial beds.* Atherosclerosis, 2015. **242**(1): p. 145-154.
577. Colin, S., G. Chinetti-Gbaguidi, and B. Staels, *Macrophage phenotypes in atherosclerosis.* Immunological Reviews, 2014. **262**(1): p. 153-166.

578. Chan, W.L., et al., *Atherosclerotic Abdominal Aortic Aneurysm and the Interaction Between Autologous Human Plaque-Derived Vascular Smooth Muscle Cells, Type 1 NKT, and Helper T Cells.* Circulation Research, 2005. **96**(6): p. 675-683.
579. Viallard, et al., *Th1 (IL-2, interferon-gamma (IFN-γ)) and Th2 (IL-10, IL-4) cytokine production by peripheral blood mononuclear cells (PBMC) from patients with systemic lupus erythematosus (SLE).* Clinical & Experimental Immunology, 1999. **115**(1): p. 189-195.
580. Zhang, W., W. Xu, and S. Xiong, *Blockade of Notch1 Signaling Alleviates Murine Lupus via Blunting Macrophage Activation and M2b Polarization.* The Journal of Immunology, 2010. **184**(11): p. 6465-6478.
581. Waldo, S.W., et al., *Heterogeneity of Human Macrophages in Culture and in Atherosclerotic Plaques.* The American Journal of Pathology, 2008. **172**(4): p. 1112-1126.
582. Ricote, M., et al., *The peroxisome proliferator-activated receptor(PPARgamma) as a regulator of monocyte/macrophage function.* Journal of Leukocyte Biology, 1999. **66**(5): p. 733-9.
583. Nissen, S.E. and K. Wolski, *Effect of Rosiglitazone on the Risk of Myocardial Infarction and Death from Cardiovascular Causes.* New England Journal of Medicine, 2007. **356**(24): p. 2457-2471.
584. Singh, S., Y.K. Loke, and C.D. Furberg, *Long-term risk of cardiovascular events with rosiglitazone: A meta-analysis.* JAMA, 2007. **298**(10): p. 1189-1195.
585. Odegaard, J.I., et al., *Macrophage-specific PPAR[ggr] controls alternative activation and improves insulin resistance.* Nature, 2007. **447**(7148): p. 1116-1120.
586. Taylor, F., et al., *Statins for the primary prevention of cardiovascular disease.* Cochrane Database of Systematic Reviews, 2013. **1**: p. CD004816.
587. Szanto, A. and T. Rőszer, *Nuclear receptors in macrophages: A link between metabolism and inflammation.* FEBS Letters, 2008. **582**(1): p. 106-116.
588. Zizzo, G. and P. Cohen, *The PPAR-gamma antagonist GW9662 elicits differentiation of M2c-like cells and upregulation of the MerTK/Gas6 axis: a key role for PPAR-gamma in human macrophage polarization.* Journal of Inflammation, 2015. **12**(1): p. 36.
589. Thorp, E., et al., *Mertk Receptor Mutation Reduces Efferocytosis Efficiency and Promotes Apoptotic Cell Accumulation and Plaque Necrosis in Atherosclerotic Lesions of Apoe−/− Mice.* Arteriosclerosis, Thrombosis, and Vascular Biology, 2008. **28**(8): p. 1421-1428.
590. Feng, J., et al., *Induction of CD36 expression by oxidized LDL and IL-4 by a common signaling pathway dependent on protein kinase C and PPAR-γ.* Journal of Lipid Research, 2000. **41**(5): p. 688-696.
591. Zizzo, G., et al., *Efficient Clearance of Early Apoptotic Cells by Human Macrophages Requires M2c Polarization and MerTK Induction.* The Journal of Immunology, 2012. **189**(7): p. 3508-3520.
592. Liao, K.P., et al., *Association Between Lipid Levels and Major Adverse Cardiovascular Events in Rheumatoid Arthritis Compared to Non–Rheumatoid Arthritis Patients.* Arthritis & Rheumatology, 2015. **67**(8): p. 2004-2010.
593. Solomon, D.H., et al., *Derivation and Internal Validation of an Expanded Cardiovascular Risk Prediction Score for Rheumatoid Arthritis: A Consortium of Rheumatology Researchers of North America Registry Study.* Arthritis & Rheumatology, 2015. **67**(8): p. 1995-2003.
594. Pilones, K.A., J. Aryankalayil, and S. Demaria, *Invariant NKT Cells as Novel Targets for Immunotherapy in Solid Tumors.* Clinical and Developmental Immunology, 2012. **2012**: p. 11.