Cardiovascular Disease and Immune Mechanisms in Systemic Lupus Erythematosus

DAG LEONARD
Systemic lupus erythematosus (SLE) is an autoimmune, inflammatory disease characterized by autoantibody production and an activated type I interferon system. Cardiovascular disease (CVD) is as a major cause of morbidity and mortality. The aim of this thesis was to identify genetic risk factors for CVD in SLE. The role of T cells in regulation of the interferon-α (IFNα) production by plasmacytoid dendritic cells (pDCs) was also investigated.

In paper I, a thicker intima, thinner media and increased intima/media ratio was found in young premenopausal women with SLE compared to healthy controls indicating increased cardiovascular risk. As traditional ultrasound assessment of the common carotid intima-media thickness (CCA-IMT) in SLE has given conflicting results separate measurement of the intima and media can be a useful tool to identify SLE patients at increased risk of CVD.

In paper II, an association was demonstrated in SLE between a \textit{STAT4} risk allele and ischemic cerebrovascular disease and presence of anti-phospholipid antibodies (aPL). The association remained after adjustment for traditional CVD risk factors. A possible mechanism for this association is that the risk allele leads to increased production of aPL, which promotes thromboembolism.

In paper III, a genetic locus in \textit{IRF8} was identified to be associated to coronary heart disease (CHD) in SLE. The association remained after adjustment of other CHD risk factors. Patients with the \textit{IRF8} risk variant had increased CCA-IMT, more carotid plaques and reduced frequency of circulating B cells. Weaker binding of nuclear protein to the risk allele was demonstrated, suggesting a regulatory function of the \textit{IRF8} risk variant.

In paper IV, activated T cells were found to strongly enhance the IFNα production by pDC stimulated with RNA-containing immune complexes via GM-CSF and IL-3. Activated SLE T cells enhanced the IFNα production to the same extent as T cells from healthy controls. This finding together with previous observations in SLE of increased levels of GM-CSF and IL-3 suggests that T cells contribute to the activated type I interferon system in SLE.

In conclusion, this thesis demonstrates that genetic predisposition is important for CVD in SLE and describes a new role for T cells in the pathogenesis of SLE.

Keywords: Systemic Lupus Erythematosus, Cardiovascular Disease, Intima-Media Thickness, STAT4, IRF8, Interferon-α, Plasmacytoid dendritic cell, GM-CSF, IL-3
To Kerstin, Olof and Karin
This thesis is based on the following papers, which are referred to in the text by their Roman numerals.


Supporting publications


*These authors contributed equally to this work.

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Systemic Lupus Erythematosus ................................................................. 11
  Historical background ........................................................................ 11
  Clinical features .................................................................................. 11
  Epidemiology ....................................................................................... 12
  Classification criteria ........................................................................... 12
  Estimation of disease activity and damage ....................................... 13

The human genome and genetic variation ............................................ 15

Etiopathogenesis of SLE................................................................. 16
  Genetics .............................................................................................. 16
    STAT4 .......................................................................................... 18
    IRF8 ............................................................................................ 19
  Environmental factors ......................................................................... 19
  Autoantibodies .................................................................................. 20
  Apoptosis .......................................................................................... 20
  Immune complex handling .................................................................. 21
  Immune cells ...................................................................................... 21
    T cells .......................................................................................... 21
    B cells .......................................................................................... 22
  Cytokines .......................................................................................... 23
    GM-CSF and IL-3 ........................................................................... 23

The interferons ....................................................................................... 25
  The plasmacytoid dendritic cell and IFNα ........................................ 25
  IFNα in SLE ..................................................................................... 26
  Regulation of the IFNα production .................................................. 27

Cardiovascular disease ......................................................................... 29
  Atherosclerosis .................................................................................. 29
    Risk factors .................................................................................. 30
    Pathogenesis ................................................................................ 30

Cardiovascular disease in SLE .............................................................. 33
  Epidemiology ..................................................................................... 33
  Atherosclerosis in SLE ...................................................................... 33
  Traditional risk factors ...................................................................... 34
## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACR</td>
<td>American College of Rheumatology</td>
</tr>
<tr>
<td>ANA</td>
<td>Anti-nuclear antibody</td>
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<tr>
<td>aPL</td>
<td>Antiphospholipid antibodies</td>
</tr>
<tr>
<td>aβ2GP1</td>
<td>Antibodies to beta 2 glycoprotein 1</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic cell</td>
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<tr>
<td>CCA-IMT</td>
<td>Common carotid artery – intima media thickness</td>
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<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
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<tr>
<td>CHD</td>
<td>Coronary heart disease</td>
</tr>
<tr>
<td>CVD</td>
<td>Cardiovascular disease</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>dsDNA</td>
<td>Double-stranded DNA</td>
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<tr>
<td>EMSA</td>
<td>Electrophoretic mobility shift assay</td>
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<tr>
<td>GM-CSF</td>
<td>Granulocyte-macrophage colony-stimulating factor</td>
</tr>
<tr>
<td>GWAS</td>
<td>Genome-wide association study</td>
</tr>
<tr>
<td>HLA</td>
<td>Human leukocyte antigen</td>
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<tr>
<td>IC</td>
<td>Immune complex</td>
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<tr>
<td>ICVD</td>
<td>Ischemic cerebrovascular disease</td>
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<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IHD</td>
<td>Ischemic heart disease</td>
</tr>
<tr>
<td>IMT</td>
<td>Intima media thickness</td>
</tr>
<tr>
<td>I/M-ratio</td>
<td>Intima / media ratio</td>
</tr>
<tr>
<td>IL3</td>
<td>Interleukin 3</td>
</tr>
<tr>
<td>IPVD</td>
<td>Ischemic peripheral vascular disease</td>
</tr>
<tr>
<td>IRF8</td>
<td>Interferon regulatory factor 8</td>
</tr>
<tr>
<td>LD</td>
<td>Linkage disequilibrium</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>MHz</td>
<td>Megahertz</td>
</tr>
<tr>
<td>ODN</td>
<td>Oligodeoxynucleotide</td>
</tr>
<tr>
<td>PBMC</td>
<td>Peripheral blood mononuclear cell</td>
</tr>
<tr>
<td>pDC</td>
<td>Plasmacytoid dendritic cell</td>
</tr>
<tr>
<td>RNA-IC</td>
<td>RNA-containing immune complex</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
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<tr>
<td>SLE</td>
<td>Systemic lupus erythematosus</td>
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<tr>
<td>SLEDAI</td>
<td>SLE disease activity index</td>
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<tr>
<td>SLICC</td>
<td>Systemic lupus international collaborating clinics</td>
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<tr>
<td>SNP</td>
<td>Single nucleotide polymorphism</td>
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<tr>
<td>STAT4</td>
<td>Signal transducer and activator of transcription 4</td>
</tr>
<tr>
<td>TIA</td>
<td>Transitory ischemic attack</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll like receptor</td>
</tr>
<tr>
<td>VTE</td>
<td>Venous thromboembolism</td>
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</table>
Systemic Lupus Erythematosus

Historical background
The word lupus, Latin for wolf, was used during the Middle ages to describe severe skin lesions in the face that reminded of a wolf’s bite. In 1846 Von Herba described the malar rash over the face and nose and introduced the butterfly metaphor. A few years later Cazenave introduced the term lupus érythémateux and separated it from skin lesions caused by tuberculosis. Kaposi recognized lupus as a systemic disease in 1872, suggesting the name lupus erythematosus disseminates. The systemic nature of the disease was further established by Sir William Osler who introduced nephritis and vasculitis as manifestations of lupus.

The modern era of lupus started with the discovery of the LE cell (lupus erythematosus cell) in the bone marrow of patients with SLE by Hargraves in 1948. A few years later Miescher and Fauconnet demonstrated that isolated cell nuclei can absorb the serum factor that induces the LE phenomenon, suggesting that the serum factor was an antibody against components of the cell nucleus. This was followed in 1957 by the development of the immunofluorescence techniques and detection of anti-nuclear antibodies by Friou and anti-DNA antibodies by Ceppellini and Robbins.

Clinical features
SLE is a chronic autoimmune inflammatory disease clinically characterized by involvement of multiple organ systems. Patients can have a mild disease affecting only a limited number of organs or present with life threatening involvement of multiple organ systems. The organs most often affected in SLE are the joints, skin and blood (Table 1). Other manifestations of SLE are serositis, glomerulonephritis and involvement of the central nervous system. General symptoms such as muscle pain, fatigue and fever are also very common. Some patients, in addition to SLE also have other autoimmune diseases such as Sjögrens syndrome or Antiphospholipid syndrome (APS). SLE is 5 to 6 times more common in women than in men with the largest gender difference during childbearing years. During prepubertal and postmenopausal ages the gender difference is smaller. The average age at diagnosis was 34 years in a recently published study.
Epidemiology

Epidemiological studies of SLE have been conducted worldwide and figures of incidence and prevalence vary considerably\textsuperscript{16}. This may be due to differences in recruitment methods and case assessment. Incidence rates of 1-10 cases per 100,000/year and prevalence from 20-70 cases per 100,000 have been described\textsuperscript{14}. SLE is more common in certain ethnic groups. In the United States SLE is 3 times more common among African American women compared to Caucasian women\textsuperscript{17-19}. In an epidemiological study conducted in a geographically defined area in southern Sweden, during the years 1987 to 1991, an incidence rate of 4.8 cases per 100,000/year and a prevalence rate of 68 cases per 100,000 was observed\textsuperscript{20}. Consecutive studies conducted in the same region have shown constant incidence rates indicating that there is no increase in SLE incidence in Sweden\textsuperscript{20,21}.

In 1955 the reported 4 year survival rate for a group of patients with SLE followed at the Johns Hopkins Hospital was 51\%\textsuperscript{22}. With the introduction of corticosteroids survival improved and in 1966 Leonhardt reported a 5 year survival rate of 70\% for a group of SLE patients in Sweden\textsuperscript{23}. Today the reported 5 year survival rate is 95\% and the 10 year survival rate 91\%\textsuperscript{24}. Nevertheless, life expectancy for patients with SLE is still below that of the general population with an overall standardized mortality rate between 2.4 and 4.6\textsuperscript{25-27}. This increased mortality risk is mainly due to CVD, infections and manifestations of the SLE disease\textsuperscript{25,27}.

Classification criteria

The diagnosis of SLE is based on clinical manifestations in combination with presence of typical immunological abnormalities. In clinical practice the best definition may be an autoimmune disease with involvement of at least two organ systems together with the presence of typical autoantibodies and the absence of a better alternative diagnosis explaining the symptoms. A similar diagnostic criteria was proposed by Fries in 1975. This included presence of a multisystem disease in at least two out of seven specified organ systems and a positive ANA test\textsuperscript{28}. For clinical research purposes the American College of Rheumatology published preliminary classification criteria for SLE in 1971\textsuperscript{29}. These were later revised by Tan et al in 1982 and have become widely used in most SLE studies\textsuperscript{30}. The criteria include nine clinical and two immunological criteria and SLE is present if a patient serially or simultaneously fulfills four or more of the eleven criteria (Table 1). These criteria were modified in 1997\textsuperscript{31} and validated 2012\textsuperscript{32}, excluding the LE cells and including anti-cardiolipin antibodies and the lupus anticoagulant test as parts of the tenth criteria. Recently the Systemic Lupus International Collaborating Clinics (SLICC) group proposed new classification criteria for SLE
including 11 clinical and 6 immunological criteria. According to this classification patients must fulfill four criteria including at least one clinical and one immunological criteria or must have a biopsy proven lupus nephritis in the presence of ANA or anti-dsDNA antibodies. It remains to be seen whether these new criteria will eventually replace the ACR classification criteria. As of today, most clinical studies still use the ACR criteria from 1982 or the revised ACR criteria from 1997.

Table 1. The 1982 American College of Rheumatology Criteria for Classification of SLE and their frequency

<table>
<thead>
<tr>
<th>Criteria</th>
<th>Definition</th>
<th>Tan* (n=177)</th>
<th>Paper III** (n=814)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Malar rash</td>
<td>Fixed erythema over the malar eminence</td>
<td>101(57)</td>
<td>470(58)</td>
</tr>
<tr>
<td>2. Discoid rash</td>
<td>Erythematous raised patches</td>
<td>31(18)</td>
<td>209(26)</td>
</tr>
<tr>
<td>3. Photosensitivity</td>
<td>Rash caused by unusual reaction to sunlight</td>
<td>76(43)</td>
<td>516(63)</td>
</tr>
<tr>
<td>4. Oral ulcers</td>
<td>Oral or nasopharyngeal ulceration</td>
<td>47(27)</td>
<td>203(25)</td>
</tr>
<tr>
<td>5. Arthritis</td>
<td>Nonerosive arthritis involving two or more peripheral joints</td>
<td>152(86)</td>
<td>635(78)</td>
</tr>
<tr>
<td>6. Serositis</td>
<td>Pleuritis or pericarditis</td>
<td>99(56)</td>
<td>361(44)</td>
</tr>
<tr>
<td>7. Renal disorder</td>
<td>Persistent proteinuria &gt;0.5 g/day or cellular casts</td>
<td>91(51)</td>
<td>277(34)</td>
</tr>
<tr>
<td>8. Neurologic disorder</td>
<td>Seizures or psychosis</td>
<td>35(20)</td>
<td>90(11)</td>
</tr>
<tr>
<td>9. Hematologic disorder</td>
<td>Hemolytic anemia, leukopenia, lymphopenia or thrombocytopenia</td>
<td>105(59)</td>
<td>483(59)</td>
</tr>
<tr>
<td>10. Immunologic disorder</td>
<td>Positive LE-cell preparation, anti-dsDNA, anti-Sm or false positive test for syphilis</td>
<td>149(85)</td>
<td>590(72)</td>
</tr>
<tr>
<td>11. Positive ANA</td>
<td>Abnormal titer of ANA</td>
<td>174(99)</td>
<td>804(99)</td>
</tr>
</tbody>
</table>

Data are number (%). A person shall be said to have SLE if any 4 or more of these 11 criteria are present, serially or simultaneously, during any interval of observation. Frequency of manifestations in *Tan et al*30, **Leonard et al**33. SLE criteria as defined in Tan et al30.

Estimation of disease activity and damage

Over the years patients with SLE may have periods of disease exacerbation (flares) and also periods with fewer symptoms (remission). Further, some patients only have one flare at onset of disease while others have persistently active disease. Several instruments have been developed to measure disease activity. One index that is often used in clinical studies to measure persistently active disease is the SLE Disease Activity Index 2000 (SLEDAI-2K)34. This is a global index based on 24 weighted clinical and laboratory variables. Other instruments to measure disease activity are the British Isles
Lupus Assessment Group Index (BILAG)\textsuperscript{35}, the European Consensus Lupus Activity Measurement (ECLAM)\textsuperscript{36} and the Systemic Lupus Erythematosus Activity Measure (SLAM)\textsuperscript{37}. In recent clinical trials a new index called the SLE responder index (SRI) has been used\textsuperscript{38}.

To measure the long term impact of SLE an instrument called the Systemic Lupus Erythematosus International Collaborating Clinics/American College of Rheumatology Damage Index (SLICC/ACR-DI) is often used\textsuperscript{39}. This cumulative organ damage index includes twelve different organ systems and measures the chronic damage of the disease.
The human genome and genetic variation

The genetic information is encoded in our DNA sequence and is organized into 22 pairs and the X and Y chromosomes\(^40\). The haploid human genome consists of about three billion base pairs and contains approximately 21,000 genes\(^41\). The protein coding sequence makes up less than 2% of our genome, and the rest of the DNA sequence includes non-coding RNA genes, regulatory DNA sequences and sequences of unknown function\(^40\).

The DNA sequence is 99.9% identical by any two individual human beings. It is the remaining 0.1% that makes us unique and contributes to our personalities, appearance and susceptibility to diseases. Single nucleotide polymorphisms (SNPs) are the most common form of genetic variation and are mutations affecting a single base of the genetic code. Once the new variant has spread in the population to a frequency of approximately one percent or higher, it is considered a SNP. Currently there are approximately 60 million human SNPs registered in dbSNP build 138 (http://www.ncbi.nlm.nih.gov). The majority of the identified SNPs are localized outside of protein coding regions and may be important for regulation of gene transcription.

There are three main strategies for identifying genes relevant for the development of a disease; linkage studies, candidate gene association studies and genome-wide association studies (GWAS). Linkage studies can be used to identify regions of the genome that predispose to disease by use of observations in related individuals\(^42\). This method has successfully identified many genes for monogenic disorders. In candidate gene association studies polymorphisms within a candidate gene or region are genotyped in a set of cases and controls and allele frequencies are compared. SNPs are the most common type of genetic variation studied. Genome-wide association studies were initiated as a result of the “common disease – common variant” hypothesis\(^43\). This hypothesis proposes that if a heritable disease is common in the population then the contributing genetic variations will also be common in the population. In a GWAS several hundred to a few million SNPs are genotyped across the genome in cases and controls. To achieve significant results large number of cases and controls are needed. During the last few years, GWAS has been used to identify disease associated genes for a large number of complex diseases and to date more than 1800 GWAS have been published in (www.genome.gov/gwastudies).
Etiopathogenesis of SLE

SLE is a complex systemic autoimmune disease that results from interaction of genetic and environmental factors. The hallmark is the production of autoantibodies predominantly directed towards nuclear macromolecules. The resulting immune complexes (ICs) cause potent stimulation of the immune system including production of type I interferon (IFN). In addition, these ICs deposit in tissue and incite inflammation and damage.

Genetics

Genetic epidemiologic studies have demonstrated that SLE is more prevalent among relatives to patients affected by SLE\(^44\). One study showed that 10% of patients had a relative also affected by SLE\(^45\). The λ\(_s\) (sibling risk divided by the risk in the general population) in SLE is estimated to be 20 which is higher than for some other autoimmune diseases including rheumatoid arthritis (RA) (λ\(_s\)=8) and diabetes mellitus type I (λ\(_s\)=15)\(^46\). The disease concordance rate for monozygotic twins is 24-58% and 2-3% for dizygotic twins\(^47,49\). This 10-fold difference indicates that genetic factors shared between each pair of twins influence the susceptibility to SLE and support the importance of genetic influence in the etiology of SLE.

The impact of individual genetic risk factors is relatively weak in SLE but there are a few exceptions. Mutations of components in the classical complement pathway are rare but show strong association with SLE\(^46\). Over 90% of individuals with homozygote C1q-deficiency develop SLE\(^50\). Other complement deficiencies include homozygote C4 deficiency where it is estimated that 75% develop SLE and homozygote C2 deficiency where 10-20% develop SLE\(^51,52\). This may be due to the important role of complement in removing apoptotic cell debris and clearance of ICs\(^52\). Further, there are a couple of rare monogenic deficiencies involved in DNA degradation associated to SLE. These include mutations in the three prime repair exonuclease 1 (\(TREX1\)) gene, involved in the degradation of cytosolic DNA during apoptosis\(^53\) and a mutation in the deoxyribonuclease I (\(DNASE1\)) gene, involved in chromatin breakdown during apoptosis\(^54\).

In recent years, considerable progress in defining risk loci for SLE has been made through candidate-gene studies and GWAS and today more than 40 SLE risk genes have been identified at genome-wide significance (\(p<5\times10^{-8}\))\(^55\). These genetic associations are restricted to moderate effects,
with odds ratios of non-HLA loci between 1.15 to 2.3. Most risk genes are located in immune related pathways, such as type I IFN signaling, IC clearance, antigen presentation, T and B cell signaling (Table 2).

Table 2. Immune-related pathways in the pathogenesis of SLE highlighted by identified susceptibility genes.

<table>
<thead>
<tr>
<th>Pathway</th>
<th>Gene</th>
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<tbody>
<tr>
<td>DNA degradation</td>
<td>TREX1, DNASE1, DNASE1L3</td>
</tr>
<tr>
<td>Phagocytosis of cellular debris</td>
<td>FCGR2A/B, FCGR3A/B, ATG5, ITGAM</td>
</tr>
<tr>
<td>Processing of ICs</td>
<td>C1Q, C2, C3, C4A/B, C1R/C1S</td>
</tr>
<tr>
<td>Antigen presentation</td>
<td>HLA-DRB1<em>03:01, HLA-DRB1</em>15:01</td>
</tr>
<tr>
<td>TLR/Type I IFN signaling</td>
<td>IRF8, STAT4, IRF5, IRF7, TLR7, PHRF1, IRAK1, IFIH1, TYK2, PRDM1, ETS1, ELF1, UBE2L3</td>
</tr>
<tr>
<td>T cell function and signaling</td>
<td>STAT4, PTPN22, TNFSF4, CD44, ETS1, TYK2, HLA-DR2, PRDM1, AFF1, IKZF1</td>
</tr>
<tr>
<td>B cell function and signaling</td>
<td>FCGR2B, BANK1, BLK, LYN, ETS1, PRDM1, IKZF1, AFF1, RASGRP3, NCF2, IRF8, HLA-DR</td>
</tr>
<tr>
<td>Cytokines</td>
<td>IL10, IL21</td>
</tr>
<tr>
<td>Epigenetic modifications</td>
<td>MECP2, MIRNA146A</td>
</tr>
</tbody>
</table>

ICs, immune complexes; TLR, Toll like receptor; IFN, interferon.

Many of the identified risk genes are shared between SLE and other autoimmune diseases suggesting common molecular pathways among different autoimmune disorders. One example is the tyrosine-protein phosphatase nonreceptor type 22 (PTPN22) gene. This gene encodes a protein that inhibits T cell activation and variants of the gene have been associated with a number of autoimmune diseases including diabetes type I, RA, Addison’s disease and myasthenia gravis.

Similar to many other autoimmune diseases a strong genetic association with SLE is seen for haplotypes in the HLA region on chromosome 6. However, the extensive LD that exists among alleles throughout this locus has made it difficult to determine the true causal loci. In Europeans the strongest SLE associated MHC haplotypes contain the class II alleles DRB1*03:01 or DRB1*15:01. In addition DRB1*08:01, DQA1*01:02 and two SNPs, rs8192591 (NOTCH4 in class III) and rs2246618 (MICB in class I), has shown association to SLE in a recent meta-analysis. The mechanisms underlying MHC association in autoimmune diseases is not clearly understood. It is suggested that aberrant class II presentation of self or foreign peptides to auto-reactive T cells leads to breach of tolerance to self-antigens.
Increased expression of type I IFN is found in patients with SLE\textsuperscript{61} and in recent years a number of gene variants involved in IFN signaling have shown association to SLE\textsuperscript{57}. One of these genes, the IFN regulatory factor 5 (IRF5) gene, is one of the most strongly SLE-associated genes outside of the HLA region (Figure 1)\textsuperscript{62}. IRF5 is a transcription factor and has a number of functions including regulation of type I IFN expression\textsuperscript{63}. SLE patients carrying the risk haplotypes for IRF5 express higher levels of IRF5 mRNA and interferon-α (IFNα) induced genes\textsuperscript{64}.

**STAT4**

Variants of the Signal transducer and activator of transcription 4 (STAT4) gene are associated to SLE\textsuperscript{62,65,66} (Figure 1) but also to other autoimmune diseases such as Sjögren’s syndrome, RA and APS\textsuperscript{55,67,68}. STAT4 is a transcription factor and is mainly expressed in a lymphoid and myeloid tissue\textsuperscript{69}. It transmits signals from the receptors of type I IFN, IL-12 and IL-23 and modulates expression of a number of genes\textsuperscript{69}. In NK cells and DCs STAT4 is important for production of cytokines including IFN-γ and TNF-α. STAT4 is also required for IL-12-dependent polarization of naïve CD4 T cells to fully functional T helper 1 cells\textsuperscript{69} and post-transplantation patients deficient of STAT4 have impaired Th1 cell development and reduced IFN-γ production\textsuperscript{70}. Furthermore, STAT4 is important for production of autoantibodies as demonstrated in an lupus mouse model\textsuperscript{71}.

In humans the STAT4 risk variant is associated with a more severe SLE phenotype, characterized by development of disease at an early age, a high frequency of nephritis, a worse nephritis outcome and presence of anti-dsDNA antibodies\textsuperscript{66,72,73}. In addition it has been shown that the STAT4 SLE-risk allele is associated to increased sensitivity to IFNα signaling\textsuperscript{74}. Fine-mapping of STAT4 has led to the identification of several functional variants.
affecting the expression of STAT4. In addition, the IRF5 and STAT4 SLE-risk alleles have been demonstrated to act additively to increase the risk of SLE.

**IRF8**

Variants of IFN regulatory factor 8 (IRF8) have been associated to SLE but also to other autoimmune diseases such as multiple sclerosis and systemic sclerosis. IRF8, previously known as Consensus Sequence-binding protein (ICSBP), is a transcriptional activator or repressor that forms DNA binding complexes with several partners. These complexes regulate the expression of multiple genes involved in, for example, cytokine signaling, cell cycle regulation, and differentiation. In B cells, there are a large number of IRF8 target genes, many of importance in the molecular crosstalk among germinal center immune cells. IRF8 also binds to TRAF6, and is required for TLR9 signaling in pDCs where it promotes type I IFN production. In addition, IRF8 helps to prolong recruitment of transcription factors to the IFN promoters thereby amplifying production of IFN.

**Environmental factors**

Pathological cutaneous reaction to sunlight, photosensitivity is a common feature of SLE and one of the 11 ACR diagnosis criteria for SLE. Ultraviolet radiation (UVR) of the sunburn spectrum and long wave can induce cutaneous lupus and systemic flares have also been described. Outdoor work has been associated to development of SLE and in Scandinavia disease exacerbations are more common in the spring and summer. UVR induces apoptosis of keratinocytes and release of cytokines leading to activation of the disease.

Certain drugs might exacerbate an underlying idiopathic SLE, induce SLE in a predisposed individual or cause a syndrome called drug-induced lupus (DIL). More than 80 different drugs have been associated to DIL including procainamide and hydralazine. Studies have demonstrated that procainamide and hydralazine inhibit DNA methylation in T cells resulting in enhanced auto-reactivity.

Viral infections including Epstein-Barr virus (EBV), Parvovirus B19 and cytomegalovirus have long been suspected in the etiology of SLE. Increased prevalence of EBV infection in young patients with SLE have been reported and there are also case reports of development of SLE following EBV infections. Molecular mimicry between Smith antigen B (SmB), Sjögren’s syndrome antigen (SSA) and an epitope of EBV have been suggested. Besides molecular mimicry, epitope spreading and bystander
activation could also be potential mechanisms by which viral infections contribute to disease.

Sex hormones are important in the pathogenesis of SLE. This is illustrated by the marked preponderance of female SLE during reproductive years \(^{13}\). Disease flares are also common during pregnancy \(^{96}\) and there are case reports of disease exacerbations following ovarian stimulation \(^{97}\). Use of contraceptives with high doses of estrogen have been reported to stimulate flares \(^{3}\) whereas low dose estrogen-containing contraceptives in low risk SLE patients shows no increased risk \(^{98}\).

Cigarette smoking is associated with a modest increased risk of SLE \(^{99}\) and moderate alcohol consumption might be protective but there conflicting results \(^{100,101}\). Moreover, ingestion of alpha-alpha sprouts has been reported to induce and exacerbate SLE disease, possibly via the effects of L-canavanine \(^{102}\).

Autoantibodies

A hallmark of SLE is hyperactive B cells producing antinuclear autoantibodies (ANA) \(^{56}\). These autoantibodies typically recognize nucleic acid or nucleic-acid associated proteins. Usually autoantibodies precede the first clinical symptoms by several years and appear in a temporal hierarchy with anti-SSA and ANA first, then anti-dsDNA, and finally anti-Sm and anti-RNP \(^{103}\). ANA is one of the diagnostic criteria of SLE and is present in 97 to 99% of patients \(^{30,32}\). However, a positive ANA is also found in patients with other autoimmune diseases, chronic infections and at lower levels in up to 5% of a normal healthy population \(^{104}\).

Today over 100 different autoantibodies have been described in SLE \(^{105}\). Two of these antibodies, anti-dsDNA and anti-Sm have a high specificity for SLE and are represented in the classification criteria \(^{30}\). Elevated levels of anti-dsDNA is associated with active disease and nephritis \(^{106}\). Presence of antibodies to RNA-containing antigens such as anti-Sm, anti-RNP, anti-SSA and anti-SSB is associated to increased activation of the type I IFN system \(^{107}\). Antibodies to SSA and SSB are associated to clinical features such as cutaneous lupus, Sjögren’s syndrome and can cause fetal cardiac conduction defects \(^{56}\). Besides the ANAs, antibodies to C1q, phospholipids and related proteins, erythrocytes and platelets are also found in patients with SLE \(^{105}\).

Apoptosis

Apoptosis is the continuous process of programmed cell death that occurs naturally throughout the body to eliminate damaged cells and maintain ho-
meostasis. During apoptosis nuclear molecules undergo extensive cleavage and translocation and appear on the surface as well as in small subcellular structures called blebs. These apoptotic blebs contain known SLE auto-antigens such as SSA, SSB and U1-RNP. Normally these apoptotic cells/blebs are rapidly removed via binding of complement, IgG and C-reactive protein, followed by phagocytosis of macrophages and dendritic cells. In SLE, the apoptotic mechanisms are disturbed due to increased apoptosis\textsuperscript{108,109} and impaired clearance\textsuperscript{110,111} of apoptotic material. This leads to prolonged exposure of nuclear antigens to the immune system and possibly to the breach of tolerance and activation of auto-reactive B cells\textsuperscript{110}.

Another mechanism whereby nuclear antigens are exposed to the immune system is via NETosis. During NETosis neutrophil extracellular traps (NETs) are released by activated neutrophils in response to pathogens. These NETs consists of antimicrobial proteins and histone bound DNA. In a subset of patients with SLE impaired degradation of NETs have been described\textsuperscript{112}.

**Immune complex handling**

Nuclear antigens are exposed to the immune system after apoptosis, necrosis or NETosis. The exposed antigens bind to the continuously produced ANAs resulting in ICs formation. The mononuclear phagocyte system normally removes ICs from the circulation. ICs activate the classical complement pathway, are opsonized by C3b and bind the complement receptor 1 (CR1) receptor on erythrocytes\textsuperscript{56}. The erythrocytes transport ICs to the liver and spleen where the ICs are phagocytized by macrophages. This is mediated via binding of Fcγ receptors to the Fc-portion of the IgG. In SLE a decreased expression of CR1 on erythrocytes has been described and may be due to repeated IC/CR1 interactions or inherited deficiency of CR1\textsuperscript{113}. Furthermore, the low levels of classical pathway components seen in SLE, due to consumption, autoantibodies (anti-C1q) or inherited deficiency may lead to less opsonization and poor binding of ICs to erythrocytes\textsuperscript{52}. In addition, both genetic and acquired FcγR clearance defects have been described in SLE resulting in impaired phagocytosis of IC by macrophages\textsuperscript{114,115}.

**Immune cells**

**T cells**

Several alterations in T cells from patients with SLE have been described indicating that T cells are involved in the pathogenesis of SLE. One prominent feature of SLE T cells is persistent hyper activation\textsuperscript{116,117,118,119}. This is
possibly due to exaggerated response to stimulation through the T cell receptor (TCR)\textsuperscript{120} or comprised activation-induced cell death\textsuperscript{121}. Abnormally low levels of DNA methylation\textsuperscript{122} and an altered gene expression profile have been described in SLE T cells\textsuperscript{123} possibly also affecting the activation status. Further, the breach of tolerance and self-directed response seen in SLE, have the characteristics of a T cell driven response.

Th17 cells are a T cell subset generated from naive CD4 T cells and induce inflammation via release of IL-17 and IL-22. Th17 cells have been found in the kidneys of SLE patients with nephritis\textsuperscript{124}. This is possibly due to altered cytokine profile in SLE with elevated levels of IL-6 and IL-21 and low levels of IL-2. Another T cell subset affected in SLE is the regulatory T cells (Tregs). Tregs are important for suppressing immune response, including activated T cells and have been found in reduced numbers in patients with active SLE. Studies have also demonstrated that the suppressing capability of SLE-Tregs is reduced\textsuperscript{125} but there are conflicting results\textsuperscript{126}. The impaired function of Tregs has been attributed to the low levels of IL-2 and elevated levels of IFN\gamma in SLE\textsuperscript{127}.

T cell activation and signaling is altered in a number of ways in SLE. Pre-clustering of lipid rafts on the surface of T cells have been demonstrated\textsuperscript{128}. This allows adhesion and co-stimulatory molecules to be drawn together making signal transduction occur more effectively. Further, the expression of the TCR \( \zeta \) chain is decreased and replaced by the common \( \gamma \) chain of the Fc\gamma\textsubscript{R}129. As a consequence signaling trough the TCR is followed by a high influx of calcium resulting in altered expression of genes including CD40L\textsuperscript{130}. This co-stimulatory molecule is important for activation of dendritic cells and for antibody production by B cells.

T cells are also linked to SLE by genetic associations including variants of the gene \textit{PTPN22}, TNF ligand superfamily member 4 (TNFSF4) also known as \textit{OX40L} and the gene \textit{CD44}. The proteins encoded by these genes have different functions and are involved in TCR signaling, T cell - B cell signaling and lymphocyte activation\textsuperscript{56}.

\textbf{B cells}

The role of B cells has been thoroughly investigated in SLE given their central role as antibody producers. In SLE B cells are hyperactive and produce a spectrum of autoantibodies and cytokines\textsuperscript{131}. The number of plasma cells are increased in patients with active disease and titers correlate with the levels of anti-dsDNA antibodies\textsuperscript{132}. Due to defective mechanisms in check-points for central B cell tolerance, SLE patients have an increased number of B cells that express an auto-reactive B cell receptor (BCR)\textsuperscript{133}. In addition, the BCR in SLE need a smaller than normal stimuli to be activated\textsuperscript{134}. Recent GWAS in SLE have identified a number of gene variants within the B cell signaling pathways including B lymphocyte kinas (BLK) and B cell scaffold protein
with ankyrin repeats 1 (BANK1). The BANK1 variant results in altered expression of the gene and has been suggested to contribute to sustained B cell receptor signaling and B cell hyperactivity in SLE\textsuperscript{135}.

Cytokines

Cytokines are small soluble proteins that are produced by immune cells and regulate the function of nearby cells via binding to surface receptors. In SLE, altered expression of a number of cytokines has been described but it has been difficult to determine which of those altered functions that is a primary contributor to the disease. However, the important role of IFN\(\alpha\) in the pathogenesis is well established and will be described in detail in later chapters.

B-Lymphocyte Stimulator (BLyS), also called B cell-activating factor (BAFF) is produced by myeloid lineage cells and acts exclusively on B cells through several receptors\textsuperscript{136}. It enhances differentiation of B cells into plasma cells, supports immunoglobulin class switching and improves the survival of B cells\textsuperscript{137-139}. Patients with SLE express elevated levels of BLyS and a higher level is associated with increased serum IFN\(\alpha\) activity and with active disease\textsuperscript{140,141}. In clinical trials a mAb to BLyS, belimumab reduced the number of activated B cells and plasma cells and reduced auto-antibody titers\textsuperscript{142}. Accordingly, as the first drug in 50 years belimumab is now approved for the treatment of SLE.

IL-6 is mainly a pro-inflammatory cytokine produced by monocytes, fibroblasts endothelial cells, B cells and T cells. It has a number of effects including differentiation of B cells and enhanced antibody production\textsuperscript{143}. In addition IL-6 promotes differentiation of T helper 17 cells, suppresses differentiation of regulatory T cells\textsuperscript{143,144} and induces acute-phase proteins such as C-reactive protein\textsuperscript{56}. Elevated level of IL-6 is found in patients with SLE\textsuperscript{145} and clinical trials with mAb against the IL-6 receptor are ongoing\textsuperscript{146}.

TNF-\(\alpha\) is produced early during immune responses and is effective at promoting influx of inflammatory cells into sites of microbial invasion\textsuperscript{56}. It is produced by a number of cells including activated monocytes and macrophages\textsuperscript{147}. In SLE studies have shown elevated levels of TNF-\(\alpha\)\textsuperscript{148}, however clinical trials with mAbs against TNF-\(\alpha\) in SLE have been terminated due to severe adverse events\textsuperscript{149}.

GM-CSF and IL-3

Granulocyte-macrophage colony-stimulating factor (GM-CSF) is produced by a number of different cells including activated T cells, B cells, endothelial cells, macrophages, fibroblasts and epithelial cells\textsuperscript{150}. Bacterial endotoxins, IL-1, IL-6 and TNF-\(\alpha\) are potent inducers and IL-10, IFN-\(\gamma\) and IL-4 inhibits expression of GM-CSF\textsuperscript{150}. Resting T cells do not produce GM-CSF but dur-
ing viral infections both CD4+ and CD8+ T cells produce GM-CSF. Further, CD4+ T helper cells of both Th1 and Th2 type have been shown to produce GM-CSF.

Besides differentiation of hematological cells GM-CSF has a number of functions including promoting production of inflammatory cytokines by monocytes and macrophages. Further, GM-CSF enhance antigen presentation, promotes complement- and antibody-mediated phagocytosis and promote chemotaxis and adhesion of leukocytes. T cell derived GM-CSF has been demonstrated to be crucial for the local differentiation of monocyte derived inflammatory dendritic cells in synovial tissue in a model of inflammatory arthritis. Patients with active SLE have increased frequency of GM-CSF secreting PBMC and elevated levels is found in plasma. Recently, promising results have been reported in clinical trials with mAb against GM-CSF in patients with RA.

Interleukin 3 (IL-3) is expressed mainly by activated T lymphocytes and mast cells. It regulates the production of a wide spectrum of hemopoietic cells including both myeloid and lymphoid cells and a clinical trial using anti-IL3Rα in patients with acute myeloid leukemia (remission) is ongoing. The IL-3 receptor consists of two subunits, the IL3Rα chain and the βc chain, where the βc chain is shared with GM-CSF and IL-5.
The interferons

In 1957 a soluble substance that could interfere with virus replication and protect cells from virus infections was described by Isaacs and Lindenmann\(^1\). Because of this interference, the proteins were called IFNs. The IFNs can be grouped into three types: Type I (IFN-\(\alpha\), -\(\beta\), -\(\omega\), -\(\kappa\), -\(\epsilon\)), Type II (IFN-\(\gamma\)) and Type III (IFN-\(\lambda\)) based on their structural features and receptor usage\(^2\). All type I IFNs bind to the same heterodimeric type I IFN receptor (IFNAR)\(^3\). In this thesis I have studied the regulation of IFN-\(\alpha\) production by pDCs, why I will focus on this cytokine.

The plasmacytoid dendritic cell and IFN-\(\alpha\)

Most cells can produce small amounts of IFN-\(\alpha\) but the principal producer is the plasmacytoid dendritic cell (pDC) previously called natural IFN-producing cell (NIPC)\(^4\). In peripheral blood less than one percent of PBMCs are pDCs and they can be identified by their expression of blood dendritic cell antigen (BDCA)-2 or (BDCA)-4. Upon stimulation pDCs produce very high levels of IFN-\(\alpha\), but also other cytokines such as TNF-\(\alpha\) and IL-6. Following activation, pDCs lose their ability to produce IFN-\(\alpha\) and instead acquire a mature dendritic cell phenotype\(^5\).

The secretion of IFN-\(\alpha\) is induced after endocytosis via Fc\(\gamma\) receptor IIa (Fc\(\gamma\)RIIa) and activation of endosomal toll like receptors (TLR). DNA is recognized by TLR9 and RNA by TLR7. This initiates signaling via myeloid differentiation factor 88 (MyD88) which associates with a complex comprised of Bruton’s tyrosine kinas (BTK), TNF receptor associated factor 6 (TRAF6), IL-1R associated kinas (IRAK) 1 and 4. Subsequent phosphorylation and nuclear translocation of IFN regulatory factor (IRF) 3, 5 and 7 results in transcription of IFN-\(\alpha\)\(^6\). In addition, IRF8 prolongs the production of IFN-\(\alpha\) via binding to the promoter region\(^7\).

In virus infected cells IFN-\(\alpha\) inhibits viral replication and induces apoptosis\(^8\). IFN-\(\alpha\) also has indirect antiviral properties by inducing antiviral peptides and generally by activating the immune system (Figure 2). IFN-\(\alpha\) promotes differentiation of monocytes to antigen presenting cells\(^9\) and induce expression of co-stimulatory molecules on DC\(^\)\(^1\)\(^0\). Further, IFN-\(\alpha\) promotes polarization of helper T cells into Th1 cells,\(^1\)\(^1\) expands and activates cytotoxic T cells,\(^1\)\(^2\) prevents activated T cells from undergoing apoptosis\(^1\)\(^3\) and
promotes the development of long-lived central memory CD4+ and CD8+ T cells\textsuperscript{168,171,172}. In addition, IFN\textalpha enhance B cell differentiation, increase antibody production, promote Ig class switching and maturation of dendritic cells\textsuperscript{173,174}. Furthermore, IFN\textalpha enhances cytotoxicity of NK cells\textsuperscript{175}. This broad activation of the immune system is naturally a great advantage when clearing pathogens. However, if not adequately terminated, the IFN\textalpha driven immune response may lead to development of disease.

\textbf{Figure 2.} Activating effects of interferon-\textalpha on different cells types.

**IFN\textalpha in SLE**

Patients with SLE have increased levels of IFN\textalpha in serum, and the levels correlate with disease activity\textsuperscript{61,176,177}. Further, in the majority of patients, an increased expression of typ I IFN inducable genes, termed IFN signature is seen in peripheral blood\textsuperscript{178-180} and affected tissues such as the kidneys\textsuperscript{181}. This IFN siganture is associated with a more severe disease phenotype including nephritis and hematological manifestations\textsuperscript{178-180,182}. Further, several of the risk gene variants identified in recent GWAS reside in genes belonging to the type I IFN system\textsuperscript{183}. Together these observations indicate a central role for IFN\textalpha in the pathogenesis of SLE.

IFN\textalpha is a powerful stimulator of the immune system and has been used in treatment of certain viral infections and malignancies\textsuperscript{184}. Therapeutic administration of IFN\textalpha to these patients occasionally results in development
of typical SLE autoantibodies\textsuperscript{184} and autoimmune diseases including SLE\textsuperscript{185-187}. Thus, IFNα has the capability of breaching immune tolerance and promote development of autoimmune disease.

Another key finding to understanding the mechanisms behind the ongoing IFNα production in SLE, was that sera from patients with SLE could induce IFNα production in PBMC\textsuperscript{188}. This IFNα inducing factor was later shown to consist of ICs containing nucleic acid or nucleic acid-containing proteins and autoantibodies directed against these structures\textsuperscript{189}. Such ICs can be created \textit{in vitro} by SLE-IgG and apoptotic or necrotic material\textsuperscript{190,191}, or purified nucleic acid containing auto-antigens, such as small nuclear ribonucleoproteins (snRNP)\textsuperscript{192}. Both DNA and RNA containing ICs can induce production of IFNα, but ICs containing RNA are particularly effective inducers\textsuperscript{107}.

The pDCs are found in low numbers in peripheral blood of patients with SLE\textsuperscript{193}. This is probably due to migration to affected tissue such as the kidneys\textsuperscript{194}, the skin\textsuperscript{195}, or lymph nodes\textsuperscript{196}. However, upon stimulation pDCs from SLE patients are functionally competent and produce normal levels of IFNα.

\section*{Regulation of the IFNα production}

A complex network of cells, cytokines and receptors regulate the production of IFNα by pDCs (Figure 3). NK cells triggered by ICs are potent enhancer of IFNα production. This effect is mediated via cell-cell contact through binding of lymphocyte function-associated antigen (LFA-1), and to some extent by secretion of macrophage inflammatory protein 1 beta (MIP-1β)\textsuperscript{197}. Another cell type involved in the regulation of the IFNα response is the B cell. When triggered by IC B cells enhance the IFNα production. This effect is mediated via cell-cell contact and the enhancing effect can be reduced by blocking of CD31\textsuperscript{198}.

Platelets activated by IC also enhance the IFNα production via CD154-CD40 interaction\textsuperscript{199}. Monocytes potently inhibit IFNα production by pDCs via secretion of TNF-α, prostaglandin E2 and reactive oxygen species\textsuperscript{200}. Notably, the suppressive effect of monocytes from patients with SLE is reduced\textsuperscript{200}. Another negative modulator of the IFNα response is the complement system. C1q binds to leukocyte-associated immunoglobulin-like receptor 1 (LIAR-1) on the pDC thereby inhibiting IFNα production\textsuperscript{201,202}.

In this thesis the role of T cells in the regulation of the IFNα production has been studied and the results will be described below.
Figure 3. An etiopathogenic model of the regulation of the IFNα production by plasmacytoid dendritic cells (pDC) in SLE. In genetically predisposed individuals, tolerance is broken and B cells produce antibodies to nucleic acid containing autoantigens and ICs are formed. These interferogenic ICs stimulate the pDC to IFNα synthesis and the synthesis is enhanced by NK cells and B cells also triggered by ICs. Monocytes inhibit IFNα synthesis.
Cardiovascular disease

Cardiovascular diseases (CVDs) are the leading cause of morbidity and mortality worldwide\textsuperscript{203} and in Sweden 38 percent of all deaths are caused by CVD\textsuperscript{204}. CVD refers to a spectrum of diseases involving the heart and the circulatory system. Among the diseases included are coronary heart disease (CHD), and ischemic cerebrovascular disease (ICVD), both of which are examined in the context of SLE in this thesis.

CHD is a condition where the heart arteries are narrowed due to buildup of atherosclerotic plaque along the inner wall of the arteries. This reduces blood flow and thereby supply of oxygen to the muscle cells sometimes resulting in chest pain known as angina pectoris or myocardial infarction. In Sweden the average age for myocardial infarction is 70 years for men and 75 years for women\textsuperscript{205}. During the last 25 years modern treatment of acute coronary syndromes and actions taken to reduce the impact of CVD risk factors has improved prognosis. However, morbidity and mortality due to CHD still remains high\textsuperscript{206}.

ICVD occurs as a result of an obstruction within a blood vessel supplying the brain. The underlying condition for ICVD is atherosclerosis and can cause two types of obstruction, cerebral thrombosis and cerebral embolism. Cerebral thrombosis refers to development of a blood clot at the clogged part of the vessel and cerebral embolism refers to blood clots formed at another location, for example the carotid arteries. Another cause of cerebral embolism is irregular heartbeat, where clots can form in the heart, dislodge and travel to the brain. The average age for ischemic stroke in Sweden is 73 years for men and 78 years for women\textsuperscript{206}.

Atherosclerosis

Atherosclerosis is a slowly progressing chronic inflammatory condition in the intima layer of large and medium-size arteries\textsuperscript{207}. Already during childhood the first signs of atherosclerosis, fatty streaks can be seen\textsuperscript{208}. Over time, lesions develop forming a necrotic core covered by a fibrous cap. This plaque can cause narrowing of the lumen and if rupturing trigger formation of a thrombus.

The artery wall consists of three different layers, the intima, the media and the adventitia, in order from the inside out (Figure 4a). The intima is
composed of the endothelium and a thin layer of fibrocollagenous tissue. The media is composed of smooth muscle cells and elastic fibers and the adventitia is mainly composed of collagen.

Risk factors

Traditional risk factors for development of atherosclerosis include both non-modifiable and modifiable risk factors. The non-modifiable conditions include old age, male gender and a family history of CVD. The modifiable conditions include smoking, dyslipidemia, hypertension, diabetes mellitus, overweight, sedentary lifestyle, stress, alcohol, low consumption of fruits and vegetables. In addition, a number of markers of inflammation have been associated to CVD, including CRP.

Twin and family studies have shown that a significant proportion of susceptibility to CVD is heritable. Via candidate gene studies a number of gene variants associated to CVD have been described, these include variants of the angiotensin converting enzyme gene, the coagulation factor VII gene and the IL-1β gene. With the introduction of GWAS, SNPs located in the 9p21.3 region were found to be associated to CVD. Recently, the CARDIOGRAMplusC4D consortium increased the total number of coronary artery disease susceptibility loci to 46 and via network analysis a strong link to pathways involved in lipid metabolism and inflammation was demonstrated. Further, the META-STROKE collaboration recently verified two gene variants previously associated to atrial fibrillation, Paired-Like Homeodomain 2 (PITX2) and Zink Finger Homeobox 3 Gene (ZFHX3) to be associated with cardioembolic stroke. In addition large vessel stroke was associated to variants at the 9p21.3 locus and variants of the histone deacetylase 9 (HDAC9) gene. Thus, demonstrating that different risk genes are associated to different stroke subtypes. When combining the genetic data from these two meta-analysis a number of loci that influence the risk of both CHD and ischemic stroke were demonstrated with the strongest signal for the 9p21.3 locus. However, several of the top CAD associated SNPs displayed no association to ischemic stroke, suggesting both general and disease specific genetic influence in the development of CVD.

Pathogenesis

The development of atherosclerosis is initiated by activation of the endothelium by irritating stimuli such as pro-inflammatory cytokines, smoking and hypertension. This enhances expression of adhesion molecules that capture leukocytes and also increases endothelial permeability. Low-density lipoproteins (LDL) enter the intima of the artery wall, are retained via binding of apolipoprotein B100 to the extracellular matrix and exposed to oxidative modifications caused by different enzymes. This leads
to formation of oxidized-LDL and release of bioactive lipids and further activation of endothelial cells and macrophages\textsuperscript{207}.

Expression of adhesion molecules and chemokines attracts monocytes, DC and T cells to the intima. In the intima macrophages up regulate the scavenger receptors enabling uptake of oxLDL which eventually turns the macrophages into foam cells. In addition, cells of the innate immune system including monocytes, DC and mast cells are activated via pattern recognition molecules, resulting in production of pro-inflammatory molecules and cytokines, including IL-1\textbeta and TNF\textsuperscript{215}. Disease related antigens, such as oxLDL presented by macrophages and DCs can trigger the activation of antigen-specific T cells in the atherosclerotic lesion. Most of these activated T cells produce Th1 cytokines, including IFN-\gamma which further activates macrophages and endothelial cells driving the inflammatory process further\textsuperscript{207}.

In response to the inflammatory process smooth muscle cells from the artery muscle layer migrate to the intima and proliferate (Figure 4c). Moreover extra-cellular matrix production is stimulated and a fibrous cap surrounding the cells is formed. Some foam cells undergo apoptosis and release lipids that form the necrotic core of the plaque. Thus the atheroma has a core of lipids including cholesterol crystals, living and apoptotic cells and a fibrous cap containing smooth muscle cells and collagen\textsuperscript{214}.

The stability of the plaque can be altered by secretion of cytokines such as TNF-\alpha and IFN-\gamma secreted by activated T cells, macrophages and mast cells. Activated macrophages and mast cells also produce metalloproteinases that degrade the fibrous cap, making the plaque unstable. If the plaque ruptures, pro-thrombotic substances of the necrotic core such as tissue factor are exposed in the vessel lumen (Figure 4d). This leads to platelet aggregation, activation of the coagulation cascade and the formation of a thrombus\textsuperscript{215}.
Figure 4. Stages in the development of atherosclerotic lesions. a. The normal artery contains three layers; the intima, media and adventitia. b. The initial step of atherosclerosis include adhesion of blood leukocytes to activated endothelial cells, migration of leukocytes into the intima, maturation of monocytes into macrophages, uptake of lipids, yielding foam cells. c. Lesion progression involving migration of smooth muscle cells from the media into the intima, synthesis of extracellular matrix proteins. Extracellular lipids derived from dead and dying cells accumulate forming a necrotic core. The advanced plaque also contains cholesterol crystals and microvessels. d. Fracture of the fibrous cap enabling blood coagulation components to come in contact with tissue factors, triggering formation of a thrombus. Reprinted by permission from Nature Publishing Group: Nature, Libby et al. copyright 2011.
Cardiovascular disease in SLE

Epidemiology

Patients with SLE have premature onset of CVD. In 1976 a bimodal mortality pattern was seen in SLE with early deaths due to active SLE disease complicated by infections, and a later peak of mortality due to myocardial infarction. Subsequent studies have demonstrated a 2 to 10-fold increased risk of myocardial infarction in SLE compared to the general population. The relative risk is especially high among young patients, whereas the absolute risk is higher among older patients. This increased risk was demonstrated in a study of young women with SLE, and showed that women with SLE between the age of 35-44 years had a 50 fold increased risk of myocardial infarction compared to women of similar age in the Framingham study.

Modern treatment of SLE has reduced the risk of death due to inflammation in major organs, but death caused by CVD shows no such decline. The standard mortality rated due to CVD has been reported to be between 1.7 and 3.0. In a recent prospective study CVD was found to be the most common cause of death in SLE patients, being the primary cause of death in 48% of deceased patients.

Atherosclerosis in SLE

Multiple studies have demonstrated accelerated atherosclerosis in patients with SLE. Histopathologic studies of tissues obtained post mortem has shown more extensive and more severe atherosclerotic lesions in patients with SLE compared to healthy controls. Patients also more frequently have coronary artery calcification as determined by electron beam computer tomography. Further, impaired endothelial function, assessed by flow mediated dilation has been demonstrated in SLE patients compared to controls.

Measurement of the common carotid intima media thickness (CCA-IMT) by ultrasound is a widely used method to study early subclinical atherosclerosis and presence of plaques. Increased IMT is associated with cardiovascular risk factors, presence of atherosclerosis and is a strong predictor of future vascular events including MI and stroke. In SLE numerous studies have
shown increased prevalence of carotid plaques\textsuperscript{225-228} but results of IMT measurements vary. Some studies report increased IMT\textsuperscript{228} while other find reduced IMT\textsuperscript{225} in SLE patients compared to healthy controls. However, a number of large studies find no difference between patients and controls\textsuperscript{226,227,229,230}. The reason for this is unclear and will be discussed further in paper I.

**Traditional risk factors**

Traditional CVD risk factors are more prevalent in patients with SLE and accumulate over time. Patients are more likely to have hypertension\textsuperscript{231}, diabetes mellitus\textsuperscript{232} and dyslipidemia\textsuperscript{233-236}. Other traditional risk factors such as age\textsuperscript{237,238}, male sex\textsuperscript{239}, high BMI\textsuperscript{239} and smoking\textsuperscript{235,240} also contribute to the increased CVD risk\textsuperscript{241}. In a large longitudinal study age, male sex, elevated systolic blood pressure and elevated cholesterol independently predicted cardiovascular events in a multivariable model\textsuperscript{236}. Thus, as in the normal population these risk factors are also important in SLE. However, after adjusting for these traditional CVD risk factors SLE patients still have an excess risk of CVD\textsuperscript{217,236}.

**SLE related risk factors**

Several SLE related factors have shown association to CVD. These include pro-thrombotic antiphospholipid antibodies (aPL)\textsuperscript{26,218}, impaired renal function\textsuperscript{242}, increased endothelial cell apoptosis\textsuperscript{243}, improper endothelial repair\textsuperscript{244}, decreased activity of lipoprotein lipases\textsuperscript{245}, SLE related treatment such as high doses of steroids\textsuperscript{225,236} and the impact of an ongoing production of type I IFN\textsuperscript{246,247}.

**IFNα and development of CVD**

In recent years IFNα has been implicated in the pathogenesis of CVD in SLE. Presence of an IFN signature has been associated to both impaired endothelial function\textsuperscript{244} and to more severe coronary calcification in patients with SLE\textsuperscript{246}. A number of different mechanisms for this association have been described. Studies have shown an imbalance between endothelial cell (EC) damage and repair, with increased number of circulating apoptotic ECs\textsuperscript{243} and decreased number of endothelial progenitor cells (EPC)\textsuperscript{244,248}. The number of circulating EPC have been demonstrated to correlate both with disease activity and to levels of type I IFN in patients with SLE\textsuperscript{244,248}. Further, IFNα have been demonstrated to have a direct effect on EPC by
reducing the capacity to form new endothelial cells, resulting in impaired vascular repair.\textsuperscript{248}

IFNα producing pDCs are found in the shoulder region of plaques and IFNα in combination with bacterial products have been demonstrated to increase synthesis of TNF-α, IL-12 and MMP9 in plaque tissue.\textsuperscript{249} Thus, IFNα can function as an inflammatory amplifier and thereby possibly altering the stability of the plaque. In addition, IFNα up-regulates macrophage scavenger receptor A, thereby enhancing uptake of oxLDL and foam cell formation.\textsuperscript{250}

An interferon signature is seen in platelets of patients with SLE and this signature is associated with a history of CVD.\textsuperscript{251} Further SLE platelets display an activated phenotype and may be more prone to take part in thrombotic events.

**Antiphospholipid antibodies**

Antiphospholipid antibodies (aPLs) include a broad range of antibodies directed against phospholipids (PLs), PL-binding proteins or PL-protein complexes. Both venous and arterial thrombosis are closely related to presence of aPLs. This increased risk of thrombosis occurs as a result of activation of monocytes, thrombocytes and endothelial cells resulting in enhanced expression of tissue factor and production of pro-inflammatory cytokines. Further, aPLs interact with different factors in the coagulation cascade shifting the equilibrium in favor of a pro-thrombotic state.\textsuperscript{252}

Prospective studies have shown an association between aPL and future cardiovascular events in SLE.\textsuperscript{26,235} However, the role of aPL in development of atherosclerosis in SLE is unclear. Some studies have reported an association to atherosclerosis\textsuperscript{253} while others have been unable to do so.\textsuperscript{225} Further, patients with recurrent thrombosis often have thrombosis of the same type, either venous or arterial.\textsuperscript{254} Thus, indicating potentially different underlying pathogenic mechanisms between these two phenotypes.

**Lipid dysregulation**

Dyslipidemia in SLE is characterized by elevated levels of very low-density lipoprotein (VLDL) and triglycerides (TG) and low levels of high-density lipoprotein. This lipid profile is more pronounce during disease flares. The underlying mechanisms involve reduced lipoprotein lipase (LPL) activity, effect of cytokines such as TNF-α and different antibodies including anti-LPL and aPL.\textsuperscript{245} Further, elevated levels of pro-inflammatory HDL (piHDL) and oxLDL have been observed in SLE.\textsuperscript{88}
Glucocorticoids

A large longitudinal study recently showed that SLE patients currently treated with a prednisone dose of 20 mg/day or more have a substantial increased risk of CVD events after adjustment for disease activity\(^\text{236}\). Others have shown longer duration and higher cumulative dose of prednisone to be associated with atherosclerosis\(^\text{231,255}\). On the other hand, a low to average prednisone dose has been associated to reduced atherosclerosis\(^\text{225}\). Thus prednisone possibly has dual roles in CVD risk. Aggressive control of disease activity and reduction of inflammation is important. Nevertheless, high glucocorticoid doses can influence traditional CVD risk factors such as hypercholesterolemia, hypertension, obesity, insulin resistance and diabetes thereby enhancing development of atherosclerosis. Further, the increased risk of CVD associated with high prednisone doses could be attributed to a higher SLE disease activity.

Inflammation and other risk factors

Inflammatory mediators play a key role both in development of atherosclerosis and by activating the coagulation cascade. A number of cytokines, in addition to IFN\(\alpha\) have been associated with CVD in SLE, including TNF-\(\alpha\)\(^\text{256}\) and IL-\(\beta\)\(^\text{257}\). In addition, elevated levels of high-sensitivity CRP have been associated to progression of IMT in patients with SLE\(^\text{235,258}\).

Both a higher SLE disease activity and a higher SLICC damage index have been associated to atherosclerosis\(^\text{225}\). In addition impaired renal function is associated with CVD events in SLE\(^\text{242}\).

In recent years research has shown that a subset of patients with SLE has impaired degradation of NETs\(^\text{112}\). These NETs may, when in contact with the endothelium provide cytotoxic signals resulting in damage of endothelial cells\(^\text{259}\).

Genetics of CVD in SLE

Mannose binding lectin (MBL) recognizes carbohydrates on pathogenic micro-organisms, activates complement and is important in host defense against bacteria. Gene variants of MBL, resulting in low expression of MBL has been associated to CVD\(^\text{260,261}\) in patients with SLE but also in other populations\(^\text{262}\). Further, SLE patients with MBL gene variants resulting in low expression have increased IMT\(^\text{263}\). Others genes suggested to be associated with CVD in SLE include variants of the CRP\(^\text{264}\) and matrix metalloproteinase-2 (MMP-2) gene\(^\text{265}\).

Recently we have demonstrated that SLE patients carrying the HLA-DRB1*04 and/or HLA-DRB1*13 alleles have increased risk of vascular events and presence of aPLs\(^\text{266}\). HLA-DRB1*04 was associated to ICVD.
and remained as a risk factor after adjusting for traditional CVD risk factors and aPLs. Neither HLA-DRB1*04 or HLA-DRB1*13 were associated to SLE.
Present investigation

Aims of the thesis

The general aim of the thesis was to study different aspects of cardiovascular disease in SLE with a focus on genetic risk factors. Further, the role of T cells in regulating the IFNα production in SLE was investigated.

The specific aims were:

- Paper I - To separately assess the thickness of the carotid intima and media layers in premenopausal women with SLE and compare the results with healthy controls.

- Paper II - To investigate whether the SLE risk allele rs10181656 (G) located in the signal transducer and activator of transcription 4 (STAT4) gene is associated with vascular events and/or the presence of aPL in SLE.

- Paper III - To identify gene variants important for the development of coronary heart disease in SLE.

- Paper IV - To investigate the role of T cells in regulation of the IFNα production by pDCs stimulated with RNA-ICs.
Patients and methods

Patients and controls
All patients participating in the four studies (paper I-IV) fulfilled four or more of the 1982 revised criteria for classification of SLE\textsuperscript{30} and all participants gave informed consent.

Participants in the first study (Paper I) included 47 premenopausal women with SLE from Uppsala University hospital, 20 healthy women of similar age and 17 postmenopausal women\textsuperscript{267}. Both control groups had participated in a previous study\textsuperscript{268}. The second study (Paper II) included 424 patients from the rheumatology clinics at Uppsala University hospital and Karolinska hospital, Stockholm and 154 patients from the rheumatology clinic at Lund University hospital\textsuperscript{269}. In addition 656 controls, matched for ethnicity and region of living were included. The third study (Paper III) included 575 patients from the rheumatology clinics in Uppsala, Stockholm and Lund and 239 patients from Umeå, collected in the four northern most counties of Sweden\textsuperscript{33}. The population controls included 532 persons matched for ethnicity and region of living.

Patients participating in the fourth study (paper IV) were all from the rheumatology clinic at Uppsala University hospital. Healthy blood donors at Uppsala University hospital and healthy controls working at Uppsala University served as controls.

Disease activity and damage indices
SLE disease activity in paper I and IV was determined with the modified Systemic Lupus Erythematosus Disease Activity 2000 Index (SLEDAI-2K)\textsuperscript{34}, where complement levels and anti-dsDNA antibodies were omitted. Cumulative disease damage was measured using the Systemic Lupus International Collaborating Clinics /American College of Rheumatology Damage Index\textsuperscript{39} (paper I-IV).

CVD definitions
Paper II
Ischemic heart disease (IHD): Myocardial infarction (MI), confirmed by electrocardiography and rise in plasma creatinine kinase, muscle and brain fraction (CKMB) or troponin T and/or angina pectoris confirmed by exercise stress test.

Ischemic cerebrovascular disease (ICVD): Stroke including cerebral infarction, confirmed by computer tomography or magnetic resonance imaging and/or transitory ischemic attacks (TIA), defined as transient focal symptoms from the brain or retina with a maximum duration of 24 hours.
Ischemic peripheral vascular disease (PIVD); Intermittent claudication and/or peripheral arterial thrombosis or embolus confirmed by angiogram or doppler flow studies.

Any arterial event; Occurrence of one or more of the above.

Venous thromboembolism (VTE); Deep vein thrombosis, confirmed by venography or ultrasonography and/or pulmonary embolism, confirmed by radionuclide lung scanning or angiogram.

Paper III

Coronary heart disease (CHD); MI, diagnosed by increased plasma troponin T or CKMB, and 1 of the following: symptoms of ischemia, ECG changes, development of pathological Q wave or new regional wall motion abnormality, or angina pectoris confirmed by exercise stress test.

Carotid ultrasound

In paper I the common carotid artery (CCA) wall layers were imaged using high resolution equipment fitted with a probe at 22MHz center frequency (Osteoson®, Minhorst Company, Meudt, Germany) as previously described\textsuperscript{268,270}. The artery walls were examined with the person sitting upright and looking straight ahead following a 15 minute rest. The transducer was applied at the point of maximal pulsation of the left CCA, in front of the sternocleidomastoid muscle. The depths of penetration was up to 20 mm. Twenty point estimates of the near artery wall, not adjusted to the cardiac cycle, were saved on a computer and measured off-line by another researcher who was blinded to the group and the time of assessment. The mean of 10 technically acceptable measurements of the intima and media layers were calculated and used in the analysis.

In paper III the CCA were imaged using a duplex scanner with a linear array transducer. Pictures were frozen with the R wave on the ECG. The CCA-IMT was defined as the distance between the leading edge of the luminal echo and the leading edge of the media/adventitia echo. Measurements were made on the far wall of both the left and right distal CCAs. The CCA-IMT is the mean calculated from the intima-media area divided by the calculated length (10 mm). Plaques were defined as local increase in wall thickness >1 mm and >100% increase in wall thickness compared with the adjacent wall.

Genotyping

In paper II the SNP rs10181656 had previously been genotyped using the GoldenGate assay (Illumina) in 497 patients and 536 controls\textsuperscript{66}. In addition, 417 partly overlapping participants were genotyped using the SNPstream system (Beckham-Coulter).

In paper III all patients had previously been genotyped using custom 12k Illumina iSelect BeadArrays\textsuperscript{271}. The SNPs included in the array had been selected from loci with a p<0.05 in a previous SLE GWAS scan\textsuperscript{62}, previous-
ly reported SLE risk genes, and confirmed risk loci in other autoimmune diseases. A total of 5676 SNPs remained for analysis after quality control and exclusion of ancestry markers.

**Antibodies**

In paper II anti-cardiolipin (IgG and IgM) and anti-beta2-glycoprotein1 (IgG) anti-prothrombin (IgG) were analyzed by ELISA (Organtec) and >2aPLs was defined as occurrence of two or more of these antibodies. Lupus anticoagulant (LAC) was determined using a modified Dilute Russell Viper Venom method (Biopool) using Bioclot LAC.

**Cell isolation and in vitro stimulation**

In paper IV, PBMCs were prepared from healthy blood donor buffy coats using Ficoll-Hypaque density gradient centrifugation. Via negative selection, pDCs, T cells and myeloid dendritic cells (mDCs) were isolated from PBMCs using magnetic bead separation technology (MACS, Miltenyi Biotec). To induce IFNα, pDCs were stimulated with RNA-IC, HSV type 1, or the CpG-containing ODN2216. The RNA-IC was generated in vitro by combining purified U1snRNP and IgG from a patient with SLE, purified by protein G chromatography. HSV was prepared by propagation of the virus in WISH cells and inactivated by UV-light. T cells cultured alone or in coculture with pDCs were activated by anti-CD3/CD28 beads (Dynabeads). In the mixed leucocyte reaction, T cells and mDCs were co-cultured for 6 days before the supernatant was added to pDC cultures.

**Detection of IFNα and other cytokines**

In paper IV, IFNα was measured with dissociation lanthanide fluoroimmunoassay (DELFIA), using anti-IFNα LT27:293 as a capture antibody and europium-labelled LT27:297 as a detection antibody. The concentrations of IFN-γ, TNF-α, IL-2, IL-5, IL-3 and MIP-1α was measured by ELISA (BioLegend and R&D Systems) and the concentration of MIP-1β was measured by AlphaLISA (Perkin Elmer).

**Statistics**

Continuous variables were analyzed by Mann-Whitney U test (paper I, II, III), Kruskal-Wallis test (paper II) or Wilcoxon signed rank test (paper IV). Categorical variables were analyzed by Chi-square test (paper I, II, III) and correlations were assessed by Spearman rank correlation test (paper I). Univariate and multivariable logistic regression analysis estimated the impact of risk factors (paper II, III). Allele frequencies between groups were compared with Chi-square test and meta-analysis were based on Mantel-Haenszel estimates (paper II, III). Independent effects were assessed using a logistic regression model including the dosage of the SNP rs925994 as a covariate (paper III).
Results and discussion

Paper I

*Increased carotid intima thickness and decreased media thickness in premenopausal women with systemic lupus erythematosus: an investigation by non-invasive high-frequency ultrasound*

Patients with SLE have increased risk of CVD and premature development of atherosclerosis. The gold standard to assess development of preclinical atherosclerosis is by measurement of the IMT and detection of plaque in the CCA. This is performed by 7-10 MHz frequency ultrasound and increased CCA-IMT or presence of plaque is associated with CVD. In SLE increased CCA-IMT and higher frequency of plaques would be expected given the increased risk of CVD. However, measurement of IMT has given varying results. One large study by Roman et al in NEJM showed that CCA-IMT was lower in patients with SLE compared to persons without SLE. Other studies have demonstrated increased CCA-IMT in SLE and a number of studies have been unable to show a difference. These inconsistent results made us ask if a novel method separately assessing the thickness of the different layers of the artery wall, *i.e.* the intima and the media, would give additional information concerning the status of the artery wall.

A total of 47 premenopausal women with SLE, mean age 37 years, 20 healthy women of similar age and 17 postmenopausal women were included in the study and separate measurements of the intima and media of the CCA by high frequency ultrasound were conducted. We found that women with SLE had a thicker intima, thinner media and a higher intima/media ratio (I/M) compared to healthy controls of similar age (*Figure 5*). Further, the I/M ratio in the SLE group was similar to the I/M ratio in the group of 30 year older postmenopausal women. In the SLE patients, a thinner carotid media was associated with a more severe SLE disease including higher SLICC/ACR damage index, a higher cumulative dose prednisone, and an increased C3d/C3 ratio. In addition, SLE patients with a history of myocardial infarction had significantly thinner media compared to patients without myocardial infarction.

Development of atherosclerosis starts with recruitment of inflammatory cells and accumulation of lipids resulting in increased thickness of the intima layer. In addition, smooth muscle cells from the media migrate into the intima further enhancing this process. Simultaneously the thickness of the media is reduced possibly due to migration of smooth muscle cells, hypoxia, mechanical compression by the intima and inflammation. The reduction of
media thickness was shown by Gussenhoven et al. which used high frequency (40 MHz) ultrasound to demonstrate that the media thickness was inversely related to an increase in atherosclerotic lesions. In the present study we show that patients with SLE have an increased intima and reduced media compared to healthy controls. Given the morphologic changes of the artery wall described above the findings are in line with increased atherosclerosis in these patients.

![Figure 5. Carotid artery near-wall dimensions assessed by 22 MHz ultrasound in premenopausal women with SLE compared to pre- and postmenopausal women without SLE. Mean ±SD. *** p < 0.001 and **** p < 0.0001 compared with SLE.](image)

In the present study we find reduced combined IMT in patients with SLE compared to controls. Similar results have been described previously in SLE suggesting a healthier artery wall in these patients. However, we could also describe divergent development of the thickness of intima and media layers suggesting development of atherosclerosis. These divergent changes have previously been described in subjects with CVD. By using the same high frequency ultrasound method persons with CVD were found to have increased intima, reduced media and a higher I/M ratio compared to persons without CVD. Further, no difference regarding combined IMT was seen in this study. Given these results it would be preferable to assess development of atherosclerosis by using high frequency ultrasound both in the general population and in patients with SLE.

In conclusion we find that young premenopausal women with SLE have morphological alterations in the carotid artery wall suggesting premature development of atherosclerosis. The high frequency ultrasound separately estimating of the thickness of the carotid intima and media layers appears to be preferable to CCA-IMT in assessing subclinical atherosclerosis in patients with SLE. This method could be useful in identifying SLE patients at increased risk of CVD.
A STAT4 risk allele is associated with ischemic cerebrovascular events and antiphospholipid antibodies in Systemic Lupus Erythematosus

Patients with SLE are at increased risk of CVD and a number of both traditional and SLE related risk factors have been described. However, little is known about possible genetic contribution to the increased risk of CVD. STAT4 has been identified as one of the strongest associated risk genes in SLE\textsuperscript{62}. This gene variant is associated to a more severe disease with high frequency of nephritis, a worse nephritis outcome and a younger age at disease onset\textsuperscript{66,72,73}. In this study we asked if the STAT4 risk allele rs10181656 (G), is associated to vascular events or aPL in patients with SLE.

Two groups of patients with SLE were genotyped (group I, n=424; group II, n=154) and the allele frequency was compared between patients with and without vascular events and aPL. Results show that the STAT4 risk allele was more common in patients with arterial events and more specifically in those with ICVD. The results were consistent in both patient groups and in the combined analysis of the two groups the association was strengthened (Table 3). Further, a dose dependent association between the number of risk alleles and manifestations of ICVD was seen, where homozygous patients had the highest risk for ICVD. When adjusting for other known CVD risk factors such as age and hypertension, the STAT4 risk allele remained as a strong independent risk factor. No significant association was seen between the risk variant and ischemic heart disease (IHD), ischemic peripheral vascular disease (PIVD) or venous thromboembolism (VTE) but presence of more than 2 aPLs showed an association to STAT4. A dose dependent association was also seen between presence of more than two aPLs and the STAT4 risk variant.

STAT4 is a transcription factor expressed in a number of different cell types and has numerous functions including regulation of antibody production by B cells\textsuperscript{71}. In SLE the STAT4 risk variant is associated with increased levels of anti-dsDNA antibodies\textsuperscript{66}. Further, STAT4 has been shown to be associated with APS\textsuperscript{67}, a condition with elevated levels of aPLs. In the present study we demonstrate an association between STAT4 and presence of more than two aPLs. Thus, one hypothesis would be that the STAT4 risk variant enhances auto-antibody production in patients with the risk allele, resulting in enhanced expression of different types of autoantibodies including aPLs.
Table 3. Association of STAT4 with vascular events (VE) and anti-phospholipid antibodies. Patients with VE are tested against patients without VE.

<table>
<thead>
<tr>
<th>Combined*</th>
<th>SLE with VE**</th>
<th>SLE without VE**</th>
<th>Controls</th>
<th>P</th>
<th>OR*** (95% C.I.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Any arterial event</td>
<td>0.41</td>
<td>0.32</td>
<td>0.22</td>
<td>0.003</td>
<td>1.5(1.1-2.0)</td>
</tr>
<tr>
<td>ICVD</td>
<td>0.52</td>
<td>0.32</td>
<td>1.6x10^-5</td>
<td>2.3(1.6-3.3)</td>
<td></td>
</tr>
<tr>
<td>Ischemic stroke</td>
<td>0.53</td>
<td>0.27</td>
<td>2.4x10^-5</td>
<td>2.4(1.6-3.6)</td>
<td></td>
</tr>
<tr>
<td>IHD</td>
<td>0.38</td>
<td>0.33</td>
<td>1.2(0.8-1.7)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PIVD</td>
<td>0.39</td>
<td>0.34</td>
<td>1.2(0.7-2.3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>VTE</td>
<td>0.38</td>
<td>0.33</td>
<td>1.3(0.9-1.7)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>≥2 aPL</td>
<td>0.44</td>
<td>0.32</td>
<td>0.003</td>
<td>1.6(1.2-2.0)</td>
<td></td>
</tr>
</tbody>
</table>

*Mid Sweden and Southern Sweden. ** Frequencies for the rs10181656 (G) allele. *** (95% C.I.)

A significant association was seen between the STAT4 risk allele and ICVD but not with IHD. This might be due to different patterns of aPLs in these two groups. We found that patients with ICVD more often had aCL IgG whereas patients with IHD did not have elevated levels of aPLs. These results are in line with findings in another study where aPLs of IgG isotype were found to be associated to ICVD but not IHD and aPLs of IgM isotype were weakly associated to IHD. APLs are known to affect the coagulation system in multiple ways resulting in enhanced thrombus formation. Thus, elevated levels of aPLs might increase the risk of a condition of thromboembolic complication such as ICVD. These findings are also in line with other studies suggesting that aPLs primarily is a risk factor for ICVD rather than IHD.

In the present study no association between the VTE and the STAT4 risk variant was seen despite elevated levels of aPLs in patients with VTE. One explanation for this discrepancy could be that different pathological mechanisms are involved in thrombosis formation in VTE and ICVD. Patients with APS experiencing recurrent thrombosis often have the same type, i.e. venous or arterial thrombosis. One possible explanation for this difference is the finding of elevated levels of endothelin-1, a marker of endothelial activation in APS patients with stroke but not in APS patients with venous thrombosis. Others have shown that STAT4 is involved in regulating the pro-inflammatory behavior of endothelial cells exposed to IFNα. Given the elevated levels of IFNα seen in SLE this role of STAT4 might be important. One hypothesis could thus be that the STAT4 risk variant affects the function of endothelial cells and in the presence of aPL this results in arterial thrombosis.

In conclusion we show that a STAT4 risk variant is associated to SLE-related ICVD and with pro-thrombotic aPLs. Genetic predisposition is thus an important risk factor of ICVD in patients with SLE and aPL may be one underlying mechanism.
Paper III

_Coronary Heart Disease in Systemic Lupus Erythematosus is associated with interferon regulatory factor 8 gene variants._

Two groups of patients with SLE were genotyped using a custom made array including 6000 SNPs (group I, n=575; group II, n=239). The allele frequency was compared between patients with and without CHD in the first group where we found 61 SNPs associated (p<0.01) to CHD. The allele frequency of these 61 SNPs was then assessed in the second group. Two SNPs both located in the IFN regulatory factor 8 (IRF8) gene, were significantly associated to CHD in both groups of patients. These two SNPs were the strongest and third strongest SNP associated to CHD in the first population (Table 4). Both identified SNPs were located in introns and in high linkage disequilibrium (LD) with each other, thus representing the same signal. All patients homozygote for the risk variant had a history of CHD. The IRF8 risk locus remained as a risk factor for CHD after adjustment for traditional CHD risk factors. Further, we could demonstrate an association between the IRF8 risk variant and increased CCA-IMT and presence of carotid plaque. To study the effect of the risk allele on different cell types PBMCs were stained for intracellular IRF8. We found that only a minority of PBMCs express IRF8 and that there was no significant difference between patients with and without the risk allele. However, a reduced frequency of B cells in patients with the risk allele was demonstrated. Further, weaker binding of nuclear protein to a highly linked SNP was demonstrated by electrophoretic mobility shift assay and potential protein binding-motifs were identified.

<table>
<thead>
<tr>
<th>SNP</th>
<th>Population 1</th>
<th>Population 2</th>
<th>Meta-analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>P OR*</td>
<td>P OR*</td>
<td>P OR*</td>
</tr>
<tr>
<td>IRF8-rs925994</td>
<td>5.5x10⁻⁵</td>
<td>3.7 (1.9-7.4)</td>
<td>1.1x10⁻²</td>
</tr>
<tr>
<td>IRF8-rs419030</td>
<td>6.2x10⁻⁵</td>
<td>2.1 (1.5-3.1)</td>
<td>0.4</td>
</tr>
<tr>
<td>IRF8-rs10514610</td>
<td>1.2x10⁻⁴</td>
<td>3.7 (1.8-7.4)</td>
<td>6.2x10⁻³</td>
</tr>
</tbody>
</table>

The 3 SNPs with the strongest association to CHD in study population 1. Unadjusted P values. *(95% C.I.)*

In this study we show that an IRF8 risk variant is associated to CHD in patients with SLE. SNPs located in IRF8 have previously shown association to increased risk of SLE. However, the IRF8 risk locus identified in the present study is not in LD with any of these SLE IRF8 risk variants. Further the IRF8 risk allele was not associated to a more severe SLE disease. Thus, indicating that the increased risk of CVD is not mediated via the SLE disease itself or by a more severe SLE disease phenotype. IRF8 gene variants have
previously not been associated to CHD in the general population as determined by results from previous GWAS. The reason for this is not clear but the IRF8 risk allele might not have been thoroughly investigated. Further, it might be a rare variant affecting only a subgroup of patients or it might enhance the risk of CHD specifically in patients with SLE or in patients with other autoimmune diseases.

In recent years a number of studies have shed light on the role of IRF8 in development of atherosclerosis. Döring et al. showed that atherosclerosis prone mice deficient of IRF8 develop more severe atherosclerosis compared to controls\textsuperscript{277}. An accumulation and apoptosis of neutrophils was seen in atherosclerotic lesions possibly enhancing inflammation in the plaque. Pourcet et al. demonstrated that macrophage arginase 1 (Arg1) expression is induced by IRF8 in synergy with purine box factor 1 (PU.1)\textsuperscript{278}. This is interesting given that Arg1 levels inversely correlate with atherosclerosis progression. Thus, IRF8 might have an anti-atherosclerotic function. Zhang et al. demonstrated that IRF8 facilitates smooth muscle cell phenotype switch and neointima formation in response to arterial damage\textsuperscript{279}, indicating an important role for IRF8 in vascular repair. In our study we show that the IRF8 risk variant is associated to increased CCA-IMT and presence of carotid plaque and thereby more severe atherosclerosis. It remains to be investigated whether the IRF8 risk locus identified by us affects the different atherosclerotic mechanisms described above.

Another important function of IRF8 is regulation of type I IFN signaling. It has been demonstrated that IRF8 prolongs recruitment of transcription factors to the IFN promoters thereby amplifying the IFN production\textsuperscript{83}. Supporting this role of IRF8 is our finding of very high expression of IRF8 in pDCs. The pDC is the main IFNα producing cell and IFNα is via several different mechanisms including, enhancing EPC apoptosis and foam cell formation important in the development of atherosclerosis in SLE\textsuperscript{247}. Further, IFNα secreted by plaque located pDCs have been implicated in the process leading to plaque rupture\textsuperscript{249}. Thus, the IRF8 risk gene variants may contribute to CHD in SLE by affecting the function of the type I IFN system.

We found a reduced frequency of B cells in patients with the IRF8 risk allele. IRF8 is known to be important in the development of B cells and their function in germinal centers\textsuperscript{82}. Thus, potential mechanisms of the IRF8 risk allele could be reducing B cell development or accumulation of B cells in lymphoid tissue. B cells are also found in atherosclerotic tissue and different B cell subtypes have been described to be both atheroprotective and proatherogenic\textsuperscript{280}. Therefore another possible effect of the IRF8 risk allele could be altering the balance of the different B cell subsets in plaque tissue thereby enhancing atherosclerosis development.

In conclusion we have identified a novel genetic locus to be associated to CHD in SLE. This study highlights that nontraditional risk factors for atherosclerosis are important in SLE.
Activated T cells enhance the interferon-α production by pDCs stimulated with RNA-IC

Patients with SLE have a prominent IFNα signature and levels of IFNα in the circulation correlate with both disease activity and severity. The pDCs are the main IFNα producing cells and produce large amounts of IFNα when triggered by ICs containing nucleic acid. IFNα has a number of immunological functions, including dendritic cell priming, Th1 polarization, activation of Tc cells and enhancing B cell differentiation and antibody production. In recent years IFNα has also been implicated in the development of CVD in SLE. The IFNα production by pDCs is regulated by a complex network of cells including NK cells, B cells and monocytes. In this study we asked if T cells can modulate the IFNα production by RNA-IC stimulated pDCs.

PBMCs were prepared from blood donor buffy coats and T cells and pDCs were isolated. RNA-IC containing U1snRNP and IgG from a patient with SLE were used to induce IFNα production by pDCs and the levels of IFNα was determined by DELFIA. In this study, we showed that activated T cells strongly promote the IFNα production by RNA-IC triggered pDCs. The effect was mediated via secretion of GM-CSF and IL-3. When GM-CSF and IL-3 was neutralized or when their receptor subunits were blocked the enhancing effect was reduced. Further, activated T cells were demonstrated to enhance the frequency of pDCs expressing the co-stimulatory molecules CD80 and CD86 also via GM-CSF and IL-3. Activated T cells from patients with SLE were demonstrated to enhance the IFNα production to the same extent as did T cells from healthy controls (Figure 6).

To activate T cells we used anti-CD3/CD28 labeled beads. This signal resemble the binding of an antigen presenting cell (APC) presenting its antigen to the T cell. However, to verify this method we also activated T cells in a mixed leukocyte reaction demonstrating similar results. Further, we showed that both activated CD4+ and CD8+ T cells could enhance the IFNα production. Next, we compared the effect of activated T cells from patients with SLE with T cells from healthy controls. Results show that activated T cells from both groups enhance the IFNα production to the same extent. Numerous studies have shown that patients with SLE have persistently activated T cells\textsuperscript{117,118,281}. This could be due to exaggerated response to stimulation through the T cell receptor (TCR)\textsuperscript{120} or due to comprised activation-induced cell death\textsuperscript{121}. Therefore, one might expect that T cells from SLE patients more strongly would enhance the IFNα production. However, the patients participating in this study were all in remission and therefore possibly exposed to less active T cells. This is supported by findings of enhanced activation of SLE T cell during periods of active disease compare to periods of...
remission. Thus, it would be interesting to study T cells from SLE patients with active disease to determine if these cells more strongly could enhance the IFNα production.

Figure 6. Activated T cells from patients with SLE and healthy controls enhance the interferon-α (IFNα) producing capacity of plasmacytoid dendritic cells (pDCs). T cells were activated by anti-CD3/CD28 (aCD3/CD28) or isotype control IgG (Iso), and co-cultivated with healthy donor pDCs in the presence of RNA-containing immune complexes. IFNα levels were measured after 20 h.

When comparing the stimulatory effect of the T cell supernatant added to pDCs to that of T cells co-cultured with pDCs a similar enhancing effect was seen. Thus, we considered the supernatant to be important in this system. The cytokine profile was analyzed and cytokines were added to the RNA-IC triggered pDCs at the same concentration as in the supernatant. In addition dose response titrations of the cytokines were performed. The results demonstrated GM-CSF and IL-3 to be responsible for the enhancing effect on the IFNα production. These two cytokines were equally potent enhancers and when GM-CSF and IL-3 were depleted from the supernatant the enhancing effect was reduced by 90 percent. However, GM-CSF was found at a much higher concentration in the supernatant than IL-3 why we conclude that it is the most important cytokine in this in vitro system. GM-CSF has a number of functions including differentiation of hematological cells. But it is also produced by T cells during viral infections resulting in activation of the immune system. However, in the presence of circulating ICs such as seen in SLE, GM-CSF might have a harmful effect by promoting production of IFNα. Both GM-CSF and IL-3 are found at elevated levels in SLE and patients with active disease have increased numbers of GM-CSF secreting PBMCs. We therefore hypothesize that activated T cells via GM-CSF and IL-3 enhance the IFNα production by paces triggered by RNA-IC in patients with SLE (Figure 7).
Figure 7. A schematic illustration of the interaction of T cells and pDCs in regulation of the interferon-α (IFNα) production in SLE. T cells are activated by antigen presenting cells and secrete GM-CSF and IL-3. PDCs triggered by immune complexes and stimulated by GM-CSF and IL-3 produce large amounts of IFNα. IFNα affects T cells in a number of ways including promotion of Th1 pathway and activation of CD8+ T cells.

The function of pDCs also includes antigen presentation. Therefor we investigated the expression of co-stimulatory molecules on pDCs. We found that supernatant from activated T cells significantly increased the frequency of RNA-IC triggered pDCs expressing CD80 and CD86. This enhancing effect was reduced when the supernatant was depleted of GM-CSF and IL-3. Furthermore, when RNA-IC was added to pDCs cultured with GM-CSF and IL-3 a significant increased frequency of CD80 and CD86 was observed. Thus one hypothesis is that a combined activation of pDCs by ICs and activated T cells occur in SLE. This eventually results in enhanced antigen presentation by the pDCs, which will promote the autoimmune process.

In conclusion we find that activated T cells enhance the IFNα production by pDC via GM-CSF and IL-3. The observations of activated T cells and elevated levels of GM-CSF and IL-3 in SLE suggest that T cells contribute to the ongoing IFNα production seen in patients with SLE.
General discussion

Cardiovascular disease is the main cause of long-term morbidity and mortality among patients with SLE. Despite better treatment of acute flares and improved overall prognosis the risk of CVD remains high. One reason for this might be insufficient treatment of the SLE disease itself. Most patients today are treated with medications that exert their effects through broad nonspecific suppression of the immune system. Patients considered in remission still often experience fatigue, pain and episodes with other milder manifestations of the disease, indicating that the SLE disease is not completely under control. Patients with RA also have increased risk of CVD. Recent studies have demonstrated that RA patients successfully treated with TNF-α inhibitors have a risk of CVD equivalent to that of the general population. These results suggest that adequate treatment of the rheumatic disease can normalize the risk of CVD. In SLE no drug so far has demonstrated positive effects comparable to the effects of TNF-α inhibitors in RA. With better treatment of SLE one might speculate whether the risk of CVD would also be reduced. Recently the first drug in over 50 years was approved for treatment of SLE, an antibody directed against BLyS. This drug reduces the effect of BLyS resulting in reduced numbers of plasmacells and lower titers of autoantibodies. Though generally demonstrating a modest clinical effect, anti-BLyS therapy has significant improved health status for a subset of patients. In addition to anti-BLyS a number of candidate drugs are being evaluated in SLE. Most of these drugs target different components of the IFN signaling pathway and have been shown to reduce the IFN signature. Initial results show modest effects but clinical trials are ongoing. These new drugs might also have positive effects on CVD given the recent findings of a possibly prominent role of type I IFN in development of atherosclerosis in SLE. However, to successfully target the IFN system further knowledge of the regulation of the IFN response is important. In this thesis the role of T cells in regulating the IFN response is demonstrated and the importance of GM-CSF and IL-3 is highlighted. Given the promising results of anti-GM-CSF treatment in RA and the relatively few side effects reported it might also be considered a candidate drug in SLE. However, studies of GM-CSF in SLE are relatively scarce and further studies are needed. Hopefully better understanding and treatment of the SLE disease itself will improve overall health but also morbidity and mortality related to CVD.

Measurement of intima-media thickness in the CCA is a widespread accepted surrogate marker for subclinical atherosclerosis in the general population. Previous studies in SLE have shown marked heterogeneity in IMT results, raising the question whether IMT measurement is generally useful in these patients. Further, a number of studies have demonstrated significantly more plaque in SLE compare to controls but at the same time finding no difference in IMT. Hence, it has been suggested that atherosclerotic
lesions might occur independently of subclinical atherosclerosis in SLE\textsuperscript{287}. In this thesis the individual artery layers of the CCA were assessed by high frequency ultrasound demonstrating significant morphological differences between SLE patients and healthy controls. These results suggest that a process involving both the intima and media is ongoing in the artery wall of these patients possibly representing development of atherosclerosis. As atherosclerosis is a chronic inflammatory process continuously affecting the artery wall, early detection of patients with an ongoing atherosclerotic process is important. The new ultrasound method described in this thesis could be preferable to the traditional CCA-IMT for identifying SLE patients at increased risk of CVD enabling early intervention and reduction of CVD risk factors.

During the last few years new genotyping technology has enabled major progress in identification of susceptibility genes for SLE. Many of these genetic risk factors contribute to the function of the immune system by affecting pathways involved in for example B and T cell function and regulation of the type I IFN system. These risk loci provide an opportunity to investigate pathogenic mechanisms contributing to the disease. Today over 40 SLE risk genes have been identified and a few of these gene variants have been associated to different manifestations of the disease\textsuperscript{57}. In this thesis two gene variants located in \textit{STAT4} and \textit{IRF8} were found to be associated to different manifestations of CVD in patients with SLE. These findings could be somewhat surprising as one might expect gene variants to affect all types of manifestations of CVD. However, our results are in line with studies in the general population demonstrating risk genes to be associated to different manifestations of CVD such as different stroke subtypes\textsuperscript{212}. Thus, indicating different underlying mechanisms to be involved in the pathogenesis of CVD.

Further, our results highlight the importance of studying association between risk genes and different SLE disease phenotypes. Risk genes only affecting a subset of patients might be missed when studying all SLE patients as a group. In the years to come a better understanding of the genetics of SLE, including investigating patients with different phenotypes will hopefully lead to development of new diagnostic and prognostic tests and provide new targets for therapy.

Concluding remarks

The focus of this thesis has been both cardiovascular disease and immunological mechanisms in SLE. The importance of genetic predisposition for development of CVD is highlighted. Two risk genes \textit{STAT4} and \textit{IRF8} were found to be associated to ICVD and CHD respectively in patients with SLE. The exact mechanisms by which these risk genes convey the increased risk of CVD is not clear. Both genes are involved in interferon signaling but if
this function is of importance for development of CVD needs further investigation. Further, these findings highlight that non-traditional risk factors are important for development of CVD in SLE. Thus, a deeper understanding of the mechanisms behind the atherosclerotic process in SLE is needed as well as new therapeutic strategies to improve morbidity and mortality due to CVD in patients with SLE.

An interferon signature is seen in the majority of patients with SLE and IFNα has been suggested to be of key importance in the pathogenesis. In the fourth paper activated T cells were demonstrated to be potent enhancers of the IFNα production via secretion of GM-CSF and IL-3. This mechanism could be important in SLE given the reports of activated T cells and elevated levels of GM-CSF and IL-3. Medical trials with anti-GM-CSF are ongoing in patients with RA and preliminary results are promising. However, to determine whether treatment with anti-GM-CSF could be beneficial for patients with SLE further investigation is needed.
Systemisk Lupus Erythematosus (SLE) är en autoimmune, inflammatorisk sjukdom som karakteriseras av produktion av auto-antikroppar, bildandet av immunkomplex och ett aktiverat typ I interferon system. Under senare år har prognosen generellt förbättrats och istället har den ökade risken för kardiovaskulär sjukdom komit i focus. Målet med denna avhandling är att studera kärlväggens olika lager samt att identifiera рискgener för kardiovaskulär sjukdom hos personer med SLE. Interferon-α (IFNα) är ett viktigt cytokin vid SLE och flera läkemedelsstudier pågår där IFNα blockeras med antikroppar. I denna avhandling studeras T-cellers roll vid reglering av plasmacytoida dendritiska cellers (pDC) produktion av IFNα.

I den första studien används en ny högfrekvent ultraljudsmetod för att studera tjockleken av de olika lagren i kärlväggen i halspulsådern, arteria carotis. Vi fann att premenopausala kvinnor med SLE, medelålder 37 år, hade en tjockare intima, tunnare media och högre intima/media kvot jämfört med friska kontroller. Detta tyder på ökad risk för kardiovaskulär sjukdom. Vid traditionell ultraljudsundersökning av halskärlen där tjockleken på intiman plus median uppskattas har man vid SLE fått olika resultat. Därför kan denna nya metod vara att föredra för att identifiera och följa patienter med SLE med ökad risk för kardiovaskulär sjukdom.

I studie nummer två studeras om en riskgen för SLE, STAT4 är associerad med ökad förekomst av kardiovaskulär sjukdom eller fosfolipidantikroppar hos patienter med SLE. Vi fann att patienter med STAT4 riskvarianten hade ökad risk att drabbas av stroke och/eller TIA. En möjlig förklaring till den ökade risken är att patienterna med STAT4 riskvarianten hade ökad förekomst av fosfolipidantikroppar vilket kan ge upphov till stroke.

I den tredje studien visar vi att en variant av genen IRF8 är associerad till hjärtinfarkt och/eller angina hos patienter med SLE. Patienter med riskgenen hade även ökad förekomst av åderförkalkning. Vidare visades att nukleärt protein band svagare till riskvarianten vilket tyder på att den kan vara inblandad i regleringen av uttrycket av IRF8. Lägre frekvens av B celler hos patienter med riskvarianten kunde påvisas men fortsatta studier krävs för att klargöra de underliggande mekanismerna för hur IRF8 ger upphov till ökad åderförkalkning och ischemisk hjärtssjukdom.

I den fjärde studien studeras T-cellers roll vid reglering av interferonproduktionen. PDC är en celltyp som producerar IFNα då den triggar av immunkomplex innehållande t.ex. RNA och antikroppar riktade mot RNA. Vi
kunde visa att aktiverade T-celler kraftigt förstärker pDCs produktion av IFNα (över 20 gånger) via sekretion av cytokinerna GM-CSF och IL-3. Vidare kunde vi påvisa att aktiverade T-celler från patienter med SLE förstärker produktionen av IFNα lika mycket som aktiverade T-celler från friska kontroller. Eftersom man påvisat förekomst av aktiverade T-celler och förhöjda nivåer av både GM-CSF och IL-3 vid SLE kan aktiverade T-celler vara viktiga för det aktiverade typ I interferon systemet vid SLE.
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