On the immunopathogenesis of systemic lupus erythematosus - Immune complexes, type I interferon system, complement system and platelets

Lood, Christian

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On the immunopathogenesis of systemic lupus erythematosus

Immune complexes, type I interferon system, complement system and platelets

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Lund University, Sweden

Doctoral dissertation
With due permission from the Medical Faculty at Lund University this doctoral thesis is to be publicly defended on the 16th of May 2012, at 9.00 in Belfragesalen, D15, Biomedical Center, Lund.

Faculty opponent
Professor Dror Mevorach
Division of Medicine
Center for Research in Rheumatology
Hadassah University Hospital Eink-Karem
Jerusalem, Israel
On the immunopathogenesis of systemic lupus erythematosus

Abstract
Systemic lupus erythematosus (SLE) is an autoimmune disorder characterized by inflammation in several organ systems. SLE patients have an impaired ability to clear dying cells which leads to the exposure of several nuclear antigens which might break self-tolerance. Autoantibodies directed against the nuclear antigens will form large immune complexes (ICs) which are central in the immunopathogenesis of SLE. Through activation of the complement system, ICs mediate tissue destruction, and through interaction with immune cells ICs induce large amounts of pro-inflammatory cytokines, including IFN-alpha. Furthermore, ICs might activate platelets and thus be involved in the development of cardiovascular diseases which is increased in SLE patients.

In this thesis I have investigated the immunopathogenesis of SLE with an emphasis on the immune complexes, complement system, type I interferon (IFN) system and the platelets. In the first paper we studied the plasmacytoid dendritic cell (pDC) and properties regulating their IFN-alpha producing ability, a central cytokine in the SLE pathogenesis. We found that C1q, a component of the classical pathway of the complement system, could regulate the type I interferon production by pDCs (Paper I). Furthermore, pDCs were able to produce a pro-inflammatory protein, S100A8/A9, which is increased in SLE patients (Paper II). Next we observed that the complement system and type I IFN system affect the platelets. Platelets isolated from SLE patients were more activated, had a type I IFN signature (Paper III), as well as complement deposition on the cell surface (Paper IV). Both the type I IFN signature and the complement deposition were associated with cardiovascular disease and venous thrombosis. In the final paper we found that EndoS, a bacterial endoglycosidase, abolished all pro-inflammatory properties of immune complexes isolated from SLE patients (Paper V). Thus, we suggest that EndoS has the potential to be developed as a new therapy against SLE considering its potent immune complex-modulating effects.

In summary, this thesis confirms the central role of immune complexes and their subsequent pro-inflammatory effects in the immunopathogenesis of SLE and development of cardiovascular disease and venous thrombosis.

Key words: Systemic lupus erythematosus, complement system, type I interferons, plasmacytoid dendritic cell, calprotectin, immune complex, cardiovascular disease, platelet.

Supplementary bibliographical information:

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On the immunopathogenesis of systemic lupus erythematosus

- Immune complexes, type I interferon system, complement system and platelets

Christian Lood

Lund University
Faculty of Medicine

Lund 2012
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Cover image:  
The most frequent non-common words used in the five papers were randomly put together in a word cloud using the Wordle™.

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To my Family

Either write something worth writing
or do something worth writing

-Benjamin Franklin
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1 Introduction

1.1 Preface

Four years ago I started a fascinating journey into the field of clinical science. Never could I have imagined the joy and excitement I was about to experience working with the autoimmune disease systemic lupus erythematosus (SLE). It has truly been inspiring to work with patient material with the aim to reveal new pathogenetic pathways in SLE and novel targets for development of therapies. After the defense of my master thesis, young and naïve, I knew that we could solve the pathogenesis of SLE within a couple of years. Today, I am grateful that I will be able to work with this puzzling disease for many years to come. Once accepted for PhD studies we decided that the title of the thesis should be "On the immunopathogenesis of systemic lupus erythematosus - immune complexes, type I interferon system, complement system and platelets". To scientifically prove that I indeed had been working with these topics for the last years I used a web-based software to identify the most common words of the published papers. As illustrated at the front page, all of the topics of the title were highlighted as well as several others which will be discussed in further detail later in this book. The thesis consists of five original papers discussing various pathological events in the autoimmune disease SLE. I will start by presenting the titles of the papers and give you a brief summary of the investigation in Swedish. Even though only my name is printed at the front page many more have contributed to this thesis, both socially and scientifically, and are greatly acknowledged! For those of you that are unfamiliar with the subject of the thesis, I have included some general chapters about immunology, platelets and the autoimmune disease systemic lupus erythematosus. Even though the different topics are presented as separate chapters they are all interwoven in the SLE pathogenesis and you might find some topics being discussed several times. If you are familiar with medical science, chapter 8 will give you an overview of the present investigation and the main findings. Finally I have added the original papers at the end for those of you who want to scrutinize the findings. I truly hope that you all will start to appreciate the intriguing signaling pathways and molecules of the immune system, and foremost learn more about the pathogenesis of lupus after reading this book.

Enjoy the reading,

[Signature]

8
1.2 List of papers included in the thesis


IV. **Lood C.**, Eriksson S., Gullstrand B., Jönsen A., Truedsson L., Bengtsson AA. Increased C1q, C4 and C3 deposition on platelets in patients with systemic lupus erythematosus - a possible link to venous thrombosis? Submitted manuscript.


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Paper III has been reprinted with the permission from the American Society of Hematology.
1.3 List of papers not included in the thesis


### 1.4 Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ACR</td>
<td>American College of Rheumatology</td>
</tr>
<tr>
<td>aPL</td>
<td>Anti-phospholipid</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen-presenting cell</td>
</tr>
<tr>
<td>BDCA</td>
<td>Blood dendritic cell antigen</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
</tr>
<tr>
<td>CR</td>
<td>Complement receptor</td>
</tr>
<tr>
<td>CVD</td>
<td>Cardiovascular disease</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic cell</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>HLA</td>
<td>Human leukocyte antigen</td>
</tr>
<tr>
<td>IC</td>
<td>Immune complex</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
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<tr>
<td>IFNAR</td>
<td>Interferon alpha receptor</td>
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<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
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<tr>
<td>IRF</td>
<td>Interferon regulatory factor</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>MAC</td>
<td>Membrane attack complex</td>
</tr>
<tr>
<td>MASP</td>
<td>MBL-associated serine protease</td>
</tr>
<tr>
<td>MBL</td>
<td>Mannose-binding lectin</td>
</tr>
<tr>
<td>mDC</td>
<td>Myeloid DC</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>MI</td>
<td>Myocardial infarction</td>
</tr>
<tr>
<td>NET</td>
<td>Neutrophil extracellular trap</td>
</tr>
<tr>
<td>PAMP</td>
<td>Pathogen-associated molecular pattern</td>
</tr>
<tr>
<td>PBMC</td>
<td>Peripheral blood mononuclear cell</td>
</tr>
<tr>
<td>pDC</td>
<td>Plasmacytoid DC</td>
</tr>
<tr>
<td>PMN</td>
<td>Polymorphonuclear neutrophil</td>
</tr>
<tr>
<td>PRR</td>
<td>Pattern recognition receptor</td>
</tr>
<tr>
<td>RAGE</td>
<td>Receptor for advanced glycation endproducts</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>SLE</td>
<td>Systemic lupus erythematosus</td>
</tr>
<tr>
<td>SLEDAI</td>
<td>SLE disease activity index</td>
</tr>
<tr>
<td>SOCS</td>
<td>Suppressor of cytokine signalling</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal transducer and activator of transcription</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
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</table>
1.5 Tack


Jag vill också rikta ett stort tack till mina båda biträdande handledare Lennart Truedsson och Gunnar Sturfelt. Lennart, tack för att du planterade intresset för forskning hos mig när jag kom första gången som sommarprojektsstudent och visade mig det fantastiska komplementsystemet! Du har alltid kommit med goda idéer när jag har fastnat i projekten och din metodologiska kunskap är något jag sätter ett stort värde på. Gunnar, tack för all uppmuntran och kunskap som du har gett genom åren och för ditt engagemang för SLE-forskning som har smittat av sig!

Jag har också haft möjligheten under min tid som doktorand att få jobba på en arbetsplats utan dess like med människor som har kommit att betyda mycket för mig. Först och främst vill jag tacka Birgitta Gullstrand, utan dig hade inte mycket blivit gjort! Dina hjärn- och blodceller har varit stor del i min framång. Stort tack för allt ditt engagemang och positiva tänkande när saker och ting inte gick i läs och för att du alltid har tid för att prata. Du har lärt mig mycket av det laborativa jag kan och för det är jag mycket tacksam och har alltid varit villig att hjälpa till. Det har varit en stor ära att lära känna dig och din optimistiska livsåskådning och personlighet, tappa aldrig den! Jag vill också tacka Gertrud Hellmer och Eva Holmström som alltid fanns där och stöttade upp och som ständigt och jämnt tvingade mig (?) att lära mig mer om datorer och allt lustigt som kan hända med dem. Ett speciellt tack till dig Eva som tog emot mig under mitt första projekt och gav mig viljan att jobba vidare inom detta område. Ett speciellt tack också till dig Gertrud som gav mig en djup inblick i hur pipettboxarna förflyttade sig mellan våningarna, ovärderlig kunskap!

Jag vill också tacka Lillemor Skattum! Du har en otrolig kunskap och en förmåga att leda och styra upp men ändå med en stor ödmjukhet. Jag är väldigt glad att jag har fått lov att arbeta med dig och önskar dig all lycka i framtiden!

Det har varit ett fantastiskt arbetsklimat under alla år jag har varit här och det är mycket tack vare alla underbara kollegor i byggnaden. Först och främst skulle jag vilja tacka alla forna och nuvarande rumskollegor. Det var ofta ett stort och tomt tab men Malin, Anna och alla studenter ska ha ett stort tack för att ni förgyllde mina dagar med ert sällskap och lärde mig om världen utanför! Jag har också fått ovärderlig hjälp av Maria och Micke med mikroskoperingen och med en positivism som är få

Jag vill också rikta ett stort tack till Barbro Sanfridsson på Blodcentralen för all support med Luminexen. Hade det inte varit för ditt alltid så vänliga bemötande hade jag nog inte vågat mig dit igen efter alla komplikationer jag ställde till med.

Jag har också haft möjligheten att träffa en mängd fantastiska människor på Reumatologiska kliniken i Lund. Först och främst vill jag tacka alla kliniker jag har kommit i kontakt med: Ola Nived, Andreas Jönsen, Michele Compagno, Ragnar Ingvarsson och Helena Tydén. Ola, tack för alla fina tågresor du har ordnat, och för dina goda råd. Andreas, tack för optimistiskt tänkande, all hjälp med databaserna och trevliga konferensresor. Michele, Ragnar och Helena, tack för gott sällskap generellt och i synnerhet på alla resor vi har varit igång på! Ni har alla en förmåga att entusiasmera er omgivning och sprida glädje och jag hoppas att detta är något era patienter uppskattar. Lycka till mer era avhandlingar! Tack också alla patienter som har deltagit i våra olika studier. Ni har alltid givit ställt upp även när ni har haft en period av svårare sjukdom och jag hoppas att något av det vi har gjort under dessa år ska komma er till nytta inom en snar framtid.


Under min period som doktorand har jag också fått möjligheten att lära känna kollegor utanför vår egen avdelning. Först och främst vill jag rikta ett stort tack till Svenska SLE-nätverket och alla ni som ingick i det. Det har alltid varit trevligt att träffa er på våra möten i Johannesberg och ni har varit ett stort stöd i min utveckling. Jag vill framförallt tacka Lars Rönnblom och Maija-Leena Eloranta i Uppsala för all er hjälp och uppmuntran och för att ni introducerade mig till det fascinerande typ I IFN systemet. Vidare vill jag tacka hela Linköpingsgänget, ni är helt underbara människor och jag har alltid sett fram emot konferenser där ni är med för då vet man att det blir skoj!

Jag vill också tacka alla andra medförfattare och samarbetspartners som vi har haft, dels i Lund, men även i övriga Sverige och Danmark. Det har varit roligt att lära känna er var och en och jag är tacksam för all uppmuntran och inspiration som ni har gett. Framförallt vill jag lyfta fram David Erlinge som var orsaken till att jag började studera blodplättar. Även om jag initialt grymtade till lite över att behöva jobba med några ”cellfragment” så är jag idag mycket tacksam över att ha fått upp ögonen för dem.


Min sista dedikation går till min lillebror Rolf. Även om jag som sex minuter äldre, med betydligt mer livserfarenhet och vishet, borde ha visat dig vilken väg du skulle gå har du ofta istället gett mig en hjälpende hand! Du är som jag brukar säga en förbättrade version av mig och det stämmer med råge. Du betyder väldigt mycket för mig och jag är mycket tacksam för all hjälp du har gett mig under doktorandperioden. Jag har svårt att se att jag skulle ha kunnat få en bättre bror att dela vardagen med! Lycka till i stora världen och glöm inte mig till Nobelfesten!
1.6 Swedish summary

Människokroppen är fantastisk! Trots att vi dagligen utsätts för mängder av bakterier och virus blir vi sällan sjuka. För att behålla denna balans har vi många primära försvarsnätverk så som vårt hudlager och slemhinnor, men ibland krävs det mer konkreta åtgärder för att försvara oss. Till vårt mer specifika försvar har vi en mängd olika immunceller, flera miljoner i bara några droppar blod, som patrullerar i vår kropp och avvärnar alla potentiellt farliga mikrober. Detta ställer mycket höga krav på vårt immunförsvar. Samtidigt som immuncellerna måste vara redo att snabbt identifiera och neutralisera alla farliga mikrober får de aldrig uppleva någon av våra kroppsegna molekyler som fientliga. För att undvika att immuncellerna reagerar mot något kroppssegget får de genomgå en inskolning där de lär sig att skilja på eget och främmande innan de släpps fria i cirkulationen. Immunceller som under sin utbildning reagerar mot våra egna molekyler förstörs omgående. För merparten av oss kommer immunförsvarat att sköta sin uppgift med bravur, men för några individer kommer immunförsvarat att angripa den egna vävnaden och vi utvecklar en så kallad autoimmun sjukdom, såsom typ I diabetes, multipel skleros, reumatoid artrit och systemisk lupus erythematosus (SLE). Även om de yttar sig väldigt olika kliniskt och drabbar olika organism så har sjukdomarna gemensamt att de som skulle försvara vår kropp istället anfaller den. Vid dessa sjukdomar också har gemensamt är att de orsakas av både ärtliga (genetiska) faktorer och vår omgivning och livsstil (Figuur 1.1).

Vid den autoimmuna sjukdomen SLE har man länge ansett att det är ett dåligt städteam som är en bidragande orsak till utveckling av sjukdomen. Cellerna i vår kropp är nämligen inte odödliga utan omsätts ofta: några lever endast någon dag, medan andra lever i flera år. För att ta hand om de döende cellerna måste vi ha ett effektivt städteam som först och främst kan markera de döende cellerna (komplementsystemet) och sedan låta specifika ätarceller (makrofager) plocka upp dem och forsla bort alla cellrester. Tyvärr har makrofagerna hos SLE-patienter en försämrad förmåga att äta upp de döende cellerna. Dessutom har SLE-patienters celler generellt en kortare livslängd, bland annat på grund av en oidentifierad celldödsfaktor. Detta gör att där ansamlas döende celler hos SLE-patienterna. Döende celler är i sig självt inget problem då detta är en aktiv tyst process som inte ger uppfo till inflammation. Om cellerna däremot skulle hinna dö innan de blivit uppätna av makrofagen så börjar de läcka ut olika molekyler som inte borde visas upp för vårt immunförsvarat, såsom vårt genetiska material (DNA). Dessa döda celler ger uppfo till en inflammatorisk process och i ett sådant sammanhang kan några immunceller reagera på dessa celler som kroppsrörande. Där börjar de producera antikroppar mot våra egna celler vilket per definition ger uppfo till en autoimmun sjukdom. Vid SLE hittar man många olika autoantikroppar, främst riktade mot molekyler från cellkärnan såsom DNA och RNA.
När väl dessa molekyler uppvisas på döda celler kommer autoantikropparna att binda in och skapa ett stort kompleks, ett så kallat immunkomplex. Dessa immunkomplex kan sedan fastna i vävnad, exempelvis hud och njurar, och där aktivera olika delar av immunförsvaret och orsaka stor vävnadsskada. Dessutom kan dessa immunkomplex förbruka många av de komponenter som är viktiga vid markeringen av döende celler, det så kallade complementssystemet. Detta medför att ytterligare fler celler hinner dö innan de blir omhändertagna och en ond cirkel har uppstått (Figur 1.1).


Figur 1.1. En illustration över bakomliggande immunologiska reaktioner vid uppkomsten av SLE. Virus, UV-ljus eller "celldödsfaktor" kan inducera celldöd hos SLE patienter. På grund av en dålig funktion hos komplementsystemet städas inte de döende cellerna bort varför de dör och uppvisar flertalet främmande ämnen. Detta kan leda till produktion av autoantikroppar och därmed till utveckling av den autoimmuna sjukdomen SLE. Immunkomplexen som bildas mellan det främmande änet och antikroppar kan fastna i vävnad och orsaka skada genom komplementaktivering. Den vidare komplementåtgången bidrar sedan till en ytterligare minskad förmåga att ta hand om döende celler. Immunkomplexen kan också ätas upp av plasmacytoida dendritceller (pDC) och ge upphov till höga nivåer av interferon-alfa, ett cytokin som via bland annat B celler kan leda till produktion av autoantikroppar och autoimmuna sjukdomar såsom SLE.

I de fem arbeten som ingår i avhandlingen har jag studerat denna sjukdom med fokus på immunkomplex, komplementsystemet, interferon-alfa samt blodplättar. Komplementsystemet är mycket viktigt vid SLE, framförallt vid undanständning av döende celler (Figur 1.1). Dock förklarar inte detta varför en viss molekyl i komplementsystemet (C1q) verkar ha en avgörande
roll för uppkomsten av sjukdomen. Mer än 90% av alla individer med C1q-brist utvecklar SLE medan endast 10-20% av de individer med brist av C2, ett annat komplementprotein, utvecklar SLE. Där verkar alltså finnas en annan viktig funktion för C1q som reglerar uppkomsten av autoimmunitet. Då man tidigare funnit att C1q kunde reglera några signaleringsmolekyler (cytokiner) frågade vi oss om C1q kunde reglera produktionen av interferon-alfa, och därmed förhindra uppkomsten av SLE. För att undersöka om detta var fallet använde vi oss av cellodling. Framrenade immunceller stimulerades med immunkomplex innehållande genetiskt material (RNA) i närvaro av olika koncentrationer av C1q. Interferon-alfa produktionen bestämdes med en ELISA metod där man kan undersöka förekomsten av specifika molekyler i en lösning. Vi fann att C1q, och ingen av de andra komplementkomponenterna, kunde förhindra produktionen av interferon-alfa. I enlighet med den tidigare illustrationen (Figur 1.1) skulle därför C1q kunna skydda mot utveckling av SLE genom att hämna produktionen av interferon-alfa. Vi ville även förstå mekanismen bakom den hämmande funktionen och fann att C1q kunde binda till de plasmacytoida dendritcellerna. Den exakta strukturen som C1q binder till på de plasmacytoida dendritcellerna är målet för fortsatta studier. Skulle man finna hur C1q reglerar interferon-alfa kan man utnyttja detta för att skapa läkemedel med liknande funktion.

I det andra arbetet arbetade vi vidare med de plasmacytoida dendritcellerna för att lära oss mer om dem. När vissa immunceller såsom makrofager och neutrofiler plockar upp material frisläpper de ett inflammatoriskt proteinkomplex som kallas kalprotektin (S100A8/A9). Detta komplex kan ses i förhöjda nivåer hos patienter med SLE men om plasmacytoida dendritceller kunde producera dessa proteiner var inte känt. Med hjälp av genetiska metodar samt olika cellförsök kunde vi beskriva att plasmacytoida dendritceller kunde producera kalprotektin. När plasmacytoida dendritceller tog upp immunkomplex transporterades kalprotektin från insidan till utsidan av cellen. Däremot är det okänt vilken biologisk funktion som detta protei

I det tredje och fjärde arbetet studerade vi den förhöjda risken för att utveckla hjärtkärlsjukdomar hos SLE patienter. Den ökade risken är främst påtaglig hos unga kvinnor med SLE som har en 50-faldig ökad risk att få en hjärtinfarkt jämfört med unga kvinnor utan SLE. Denna skillnad kan inte endast förklaras av traditionella riskfaktorer såsom blodfetter och blodtryck utan sjukdomen i sig själv verkar också vara en viktig riskfaktor. En nyckelspelare vid hjärtkärlsjukdomar är blodplätten, det cellfragment som skapar själva blodproppen. Dock är dessa blodceller inte så väl undersökta vid sjukdomen SLE.


Sammanfattningsvis har vi identifierat nya skeenden i centrala Immunologiska reaktioner i SLE-sjukdomen och med fortsatt forskning inom dessa områden finns möjlighet att slutligen finna nya läkemedelsmål för att behandla dessa patienter.
2 The Immune System

2.1 Introduction

Every day we encounter bacteria and viruses without falling sick, we even benefit from many of our bacteria. However, the pathogens need to be tightly regulated not to cause disease and that is the objective of the immune system. In this section I will briefly discuss the basics of the immune system, how self-tolerance is regulated and the development of autoimmunity.

2.2 Basics of the immune system

The immune system consists of several different organs, tissues, cell populations and proteins all working together to defend us against pathogens. One main barrier separating us from the bacteria is physical hinder including the skin, mucous membranes and the acidic environment in the gut. However, pathogens do penetrate these layers why we need a much more active immune system as well. Upon breach of the barriers, tissue-residing macrophages and dendritic cells will recognize pathogen-associated molecular patterns (PAMPs) on the pathogen. The PAMPs are specifically expressed by pathogens and could be different sugar structures, bacterial DNA or lipopolysaccharide (LPS) for example. The PAMP binds to the pattern-recognition receptor (PRR) on the immune cell and activates it. The PRRs could be Toll-like receptors (TLRs) and complement proteins which will be more extensively discussed in later chapters.

Once the immune cell is activated it releases several signaling mediators, cytokines, to alert surrounding cells about the pathogen and recruit other immune cells such as NK cells and polymorphonuclear neutrophils (PMNs) [1]. The recruited immune cells could then help to clear the infection. All of these components: the physical barriers, the cytokines, the complement system and certain cell populations (monocytes, macrophages, dendritic cells, NK cells and PMNs) are part of the innate immune system and have been evolved to provide a first line of defense and act as soon as they recognize the specific threat. All immune cells of the innate immune system, except for the NK cells and PMNs, are antigen-presenting cells (APCs) and will phagocytose self and non-self material and present it through the major histocompatibility complex (MHC) molecule. The MHC class I molecule is expressed by all nucleated cells and the MHC class II molecule mainly by APCs and they will bind to CD8+ and CD4+ T cells, respectively. NK cells are the main cell population of the innate immune system and they eliminate all cells that do not express MHC class I molecules, such as virus-infected cells and tumors. Erythrocytes however, who do not express MHC molecules, are not targeted by the NK cells [1, 2]. Even though most often not included in the innate immune system, erythrocytes are also important in the removal of complement-opsonized particles to the liver and spleen for destruction [3]. Platelets, membrane-
enclosed residues of the megakaryocyte, are also important in the clearance of pathogens [4], and they will be discussed more extensively in further chapters.

In contrast to the broad and fast innate immune system we also have a specific adaptive immune system. However, physiologically, the distinction between the two systems is not clear and the adaptive and innate immune systems interact to efficiently remove all pathogens. Whereas the innate immune system contains many cell populations, the adaptive immune system only contains two cell populations: the T and B cells. Instead of expressing general PRRs, these cells express antigen-specific receptors: the T cell receptor (TCR) and the B cell receptor (BCR). Once the antigen is presented by the MHC molecule of the APC, the TCR will bind to it, and through the interaction of co-stimulatory molecules (CD80 and CD86) the T cell will become activated. Depending on the inflammatory environment the CD4+ T cell will develop into one of several different specialized cells: Th1, Th2, Th9, Th17 or Treg [5]. They have all different immunological properties, primarily through the different cytokines they are able to produce. The CD8+ T cell will become a cytotoxic effector T cell and eliminate virus-infected and tumor cells once activated.

The BCR is in fact a membrane-bound antibody and it is able to directly identify its antigen. However, to become activated it requires either a cross-linking of several BCRs, which is often mediated by polysaccharides, to induce a T cell-independent maturation, or an interaction with an antigen-specific activated Th2 cell to induce a T cell-dependent maturation. The activation of B and T cells will take several days and thus we rely on the innate immune system to remove the pathogen in time. However, both B and T cells develop an immunological memory for this specific antigen. Next time they encounter the same antigen they are able to respond immediately [2].

### 2.3 Self tolerance and autoimmunity

The B and T cells have to be able to recognize each and every antigenic peptide of a pathogen and still not react against self molecules. To be able to respond to all diverse antigens both the TCR and BCR are made up by rearrangement of several genes during development in the thymus and bone marrow, respectively. If the created TCR or BCR would recognize any self-molecule the cells would die by programmed cell death (apoptosis). However, for the B cell, rearrangements of the BCR might sometimes be sufficient for the B cell to make it through the central tolerance. T cells also have to be able to bind to the MHC class II adequately. During development, T cells with a low affinity for the MHC-peptide complex will not receive the necessary survival signals and die through apoptosis. This is called a positive selection to ensure the capability of the TCR, but does not control self-reactivity. The next step in the maturation of the T cell is called negative selection and ensures that the T cells do not react against self-peptides displayed by MHC molecules. Both the positive and negative selection is referred as central tolerance. Most of the B and T cells do
not pass the central tolerance, but the ones that do not react with any of the exposed antigens in the thymus and bone marrow will be released into the periphery. Some autoantigens are not present in the thymus or bone marrow and will be encountered by the naïve cells in the periphery. For this reason we also have peripheral tolerance to avoid reactivity against self-peptides. If a T cell recognizes a self-peptide it may, by unknown mechanisms, receive an alternative signal through the cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) instead of CD28 which causes anergy. There has also been described other mechanism including the ligation of programmed cell death 1 (PD-1) which induces cell cycle arrest. The interaction with a self-peptide may also lead to activation-induced cell death through up-regulation of the T cell Fas ligand and subsequent interaction with the death receptor Fas on the cell surface [6]. All of those processes ensure that we have a high capacity to find non-self molecules but will not react to self molecules [2, 7]. However, under certain circumstances B cells will become activated and produce antibodies against self molecules, resulting in an autoimmune disease. In some autoimmune diseases the autoantigens are well-defined and restricted to a certain organ system such as type I diabetes, whereas the autoantigens could be more common such as DNA and histones as in systemic lupus erythematosus (SLE). Low concentrations of autoantibodies, mainly IgM, are found in healthy individuals without giving rise to inflammatory autoimmune diseases. These are called natural antibodies and react to several microbial antigens as well as to self molecules and have been described to have an important function in the clearance of apoptotic cells [8]. However, occasionally, B cells producing natural antibodies might undergo somatic hypermutation and start to produce high-affinity IgG molecules directed to self molecules [9]. Several other possibilities exist of how autoimmunity could develop including cross-reactive antibodies if the non-self antigen resembles a self molecule [2, 7].

2.4 Conclusions

The immune system is a complex system of physical barriers, proteins, signaling pathways and immune cells. It can be divided into the innate and adaptive immune system, but they both interact to defend us against non-self. The immune system is trained not to react against self molecules but under certain circumstances cells of the adaptive immune system become self-reactive and we develop autoimmune diseases.
3 The pro-inflammatory molecule S100A8/A9

3.1 Introduction
Upon recognition of a foreign molecule many different signaling substances are produced and secreted, one of which is the heterodimeric complex S100A8/A9, also called calprotectin. S100A8/A9 is composed of two subunits: S100A8 (myeloid-related protein 8; mrp8) and S100A9 (mrp14), belonging to the calcium-binding family of proteins. The components can form homodimers, as well as higher heterodimer oligomeric forms such as tetramers depending on the presence on calcium and other ions. There exist more than 20 members of the S100-family, named so because of their solubility in 100% ammonium sulphate [10]. In this section the cellular origin, immunological properties and cardiovascular effects of S100A8/A9 will be discussed briefly.

3.2 Cellular origin of S100A8/A9
Many different cell populations, including PMNs, monocytes, DCs, B cells, MDSCs, endothelial cells and platelets, have been reported to express S100A8/A9, either on the mRNA level or the protein level [11-17]. Several reports also demonstrate that upon maturation of monocytes to macrophages or pro-inflammatory CD16+ monocytes, the expression of S100A8 and S100A9 is reduced [12, 15]. However, not all cells seem to secrete the protein complex actively. Endothelial cells, for an example, will induce the expression of S100A8 and S100A9 in an inflammatory environment but not secrete the proteins [16]. Even though many cells have expression of S100A8 and S100A9, it is generally believed that PMNs, where S100A8/A9 occupies 40% of the cytoplasm, are the major producer of S100A8/A9, as compared to monocytes who only have about 1% cytoplasmic S100A8/A9 [18]. S100A8/A9 is released from dying cells or upon activation. Once the cell is activated, S100A8/A9 is translocated to the cell surface and is eventually excreted to the plasma [19]. Furthermore, S100A8/A9 could be secreted by the recently discovered neutrophil extracellular traps (NETs), a potent anti-fungal and anti-bacterial mechanism where nuclear material together with S100A8/A9 is released from activated PMNs to catch and opsonize the microbes [20].

3.3 Immunological properties of S100A8/A9
The S100A8/A9 heterodimer has been found in increased concentrations in several inflammatory diseases including SLE, rheumatoid arthritis, Sjögren’s syndrome, cancer, sepsis and inflammatory bowel disease [12, 17, 21-24]. The association with many autoimmune diseases including SLE and rheumatoid arthritis might partly depend on the ability of S100A8/A9 to induce TLR4-mediated production of IL-17 and autoreactive CD8+ T cells as demonstrated in a mice model [25]. It is generally considered that S100A8/A9 is a damage-associated molecular
pattern molecule (DAMP) even though there are a few publications suggesting potential anti-inflammatory effects. The pro-inflammatory properties of this complex and its subcomponents, on the other hand, are well-documented [26].

Several receptors for extracellular S100A8/A9, including TLR4 [27], RAGE [28, 29], CD36 [30], heparin, heparan sulfate and chondroitin sulfate [31, 32] have been identified of which TLR4 is the best characterized and most pro-inflammatory effects seem to be mediated through TLR4-interactions. In mice, S100A8/A9, as well as the individual homodimers, increases the FcγR expression on macrophages [33]. Binding of TLR4 by either the S100A9 homodimer or the S100A8/A9 heterodimer increases the production of pro-inflammatory cytokines by monocytes [34]. Besides the extracellular pro-inflammatory effects of the S100A8/A9 complex, it has important intracellular properties in regulating calcium binding and microtubule function [35].

The increased S100A8/A9 production seen in tumors might be both beneficial and dangerous for the tumor cells. S100A8/A9 can reduce the growth of the cells and induce cell death through chelation of zinc ions. However, S100A8/A9 will also recruit myeloid-derived suppressor cells that migrate to the tumoral tissue and suppress inflammation and promote tumor growth and protection against the immune system [13]. It has also been suggested that the effect of S100A8/A9 is dose-dependent where low doses render tumor survival, whereas high concentration induce cytotoxicity. Thus, S100A8/A9 might have dual roles in the development and progression of tumors [26].

3.4 Effects on the cardiovascular system

Besides the inflammatory diseases and properties discussed above, S100A8/A9 has also been associated with development of cardiovascular diseases, and especially in the atherosclerotic process [16]. Increased concentrations of S100A8/A9 might serve as a predictor of MI [11] and acute coronary syndrome [36]. Patients with acute MI have higher concentrations of S100A8/A9 than patients with unstable angina pectoris [37]. Furthermore, S100A8/A9 expression is increased in the area surrounding the thrombus and atherosclerotic plaque [37, 38] and S100A9 expression in the plaque is associated with plaque rupture [39].

S100A8/A9 is important in the inflammatory process and infiltration of immune cells in the development of atherosclerosis. S100A8/A9, released by the activated PMNs, will bind to the carboxylated glycans on the endothelial cells and increase leukocyte extravasation [31, 32]. The exact mechanism for the increased extravasation is not known, but includes up-regulation of endothelial adhesion integrins (MAC-1) and decreased expression of cell junction proteins [40, 41].

Due to the potent pro-inflammatory properties of the S100A8/A9 heterodimer a quinoline-3-carboxamide derivate (Paquinimod) has been...
developed which could inhibit binding of S100A9 to both TLR4 and RAGE [28]. Paquinimod was able to reduce both hematuria and proteinuria in a lupus mice model and no severe side effects were observed in healthy individuals. However, no substantial effects were seen in the clinical phase I study in patients with inactive SLE. However, there was a trend towards decreased type I IFN production in patients treated with Paquinimod [42] suggesting that S100A9, and S100A8/A9 may operate in the type I IFN system.

3.5 Conclusions

Altogether, S100A8/A9 is a pro-inflammatory protein produced and released by most phagocytes upon activation. The pro-inflammatory effects are mainly mediated through TLR4 and RAGE, and the S100A8/A9-mediated extravasation of immune cells into atherosclerotic plaques might be an important mechanism in the development of atherosclerosis and cardiovascular diseases.
4 The Complement System

4.1 Introduction
The complement system is an important part of the immune system bridging the adaptive and the innate immune system. It was described in 1896 by Bordet as a heat-labile serum component with the ability to complement the antibacterial effect of antibodies. Today, the complement system comprises more than 30 soluble and membrane bound proteins involved in three main activation pathways as well as several regulatory proteins and receptors. Upon activation of the complement system, several activation split products are produced which attract immune cells, mark the target for destruction and may lyse some bacteria through the formation of cell membrane penetrating pores [43]. Genetic deficiencies in the complement system are rare, but clinically important, and associated with an increased susceptibility to bacterial infections and development of the autoimmune disease SLE [44]. In this section the different activation pathways of the complement system, the function of the activation split products, the regulation of the complement system and genetic deficiencies will be discussed in more detail.

4.2 The classical pathway
The classical pathway of the complement system was the first of the three main activation pathways to be identified. C1, the recognition molecule of the classical pathway, is composed of C1q, two molecules of C1r and two molecules of C1s. The shape of C1q is often referred to as a bouquet of tulips due to the six heterotrimeric (A, B and C chain) collagen-like fibers which form a collagen stalk and six globular heads [45]. The classical pathway of the complement system is initiated by the binding of the globular heads of C1q to the Fc-region of IgM or IgG or other activating molecules including C-reactive protein and apoptotic cells [46-49]. To become activated by antibodies, the globular heads of the same C1q molecule need to attach to two or more Fc-regions on bound antibodies. Thus, one IgM molecule can be sufficient for the activation whereas two or more IgG molecules in close proximity are needed [50]. Upon activation, the C1 molecule changes its conformation and the two attached serine proteases C1r and C1s become activated. C1r activates C1s which subsequently cleaves C4 and C2 into larger fragments (C4b and C2a) and smaller fragments (C4a and C2b). Once cleaved, C4b is able to bind covalently to the surface of the target and associate with C2a to form the classical pathway C3 convertase C4b2a [43] (Figure 4.1).

Many of the enzymatic reactions in the classical pathway are dependent on the presence of Ca$^{2+}$ and Mg$^{2+}$. The nomenclature used in the complement system is not based on the order in which the complement components are activated but instead based on when they were identified. Furthermore, when the complement components are cleaved into one smaller and one larger fragment, the larger fragment is des-
ignated “b” except for C2 where C2b is the designation for the small activation fragment [51].

4.3 The lectin pathway

The lectin pathway is the most recently discovered activation pathway of the complement system and it has many similarities to the classical pathway. In 1976 young children were investigated thoroughly due to recurrent infections [52]. Serum from those children did not support opsonization of yeast particles, a feature also observed in a small percentage of the adult population [53]. Later, the association to serum levels of mannose-binding lectin (MBL) was found and the lectin pathway discovered [54]. The lectin pathway is initiated by the binding of MBL to carbohydrate structures such as mannose and N-acetyl-glucosamine, or by the binding of ficolins to acetylated molecules, which are widely expressed on pathogens but not on human cells [55, 56]. Upon binding of ficolins or MBL to the target, MBL-associated serine proteases (MASPs) are activated and subsequently activate the complement component C4 and C2 to form the C3 convertase (C4b2a) in analogy with the classical pathway activation (Figure 4.1).

4.4 The alternative pathway

The alternative pathway of the complement system is constitutively activated through a low-level spontaneous hydrolysis of C3 to form C3(H2O) molecules. C3(H2O), which resembles C3b, associates with factor B after which factor D could cleave factor B into a Bb and Ba fragment with the subsequent formation of the alternative pathway fluid phase C3 convertase C3(H2O)Bb. This C3 convertase could convert C3 into C3a and C3b and C3b could bind to adjacent surfaces. Once bound, factor B could form complex with C3b, and after cleavage of factor B by factor D the alternative pathway C3 convertase is generated. This complex is further stabilized by the binding of properdin. The alternative pathway could also be activated through the production of C3b fragments in the classical and lectin pathway thus function as an amplification loop in the activation of the complement pathway [51] (Figure 4.1).

4.5 The terminal pathway

All C3 convertases, even though the composition is different, can cleave C3 into C3a and C3b. C3b covalently attaches to adjacent surfaces and opsonizes the target. Binding of C3b to the existing C3 convertase, forming the C5 convertases (C4b2a3b and C3bBbC3b for the different C3 convertases), changes the specificity of the convertase from C3 to C5 and initiates the common terminal pathway [57].

The C5 convertase cleaves C5 into the small anaphylatoxin C5a and the larger fragment C5b which binds to the cell membrane. The other terminal complement components C6, C7 and C8 assemble where after several C9 molecules are incorporated into the cell membrane to create cell membrane-penetrating pores in the target [58] (Figure 4.1).
Figure 4.1. An illustration of the three main activation pathways of the complement system. Upon activation of the classical or the lectin pathway, serine protease (C1s and MASP) are activated and cleave C2 and C4 to form the C4b2a C3 convertase. The C3 convertase cleaves C3 to form the C4b2a3b C5 convertase and initiate the terminal pathway and the subsequent MAC formation. The alternative pathway is constitutively activated by spontaneous hydrolysis of C3, but could also act as an amplification loop upon activation of any of the other main activation pathways. Surface-bound C3b binds to factor B which is cleaved by factor D to form the alternative pathway C3 convertase C3bBb. This complex is further stabilized by the binding of properdin. Additional C3 is then cleaved to form the alternative pathway C5 convertase with the subsequent cleavage of C5 and initiation of the terminal pathway. Enzymatic cleavage of complement components are marked with a lightning bolt. Abbreviations used in the figure; MBL: mannan binding lectin, MASP: MBL associated serine protease, fB: factor B, fD: factor D, P: properdin, and MAC: membrane attack complex.
4.6 Non-classical complement activation pathways

Even though described as a three-way activation system in Figure 4.1, the complement system is much more complex and there exist several other mechanisms to activate complement components. It is not always necessary to use the different recognition molecules in the complement system to render important complement split products. Thrombin, a molecule mostly associated with the coagulation cascade, is able to cleave C5 and produce the anaphylatoxin C5a without any prior complement activation taking place [59]. Properdin is an important part of the alternative pathway in its ability to stabilize the C3 convertase, but has recently also been described to act as a pattern recognition molecule and initiator of the alternative pathway on bacterial surfaces [60]. The importance of properdin in the initiation of the alternative pathway is further supported by knock-out mice models where a properdin deficiency abolished the ability to activate the alternative pathway for several, but not all, of the investigated stimuli [61]. There have also been described several mechanisms of how the different activation pathways of the complement system interact. In 2006, Selander et al demonstrated that MBL could activate the alternative pathway of the complement system in C2 deficient (C2D) individuals [62], a finding later verified by several other groups [63, 64]. There has also been described a similar mechanism to bypass C2 activation by the classical pathway. In 1973, May and Frank demonstrated that hemolysis could occur even in the absence of C2 or C4 and it was dependent on antibody-mediated activation of C1 [65]. This C2-bypass pathway was further verified both in human [66] and in guinea pigs [67] but the exact mechanism for the C2-bypass mediated alternative pathway activation is still not clear.

4.7 Immunological effects of the complement system

The complement system has many important immunological functions both in the protection against pathogens but also in the clearance of dying cells. During complement activation several split products are produced of which most of them have been described to have immunological properties. There exist three main mechanisms for the complement system to eliminate pathogens: chemotaxis, opsonization and lysis. Upon complement activation the split products C3a, C4a and C5a are released into the circulation and these components are anaphylatoxins and recruit immune cells to the infected area to clear the pathogen. C5a is the most potent anaphylatoxin and C4a is the least efficient [68]. Furthermore, besides the chemoattractant function, the anaphylatoxins induce histamine release from mast cells and increase the vascular permeability [69]. The C3b and C4b-opsonized pathogens are then recognized by the recruited immune cells. Complement opsonization is also very important in clearance of dying cells. If not cleared efficiently, the dying cells could expose intracellular nuclear material, which, in a pro-inflammatory environment could be identified as non-self and initiate the development of autoimmune diseases such as SLE [70]. Finally, the complement system could form pores (membrane attack complex; MAC) in the membrane
of the cell thus inducing lysis of the target [58]. In 2004, Yamada and colleagues observed that C1q could suppress the LPS-induced IL-12p40 production in murine bone marrow-derived dendritic cells [71]. Both the collagen and globular part of the C1q molecule seemed to be important in reducing the NF-κB activity. These findings were later verified by Fraser et al in human cell populations. C1q was demonstrated to increase LPS-induced IL-10 and IL-6 production and reduce IL-1 synthesis [72]. C1q is not only able to inhibit TLR4-induced cytokines but we and others demonstrated that C1q is able to inhibit TLR7 and TLR9-induced IFNα production by pDCs, thus providing a link between C1q deficiency and development of SLE [73, 74]. Furthermore, C1q might skew the immune response through the preferential uptake of C1q-opsonized complexes by monocytes instead of dendritic cells [73, 75].

### 4.8 Complement receptors

Many of the immunological functions exerted by the complement system depend on the interaction with complement receptors. C1q has for long only been seen as a part of the classical pathway activation cascade, but has recently also been identified as a modulator of several immunological responses through potential C1q binding proteins (Table 4.1). However, the area of C1q receptors is controversial and it is yet to be determined if all of the identified C1q binding proteins are signaling receptors or merely C1q binding proteins. C1q has an important role in the enhancement of phagocytosis of dying cells [76], but the exact receptor in this C1q-mediated phagocytosis is unknown. One of the first candidates was CD93 (also called C1qRp) [77, 78], but later studies demonstrated that CD93 was not involved in the C1q-mediated phagocytosis [79] and that C1q did not interact directly with this cell surface protein [80]. Recently the α2β1 integrin was demonstrated to bind to C1q, MBL and surfactant protein A and initiate mast cell activation and cytokine secretion in mice [81]. Both C1q and MBL have been shown to bind to apoptotic cells and induce uptake of the target through interaction with cell surface CD91 and the receptor for the collagen part of C1q (cC1qR) on the phagocyte [48]. The dogma has been that C1q and collectins could interact with cC1qR, located to the surface through interactions with CD91, and signal phagocytosis through CD91. Recent data, however, indicate that not only cC1qR, but also CD91 could bind directly to C1q [82].

Besides the cC1qR which recognizes the collagen part of C1q, a C1q binding protein with affinity for the globular heads of C1q (gC1qR) has also been described [83]. The gC1qR protein is expressed intracellularly in the mitochondria and has been identified on the cell surface of several different cell populations including B cells, T cells, endothelial cells and platelets. The receptor has a broad ligand specificity including several microbes as well as factors in the coagulation system as thrombin and fibrinogen [84]. The functional properties of the gC1qR is not fully understood but there are some studies indicating a gC1qR-mediated reduction of T cell activation [85] and increased platelet activation [86, 87].
Several of the complement receptors (CRs) are important for phagocytosis of opsonized targets including CR1, CR3, CR4 and complement receptor of the immunoglobulin superfamily (CRIg) which all recognize certain C3 fragments. CR1, also called CD35, is expressed on many of the peripheral blood cells and recognizes C3b and C4b fragments and perhaps C1q. One function of this receptor is the erythrocyte-mediated transport of opsonized ICs to the liver and spleen for destruction [3, 88]. Once C3b is bound to a surface it can be degraded to iC3b, C3c and C3dg fragments which shifts the affinity of the C3 fragment to CR2, CR3 and CR4 instead of CR1. CR3 and CR4 are expressed on many immune cells including monocytes, macrophages, neutrophils and dendritic cells and are important for the phagocytosis of opsonized material. In contrary to the inflammatory responses of FcγRs, phagocytosis by CRs does not need to cause inflammation [89-91] which is in concordance with the silent non-inflammatory clearance of complement-opsonized apoptotic cells [92]. CR2 is mainly expressed on B cells and recognizes break-down products of C3. Upon binding of C3d to CR2, B cells decrease their activation threshold and mature more easily.

| Table 4.1. Complement receptors, their main ligands and immunological functions. |
|---|---|---|
| **Receptor** | **Ligand** | **Function** |
| CR1 (CD35) | C3b, C4b, C1q | Clearance of ICs, phagocytosis |
| CR2 (CD21) | C3d, C3dg | B cell activation |
| CR3 (CD11b/CD18) | iC3b | Phagocytosis |
| CR4 (CD11c/CD18) | iC3b | Phagocytosis |
| CRIg | C3b | Phagocytosis |
| CD91 | C1q, MBL | Phagocytosis |
| cC1qR | C1q, MBL | Phagocytosis |
| CD93 (C1qRp) | C1q | Phagocytosis? |
| C3aR | C3a | Chemotaxis, histamine release |
| C5aR (CD88) | C5a | Chemotaxis, histamine release |
| C5L2 | C5a | C5a scavenger receptor |
| gC1qR | C1q | T cell inhibition, platelet activation |
| α2/1 integrin | C1q, MBL | Mast cell activation, cytokine secretion |
Another important function of the complement system is mediated by the anaphylatoxins C3a, C4a and C5a. To date, no receptors have been identified for C4a. However, there exist one receptor for C3a and two receptors for C5a. These receptors are widely distributed on both immune and non-immune cells and activation of C3aR or C5aR (CD88) leads to chemotaxis, release of histamine from mast cells and increased vascular permeability to attract immune cells efficiently to the inflamed area [69]. The other C5a receptor (C5L2) has been described to function as a scavenger receptor able to bind C5a but not to induce any functional responses [93].

4.9 Complement regulators
The complement system is very potent and, as such, it needs to be tightly regulated to avoid activation on inappropriate targets. Thus, there exist many complement regulatory molecules acting on several different parts of the activation cascade (Figure 4.2). One of the first inhibitors in the complement activation cascade is C1 inhibitor which binds to and inactivates C1r, C1s and MASP-2 thus inhibiting both the classical and the lectin pathway [94]. Another complement inhibitor of the classical pathway is the complement C2 receptor inhibitor trispanning (CRIT) which is a widely expressed surface receptor for C2. CRIT inhibits C1s-mediated cleavage of C2 and thus formation of the C3 convertase [95] (Figure 4.2).

Factor I is an important regulator of all complement activation through the cleavage and inactivation of C3b and C4b. However, for this process, factor I needs cofactors; CR1, C4b-binding protein (C4BP), Factor H or membrane cofactor protein (MCP; CD46) [96, 97]. All of the cofactors, except MCP, have also regulatory functions on their own by preventing the assembly of the C3 convertase, accelerating the decay of the C3 convertase and competing for the C3b binding site, respectively [96, 98, 99]. Binding of CR1g to the C3b subunit in either the C3 or the C5 convertase will inhibit further complement activation through the alternative pathway [100, 101]. Decay accelerating factor (DAF, CD55) prevents assembly and promotes decay of the C3 and C5 convertases [102] (Figure 4.2).

There also exist several inhibitors of the terminal pathway: Protein S (also called vitronectin), clusterin and CD59 (also called protectin). Vitronectin and clusterin inhibit the polymerization and assembly of C9 molecules, respectively [103, 104] whereas CD59 inhibits the formation of the MAC by binding to C8 and C9 [102] (Figure 4.2). All of the above described regulators act as inhibitors, but there is also one positive regulator of the complement system, properdin, which stabilizes the alternative pathway C3 convertase.

4.10 Complement deficiencies
The complement system is part of the first-line of defense against microbial infections. Furthermore, the complement system has several important effector functions in regulating the immune response and clearance of dying cells. Thus, deficiencies either in the activation cas-
cade or in regulatory proteins are associated with development of disease. Complement deficiencies can be genetically inherited but also acquired due to autoantibodies or complement consumption. Deficiencies in the classical pathway are associated with development of the autoimmune disease SLE, but also with an increased susceptibility to bacterial infections including meningitis and pneumonia [44, 105]. There seems to be a hierarchical association between development of SLE and the order of activation in the classical pathway. Almost all individuals (90-100%) with a C1q deficiency will develop SLE, whereas the association to C2D is much lower (20%) [44]. The decreased disease susceptibility for C2D individuals could be due to the existence of C2-bypass pathways. However, the strong association between C1qD and development of SLE could also indicate a specific role for C1q in the disease development besides the complement activation cascade, such as cytokine regulation [74]. Fortunately, complement deficiencies in the classical pathway are rare and less than 100 individuals have been reported with a homozygous C1q, C1r, C1s or C4 deficiency. C2D is more common with an estimated frequency of 1/20,000, but many individuals are apparently healthy [106, 107].

There exist several different genotypes of MBL and approximately 10% of the Caucasian population is considered to be deficient. MBL deficiency is not a problem in itself, but has been associated with an increased susceptibility to infections in young children and in immunosuppressed individuals. Furthermore, there has been suggested an association between MBL genotype and development of cardiovascular diseases [108, 109]. Deficiencies in the alternative as well as the terminal pathway are also rare and are associated primarily with meningitis and sepsis caused by Neisseria. Deficiencies in the regulatory proteins of the complement system have also been described and are mostly associated with development of glomerulonephritis, atypical hemolytic uremic syndrome and hereditary angioedema [44]. For more information about complement deficiencies and the association with infections and other diseases, the reader is referred to the recent review by Skat tum et al [44].

4.11 Conclusions

The complement system is an important part of the innate immune system and consists of three main activation pathways, complement receptors and inhibitors. Upon activation of the complement system several split products are produced which opsonize the target, attract immune cells and lyse the targeted cell or bacteria. Human complement deficiencies are rare, but clinically important, and are associated with an increased susceptibility to certain bacterial infections and development of the autoimmune disease SLE. Even though often described as a three-pathway activation system, several other pathways have been described and the components of the complement system have been assigned many new functions such as cytokine regulation. Thus, the complement system should be recognized as much more than a simple effector mechanism to destroy bacteria.
Figure 4.2. A schematic figure illustrating the major points of regulation and the complement proteins involved. The complement system is regulated at several important stages: assembly of the C3 convertase, assembly of the C5 convertase, activation of the C5 convertase and the subsequent MAC formation. The initial formation of C3 convertases is regulated by C1 inhibitor (C1 INH), CR1T and factor I (fI) in association with its cofactors C4BP and factor H (fH) as well as CR1g. The decay of the different convertases is accelerated by CR1g and DAF, as well as fI with its cofactors. The formation of the MAC is regulated by fluid phase proteins (clusterin and vitronectin) as well as the membrane-bound protein CD59.
5 The Interferon Family

5.1 Introduction
In 1892, the Russian botanist Iwanowsk identified the first virus; the tobacco mosaic virus [110]. During the following decades many new viruses were identified and it was suggested that viruses could cause disease. In 1957 Isaac and Lindenmann discovered an anti-viral substance that protected the cells against the viruses and named the substance "interferon" (IFN) [111]. Since then, many physiological functions have been ascribed to the IFN family and several different members have been identified. In this session, the IFN family members, activation pathways and functional effects will be discussed with an emphasis on the type I IFN system.

5.2 The interferon family
The IFN family can be divided into three different types of IFNs with different receptors and cellular origin (Table 5.1) and all exist in only one form, except for IFNα which have 12 different IFNα subtypes [112]. In general, viral exposure, or activation via pattern recognition receptors (PRRs) induce production of type I IFNs, but which form and amount depends on the cell population being stimulated as well as the viral challenge. Epithelial cells, fibroblasts and non-immune cells mainly produce IFNβ while immune cells (monocytes, macrophages and dendritic cells) also produce IFNα [113]. Even though most cells are able to produce type I IFNs, the immune system is equipped with a specialized cell with a high capacity to identify viral challenges and produce type I IFNs to eliminate the threat; the plasmacytoid dendritic cell (pDC) [114]. It has been estimated that one single pDC can produce up to a million IFNα molecules in 12 hours, which is 10-fold the production rate of a monocyte [115]. This highly increased production rate is mainly due to the constitutively expression of several interferon-regulatory factors (IRF) which immediately allows transcription of type I IFNs.

Table 5.1. Summary of the interferon family.

<table>
<thead>
<tr>
<th>Type</th>
<th>Form</th>
<th>Receptor subunits</th>
<th>Cellular origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>IFNα, IFNβ, IFNε, IFNκ, IFNω</td>
<td>IFNAR1 IFNAR2</td>
<td>Most cells upon viral infection</td>
</tr>
<tr>
<td>II</td>
<td>IFNγ</td>
<td>IFNGR1 IFNGR2</td>
<td>NK cells and T cells</td>
</tr>
<tr>
<td>III</td>
<td>IFNλ1, IFNλ2, IFNλ3</td>
<td>IFNλAR1 IL10R2</td>
<td>Most cells upon viral infection</td>
</tr>
</tbody>
</table>
Furthermore, pDCs are able to recognize many more different interferogenic stimuli and respond with lots of different type I IFNs as compared to other cell populations [116]. The pDCs can be easily distinguished by the unique expression of blood-dendritic cell antigen (BDCA)-2 and BDCA-4. They are able to phagocytose antibody-opsonized antigens through FcγRIIA (CD32A) and may present the antigen on the MHC class II molecule. However, unless activated by viruses or other stimuli, the pDCs do not express CD80 and CD86 and might thus not participate in T cell activation [117]. The type II IFNs consist of IFN-γ which is mainly produced by activated NK cells and T cells upon viral infection [118, 119]. The type III IFNs is the most recent addition to the interferon family and was not discovered until 2003 [120, 121]. The type III IFNs (IFNλ1, IFNλ2 and IFNλ3, or IL29, IL28A and IL28B, respectively) are produced by most cells [122].

5.3 Immunological properties of interferons

Type I IFNs are very important for the protection against viral infections as demonstrated by the incapability of type I IFN receptor (IFNAR) deficient mice to handle viral infections [123]. Signaling through the IFNAR initiates the production of hundreds of proteins which defend us against viruses by several different mechanisms, either by directly interfering with viral replication or by decreasing cell growth, protein synthesis or inducing cell death in the infected cells [124-128]. In a recent study by Schoggins et al hundreds of type I IFN-regulated proteins were analyzed for the individual contribution upon viral infection. Some type I IFN-regulated proteins had very broad anti-viral effects whereas other type I IFN-regulated proteins instead increased the viral replication [129].

<table>
<thead>
<tr>
<th>Interferon</th>
<th>Function</th>
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<tbody>
<tr>
<td><strong>Type I</strong></td>
<td>Increases production of chemoattractant chemokines [130]</td>
</tr>
<tr>
<td></td>
<td>Activates cytotoxic anti-viral CD8+ T cells by mDCs [130]</td>
</tr>
<tr>
<td></td>
<td>Increases antigen-presenting ability of mDCs [130]</td>
</tr>
<tr>
<td></td>
<td>Increases IFN-γ production from NK cells and T cells [131]</td>
</tr>
<tr>
<td></td>
<td>Induces accumulation of lymphocytes, including pDCs, in lymph nodes [132]</td>
</tr>
<tr>
<td></td>
<td>Activates B cells to become antibody producing plasma cells [133]</td>
</tr>
<tr>
<td></td>
<td>Decreases viral replication [125]</td>
</tr>
<tr>
<td></td>
<td>Increases apoptosis [134, 135]</td>
</tr>
<tr>
<td></td>
<td>Decreases angiogenesis [136, 137]</td>
</tr>
<tr>
<td></td>
<td>Differentiates T cells to Th1 cells [138]</td>
</tr>
<tr>
<td><strong>Type II</strong></td>
<td>Affects tumor growth and survival [139, 140]</td>
</tr>
<tr>
<td></td>
<td>Protects against intracellular bacteria and viruses [141, 142]</td>
</tr>
<tr>
<td><strong>Type III</strong></td>
<td>Similar to type I IFNs</td>
</tr>
</tbody>
</table>
Besides the direct cellular antiviral effects of type I IFNs they also exert many other immunological functions (Table 5.2). IFNα is used therapeutically to treat malignancies and one function of type I IFNs is to decrease angiogenesis, an important part of tumor growth [136, 137] as well as to induce cell death [134, 135]. Besides the anti-tumor and anti-viral effects of type I IFNs, they have also profound effects on several different immune cells. Monocyte-derived DCs treated with type I IFNs have an increased surface expression of CD80, CD86 and HLA-DR which are important molecules in the presentation of antigens to other immune cells. The increased antigen-presenting ability of these DCs is important to activate CD8+ T cells for identification and elimination of virus-infected cells through release of cytotoxins, an important part of the anti-viral defense. Furthermore, type I IFNs promote the production of several chemoattractant chemokines such as CXCL9 and CXCL10 which recruit immune cells to the infected area [130] (Figure 5.1). Type I IFNs increase the accumulation of pDCs and other lymphocytes in lymph nodes [132], activates B cells to differentiate into plasma cells [133] and drives the differentiation of naïve T cells to Th1 cells together with IL-27 [138]. Furthermore, type I IFNs increase NK cell- and T cell-mediated IFNγ production, a type II IFN with anti-bacterial and anti-viral effects [131] (Table 5.2). A genetic deficiency in the IFNγ pathway is strongly associated with susceptibility to mycobacterial infections [141], Listeria infections [142] and decreased macrophage function [143].

Type II IFNs have also been described to have anti-tumor effects and mice deficient in the type II IFN receptor develop more aggressive tumors [139, 140]. Type III IFNs signals through a distinct receptor with activation of IFN-stimulated response element (ISRE) in the nucleus exactly as for the type I IFNs. Thus, type I and type III IFNs share most antiviral effects even though signaling through different receptors.

5.4 Type I IFN activation pathways

Viruses and bacteria exist in many forms with differences in their surface structure and their genetic material. Thus, it is important for the immune system to possess several different receptors recognizing different elements of the microbe for the induction of type I IFNs. There are two main induction routes for type I IFNs; toll-like receptors (TLRs) and retinoic acid inducible gene 1 (RIG-1) like receptors (RLRs) [144, 145]. Several cytoplasmic DNA-recognizing molecules which could initiate the type I IFN production has also been described [145]. TLRs are important intracellular or cell surface-located PRRs recognizing different pathogen-associated molecular patterns (PAMPs). There are 10 human TLRs described and they are widely distributed among the immune cells (Table 5.3) but are also found in non-immune cells [146-152].
Figure 5.1. A schematic figure of some of the type I IFN-mediated anti-viral effects exerted by immune cells. Plasmacytoid dendritic cells (pDCs) recognize the virus and produce huge amounts of type I IFNs through different pattern recognition receptors such as Toll-like receptor 7. The type I IFNs activate antigen presenting cells (APC) and increase the surface expression of MHC molecules as well as the co-stimulatory molecules CD80 and CD86 (B7). Activated APCs recruit immune cells through release of chemokines and activate T cells through interaction with the T cell receptor (TCR) and CD28. The cytotoxic T cell identifies the infected endothelial cell and releases several cytotoxic substances which kill the infected cell. B cells are also recruited to the inflammatory site and type I IFNs increases the differentiation of B cells into mature antibody-producing plasma cells which help to neutralize the virus. Antibody-coated viral particles (ICs) are taken up by pDCs and enhance the production of type I IFNs further.

TLR1, TLR2, TLR4, TLR5 and TLR6 recognize different bacterial surface structures and lipopeptides whereas TLR3, TLR7, TLR8 and TLR9 recognize bacterial and viral RNA and DNA. The ligand for TLR10 is not known [146]. The exact distribution of the different TLRs among the immune cells is controversial and several studies have shown contradictory results probably due to differences in purity or maturation status of the cell populations in-
vestigated as well as different techniques (flow cytometry, Western blot and real-time PCR). Among others, TLR7 has been described to be expressed by myeloid DCs (mDCs) and monocytes in some investigations [153] but not in others [154-156]. Furthermore, the TLR expression of the different cell populations can change upon maturation. Monocyte-derived DCs decrease the expression of several TLRs but gain the expression of TLR3 upon maturation [157]. Non-immune cells, like epithelial cells, do not express the viral sensing TLR7, TLR8 and TLR9, and only very low or no expression of TLR3. However, upon activation with IFNα through IFNAR, the epithelial cells increase the TLR3 expression and thus the ability to sense and reply to viral infections [158]. When activated, the TLRs signal through different signaling pathways and adaptor proteins (Figure 5.2). There are four different adaptor molecules for the TLRs enabling signaling through different pathways: Myeloid differentiation primary response gene 88 (MyD88), Toll-like receptor adaptor molecule 1 (TRIF), TRIF-related adaptor molecule (TRAM) and Toll-interleukin 1 receptor domain containing adaptor protein (TIRAP). MyD88 is used by all TLRs except for TLR3, TRIF is used by both TLR3 and TLR4, TRAM is used exclusively by TLR4 and TIRAP is used by TLR2 and TLR4. When the adaptor molecule is bound to the TLR it recruits more proteins and activates different signaling pathways activating the transcription factors nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) or the interferon regulatory factor (IRF) 3 leading to the production of pro-inflammatory cytokines [159] (Figure 5.2). For a more detailed overview of the signaling cascade see the review by Lee and Kim [160].

<table>
<thead>
<tr>
<th>TLR</th>
<th>B cell</th>
<th>T cell</th>
<th>Monocyte</th>
<th>mDC</th>
<th>pDC</th>
<th>Neutrophil</th>
<th>NK cell</th>
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<tbody>
<tr>
<td>TLR1</td>
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<td>TLR4</td>
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<td>TLR5</td>
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<td>TLR9</td>
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<td>TLR10</td>
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</table>
Figure 5.2. A schematic figure demonstrating activation of a toll-like receptor (TLR) by a ligand. The activated transmembrane TLR will bind its specific adaptor protein and initiate the recruitment and activation of several signaling molecules ending up in the activation of transcription factors (TF). These factors are transported across the nuclear membrane and bind specific regions in the DNA to promote the transcription of specific genes.

All intracellular TLRs (TLR3, TLR7, TLR8 and TLR9) as well as TLR4 on the cell surface are able to initiate the production of type I IFNs upon activation (Figure 5.3). TLR3, an endosomal TLR expressed in mDCs, macrophages and NK cells, recognizes double-stranded (ds) RNA and signals through the adaptor protein TRIF. TRIF activates TRAF3 and TANK-binding kinase 1 (TBK1) which subsequently phosphorylates IRF3 to initiate the production of type I IFNs [145, 161]. TLR4 is associated with its co-receptor myeloid differentiation protein 2 (MD2) on the cell surface of several immune cells and recognizes lipopolysaccharide (LPS). Upon activation TLR4 signals through the adaptor protein TRIF and TRAM to activate the TRAF3/TBK1-mediated IRF3-initiated production of IFNβ [162]. Activation of TLR4 will also activate the MyD88 pathway by recruitment of TIRAP and assembly of a complex consisting of IRAK4, IRAK1 and TRAF6, but not TRAF3 [160]. TLR7, TLR8 and TLR9 are intracellular endosomal receptors recognizing single-stranded (ss) RNA [163], synthetic molecules as resiquimod and imiquimod [164] and unmethylated cytidine-phosphate-guanosine (CpG) rich DNA [165]. When TLR7 or TLR9 is activated, MyD88 recruits TRAF6, IRAK1 and IRAK4 and TRAF3. This protein complex could then phosphorylate IRF7 which translocate to the nucleus and initiate the transcription of type I IFNs in pDCs [160] (Figure 5.3).

Even though the same TLR is used, the signaling pathway can be skewed to IFNα, IFNβ or other pro-inflammatory cytokines in different cell populations, mainly due to the absence or presence of IRFs. As an example, TLR7 activation in mDCs gives rise to the production of IL-12, while TLR7 activation in pDCs activates huge amounts of IFNα but no IL-12 [116, 153]. The IFNα transcription is controlled by IRF3, IRF5 and IRF7. IRF3 is constitutively expressed in the cytoplasm by most cell types and initiates the production of IFNβ. IRF7 is needed to produce IFNα and is expressed at very low levels in most cells, but at high levels only in pDCs. The expression of IRF7 is enhanced by type I IFNs, acting through the IFNAR, and is part of a positive autocrine and paracrine feed-
back system amplifying the type I IFN response [166]. The binding of type I IFNs to IFNAR leads to the activation of Janus kinase 1 (JAK1) and tyrosine kinase 2 (TYK2). These proteins are kinases and recruit and phosphorylate signal transducers and activators of transcription (STAT) 1 and STAT2. These factors can then form a complex with IRF9, translocate to the nucleus and bind to genes with an IFN-stimulated response element (ISRE) and initiate the transcription of hundreds of type I IFN-regulated genes [167] (Figure 5.4).

**Figure 5.3.** A simplified figure illustrating the five type I IFN-inducing toll-like receptors (TLRs) with their respective ligand, adaptor protein, main signaling pathway and transcription factor depicted. TLR7 and TLR8 are intracellular endosomal receptors recognizing single stranded RNA (ssRNA) and TLR9 recognizes unmethylated DNA. After activation, the adaptor protein MyD88 recruits several signaling molecules, including TRAF6, IRAK1, IRAK4 and TRAF3, which activates the transcription factor NF-κB, and in pDCs IRF7 thus initiating transcription of several pro-inflammatory cytokines and type I IFNs. TLR4 is activated by LPS and has several adaptor proteins enabling signaling through the TRAF6, IRAK1, IRAK4 complex as well as through the TRAF3/TBK1 pathway. TLR3 is activated by double stranded RNA (dsRNA) and activates TRAF3 and TBK1 with the subsequent activation of the transcription factor IRF3 and transcription of type I IFNs.
5.5 Cytoplasmic RNA and DNA recognizing molecules

Retinoic acid inducible gene 1 like receptors (RLRs) are cytoplasmic proteins expressed by most cells and they recognize viral dsRNA. Two of the three described members (RIG-1 and melanoma differentiation-associated gene 5 (MDA5)) signal through IRF3 to induce type I IFN production while the third member (laboratory of genetics and physiology 2 (LGP2)) does not have the caspase recruitment domain needed for downstream antiviral signaling, but could instead regulate the other RLR activation pathways negatively or positively [168-170]. Upon activation, RIG-1 and MDA5 bind to an adaptor molecule (IPS-1), localize to the mitochondria and initiate different signaling cascades leading to the subsequent cytokine production through IRF3 and NF-κB [171] (Figure 5.5). RIG-1 and MDA5 are responsible for the major type I IFN production in response to RNA viruses in most cells, except for pDCs who rely on TLR7 [172]. There have also been described cytoplasmic DNA-recognizing molecules; DNA-dependent activator of IRF (DAI) and RNA polymerase III, which could induce type I IFN production [145, 173]. DAI acts by activating IRF3 [174] and RNA polymerase III transcribes the cytosolic dsDNA into 5’ triphosphate RNA which activate the RIG-1 pathway [175] (Figure 5.5).

Figure 5.4. The type I IFN receptor pathway. After the induction of type I IFNs by TLRs or DNA and RNA recognizing cytoplasmic receptors, the type I IFNs may bind to the type I IFN receptor in an autocrine or paracrine way to amplify the type I IFN response. The binding of type I IFNs to IFNAR initiates the phosphorylation of STAT1 and STAT2 by JAK1 and TYK2. STAT1 and STAT2 form a complex with IRF9 and induce the transcription of several hundreds of type I IFN-regulated genes. Three different inhibitors of this pathway (SOCS-1, PIAS-1 and SHP-2) are also depicted.
5.6 Regulators of type I IFN production in pDCs

As previously described, type I IFNs have a great impact on the immune system and thus, they need to be strictly regulated. One potent inhibitory family of type I IFNs is the suppressors of cytokine signaling (SOCS). The expression of SOCS is induced by different cytokines and they function as negative regulators. One of the members, SOCS-1, binds to TYK2 and inhibits the phosphorylation and nuclear transportation of STAT1 thus inhibiting IFNAR-mediated signaling and amplification of the type I IFN system [176] (Figure 5.4). The protein inhibitors of activated stats (PIAS)-1 also regulate the IFNAR signaling pathway by inhibiting STAT1 activation [177]. The JAK/STAT-pathway is inhibited by the SH2-containing phosphatase (SHP)-2 [178] (Figure 5.4).

Figure 5.5. DNA and RNA recognizing proteins. Besides the TLRs there are several DNA and RNA recognizing receptors in the cytoplasma. The RNA recognizing receptors (RIG-1 and MDA5) belong to the retinoic acid inducible gene 1 (RIG-1) like receptors (RLRs) and detect dsRNA and activate both IRF3 and NF-κB. The dsDNA recognizing molecule DAI could signal through IRF3 and NF-κB directly whereas the other dsDNA recognizing molecule, RNA polymerase III, transcribes the dsDNA into dsRNA, and thus activating RIG-1.
Besides the above mentioned families of cytokine suppressors, several molecules have been described to have type I IFN-regulatory functions. Activated monocytes produce IL-10, prostaglandin E2, TNFα and reactive oxygen species (ROS) which could inhibit IFNα production by pDCs and decrease their survival [179-181]. IFNα2b and GM-CSF, as well as the NK cell-derived MIP-1β and LFA-1 could instead increase the IFNα production from pDCs [180, 182]. Osteopontin, high mobility group box 1, 17beta-estradiol and miR-155* all seem to be important to induce a high IFNα production [183-185]. Negative regulators of IFNα production include BDCA-2 ligation [186, 187], the micro RNA miR-155 [188], the micro RNA miR-155 [188] and the classical pathway complement component C1q [73, 74].

5.7 Type I IFNs as therapeutics

Initially, interferons were produced by culturing human leukocytes with different viruses but the purity of the interferons was very low and it was not possible to characterize the protein in detail [189]. It was not until 1979 that Rubinstein et al succeeded in purifying interferons and could determine the amino acid composition [190]. Today, recombinant IFNα is produced in E. coli to gain an even higher purity and amount of the protein. Even though there are some differences in efficacy between the different subtypes of IFNα, only the three allelic variants IFNα 2a, 2b and 2c are used therapeutically. IFNα was tested as a candidate drug in clinical trials for malignancies and viral infections in the early 1980s [191-194] and is still used to treat chronic hepatitis B and C as well as several malignant diseases and Behçet disease. However, there are several severe side effects with the administration of recombinant IFNα including thrombocytopenia, leukopenia and anemia [195-197]. Furthermore, about 35% of the patients receiving IFNα therapy develop interferon-induced depression [198], possibly due to decreased platelet serotonin content [199]. In a review from 1991 Sonnenblick et al report 44 cases of cardiotoxicity including myocardial infarction due to treatment with IFNα [200]. Administration of recombinant IFNα may also lead to development of anti-nuclear antibodies and development of SLE and other autoimmune diseases [201-205] suggesting that IFNα is an important regulator of self tolerance.

5.8 Conclusions

The IFN family is an important part of the immune system protecting our cells against viral infections. Three types of IFNs have been discovered acting through different receptors and signaling systems but with similar mode of action to inhibit the spreading of the virus. Type I IFNs could be induced by several different receptors, most of which recognize viral or bacterial RNA and DNA. Several side-effects of type I IFNs have been observed after administration with recombinant IFNα including development of autoimmunity, cytopenia and myocardial infarctions. Thus, this intricate signaling system needs to be tightly regulated not to develop disease.
6 The Platelet

6.1 Introduction

Platelets, small residues of the bone marrow-derived megakaryocyte, are the second most common population in blood after the erythrocytes. Even though they do not possess a nucleus, they have mRNA and can synthesize proteins [206]. The lifespan of a platelet is highly variable but if not activated it lives for a week before being cleared from the circulation. Platelets play a crucial role in wound healing and haemostasis, but also in inflammation and thrombosis. In this chapter I will discuss the role of platelets in inflammation and the development of cardiovascular diseases.

6.2 Platelets and inflammation

Platelets have for long been recognized as an important part of our defense against pathogens and are able to recognize several pathogens as well as to modulate immune responses through interactions with leukocytes and endothelial cells. Once the platelet has recognized a pathogen it becomes activated, form large platelet aggregates and trap the pathogen. Furthermore, upon activation the platelet releases its granula containing anti-microbial substances as well as immune stimulatory substances. This reaction will clear the pathogen, but it will also cause vascular inflammation and potentially uncontrolled formation of a thrombus. Thus, bacterial-induced platelet aggregation may contribute to cardiovascular disease [4, 207].

Platelets express several PRRs including TLR1, 2, 4, 5, 6 and 9 [4]. TLR9 is located intracellularly in endosomes as described in previous chapters, but in platelets, TLR9 may also be expressed on the cell surface [208]. The ligand for TLR9, unmethylated DNA with cytosine-guanine sequences (CpG DNA) is common in bacteria, but very rare in humans. However, in SLE the DNA is less methylated [209]. It could thus be speculated that the increased risk to develop cardiovascular diseases seen in SLE [210] might depend on platelet TLR9 activation by CpG DNA. However, functional properties of TLRs in platelets are not well characterized and it is not known whether TLR9 interaction will result in platelet activation. TLR4, one of the most characterized platelet TLRs, is suggested not to induce classical platelet activation upon LPS activation [211].

Platelets do not only interact with bacteria, but also directly with endothelial cells as well as immune cells. Once activated by thrombin, immune complexes, shear stress or another activating agent, the platelets up-regulate the surface expression of several activation markers including P-selectin which is able to bind to P-selectin glycoprotein ligand 1 (PSGL-1) on immune cells. If the platelets are bound to endothelial cells, the interaction with the immune cells may be an efficient way to transfer immune cells to sites of vascular injury [4]. Several important functions are mediated by
activated platelets upon interaction with immune cells. The main immune cell targeted for platelet binding is the monocyte. Circulating platelet-monocyte complexes is a general marker of platelet activation [212] and is increased in patients with SLE [213, 214]. Once the platelet binds to the monocyte, the expression of inflammatory cytokines and tissue factor is increased [215, 216] with subsequent tissue factor-mediated formation of thrombin and blood coagulation [217]. Platelets could also interact with other immune cells including the PMNs to promote NET formation [218]. Finally, activated platelets express CD40L which may interact with CD40 on pDCs to amplify the IFNα production [219]. Thus, platelet can interact with many different immune cells in order to amplify the inflammation.

Besides P-selectin and CD40L, the platelets express several other surface markers upon activation, including phosphatidylserine and chondroitin sulphate which could mediate binding of the C1q molecule to the platelet surface [220, 221]. In SLE patients, where platelet activation is increased, we, and others [222], have demonstrated high levels of C1q, C4d and C3d on platelets and this is associated with platelet activation and cardiovascular disease and venous thrombosis (Paper IV). If the progression of complement activation is not inhibited by complement regulators (CD46, CD55 and CD59), complement activation might proceed to the formation of MAC and the subsequent release of platelet microparticles [223, 224]. Such microparticles are increased in SLE patients and are able to generate thrombin, a key component in the coagulation cascade [223]. Thus activated platelets also support complement activation which might be an important event in the establishment of cardiovascular diseases.

### 6.3 Platelets and cardiovascular disease

Platelets are important in the healing of vascular damage. If the damage is not healed properly, an ongoing inflammation could lead to development of atherosclerosis and eventually thrombosis. Atherosclerosis is the most common cause of arterial thrombosis whereas other mechanisms apply for the venous side, including trauma and immobility. In this section we will focus on the role of platelets in atherosclerosis and development of arterial thrombosis.

The initiating event in the development of atherosclerosis has been suggested to be induction of endothelial dysfunction caused by modified low-density lipoprotein (LDL), cigarette smoking, hypertension, infection or other stimuli [225, 226]. Endothelial dysfunction affects the endothelial cells in the proximity to increase their adhesiveness to leukocytes and platelets and increase their permeability to lipoproteins. Furthermore, the damaged endothelium will produce monocyte chemotactic protein 1 (MCP-1) and IL-8 which stimulates the transendothelial migration of the monocytes [227]. LDL, which is being incorporated into the arterial wall, is phagocytosed by the monocytes which eventually will transform into foam cells [228]. The foam cells, and other cells of the vascu-
lar wall, will produce cytokines which recruit smooth muscle cells and immune cells.

The healing process of the endothelium gives rise to a fibrous cap, which covers all immune cells, lipids and cell debris. The core will continue to expand since more immune cells are recruited and expand in the injured endothelium. The activated macrophages and other immune cells in the damaged endothelium will release lots of proteolytic enzymes which will degrade the extracellular matrix and thus make the plaque unstable [229]. Eventually, once the fibrous cap has become thinner due to degradation by proteases, it may rupture and expose von Willenbrand factor, collagen and other extracellular matrix molecules [225]. Once collagen is exposed, platelets could bind to it through a specific constitutively expressed receptor (GPIIb) and become activated [230] and release their granula. The granula contain several pro-coagulative factors which amplifies platelet activation; ADP, serotonin, and thromboxane A2, as well as the factors which propagates the coagulation system, including Factor V and fibrinogen [231, 232]. To stabilize the aggregated platelets the coagulation system needs to become activated and produce the fibrin network. Damaged endothelial cells, as well as activated monocytes, expose tissue factor, a potent inducer of the coagulation system. Activated platelets also expose a negative surface which could activate the coagulation system. Upon activation of this system, thrombin will be generated which will further activate the platelets. Thrombin will also cleave fibrinogen and Factor XIII to form the fibrin network which stabilizes the clot and anchors it at the vascular wall. If platelet aggregation continues the blood clot will obstruct the blood flow. The formed blood clot might also be released and stuck in tighter blood vessels and cause thrombosis [231].

6.4 Conclusions
Platelets are an important part of the immune system and the coagulation system through their diverse effects with endothelial cells, immune cells and secreted granula. The platelets are crucial in the detection of vascular damage and clot formation to hinder further blood loss. However, if the platelet activation at the site of vascular damage is not restricted and the inflammatory process not regulated, such as in atherosclerosis, large aggregates of platelets may form and occlude the artery.
7 Systemic Lupus Erythematosus

7.1 Introduction

Systemic lupus erythematosus (SLE) is an autoimmune rheumatic disorder characterized by chronic or episodic inflammation in several organ systems. The immune system is hyperactive with autoantibody-producing B cells leading to circulating immune complexes, increased complement consumption and an ongoing type I IFN production. Even though this is a highly severe disease, the development of new therapies has progressed very slowly. In this chapter I will give an introduction to the epidemiology and genetics of the disease, clinical manifestations and the underlying immunological mechanisms causing the disease. Finally I will discuss some of the commonly used therapeutics and some drugs in development.

7.2 Basics of SLE

SLE is an autoimmune disease with a prevalence and incidence of 68/100,000 and 4.8/100,000 inhabitants, respectively, in southern Sweden [233]. As for most other autoimmune diseases, SLE is more common in women than in men and often starts in childbearing age. The cause is not known, but clearly both genetic and environmental factors contribute to the development of the disease. Some environmental factors have been described to be risk factors including UV-light and smoking, whereas a modest consumption of alcohol seems to be protective against development of SLE [234]. Viral infections have also been suggested to be part of the development of SLE. SLE patients have an increased Epstein-Barr viral load in the circulation and these viruses can induce type I IFNs by pDCs, thus breaking self tolerance [235, 236]. The highly increased prevalence of autoimmune diseases among women as compared to men is thought to depend on differences in hormones. Sex hormones have been implicated in the development of SLE [237] and it was recently described that 17-beta estradiol, amplified the type I IFN production by pDCs in mice [185]. SLE is diagnosed based on both clinical manifestations and immunological abnormalities. Since patients with SLE can have a wide range of clinical manifestations, including symptoms commonly seen in other diseases as well, it is difficult to set the diagnosis. Classification criteria, as the American College of Rheumatology (ACR), are only used for scientific reports and not for clinical diagnosis. However, they give a hint of the most common clinical manifestations involved in the disease. The ACR criteria include 11 manifestations (Table 7.1) of which 4 have to be fulfilled for the disease to be classified as SLE [238]. In SLE an accumulation of ACR criteria is often seen over time, but patients with only a few ACR criteria could still have the clinical diagnosis SLE. The four first ACR classification criteria include manifestations in the skin such as sensitivity to UV-light. The four following ACR classification criteria include inflammation in different...
organ systems (joints, lung/hearth, kidney and central nervous system). The final three ACR classification criteria include immunological abnormalities (low levels of blood cells or the presence of specific autoantibodies).

To be able to follow the activity of the disease, a specific index, the SLE disease activity index 2000 (SLEDAI-2K) is used [239]. There exist some different versions, but they all include both clinical and immunological findings in the scoring system. The scoring system of SLEDAI-2K is presented in Table 7.2. The scoring of different disease manifestations is uneven and activity in the central nervous system and cardiovascular disease all give 8 points. Signs of kidney involvement (urinary casts, hematuria, proteinuria and pyuria) all give 4 points each, but it is common with several of these manifestations simultaneously. Skin involvement and immunological abnormalities are regarded as mild manifestations and are scored between 1-2 points per manifestation.

7.3 Genetics

As for many other diseases there are both environmental and genetic factors contributing to the development of the disease. The entry of the genetic era has enabled several research groups to find new associations between certain genes and the development of SLE, mainly by using genome-wide association studies. Most of the identified genes encode proteins that can be assigned one of the following major functions; clearance of apoptotic cells and immune complexes, antigen presentation or TLR signaling [240]. The strongest susceptibility genes for development of SLE are seen for the complement components of the classical pathway; C1q, C1r/C1s, C4 and C2, which are all important in the clearance of apoptotic cells and immune complexes [76]. Other susceptibility genes involved in the clearance of apoptotic cells and immune complexes include the genes encoding the FcγRIIA, FcγRIIIA and ITGAM [240]. The human leukocyte antigen (HLA) is also highly associated with SLE. It has been suggested that the susceptibility HLA-DR haplotype might contribute to an abnormal presentation of self antigens thus increasing the risk to develop autoimmunity [240]. Finally, genes involved in the TLR signaling and type I IFN signaling including IRF5, IRF7, IRAK1 and STAT4 are all associated with development of SLE [240, 241]. Abnormal activation of the type I IFN pathway might lead to development of SLE due to its potent immunostimulatory effects. There exists many more susceptibility genes in SLE which could be found in the recent review of Lee and Bae [240].

7.4 Immunological features of SLE

Our cells are not immortal and many cells die and are removed and replaced every day. The death of a cell is most often highly regulated through a process called apoptosis, a programmed cell death. Once dedicated to die the cell expose lots of 'eat me' molecules such as phosphatidylserine to which bridging molecules, including complement components, can bind. Macrophages and other phagocytes
will be recruited to the dying cell by excreted ‘find me’ signals and with the aid of the bridging molecules efficiently remove the apoptotic cell and induce an anti-inflammatory response [242]. However, the clearance of apoptotic cells is dysfunctional in SLE. First of all, SLE patients have an unidentified “cell death-inducing factor” in the circulation which amplifies the amount of dying cells [243, 244]. Secondly, complement components of the classical pathway are important in the clearance of apoptotic cells [76], and SLE patients often have decreased levels of those components due to complement consumption. Finally, macrophages from SLE patients have a reduced ability to clear apoptotic cells [245].

Taken together, SLE patients can not clear all apoptotic cells and some progress into secondary necrosis. Upon necrosis the dying cells lose their plasma membrane integrity and expose intracellular and nuclear antigens. This process is pro-inflammatory and is suggested to initiate the break of self tolerance in the development of SLE [246]. Necrotic cells are believed to be the major source of autoantigens in SLE. However, recent findings suggest that also neutrophil extracellular traps (NETs), which are released from activated neutrophils, may be a source of autoantigens in SLE [20]. In the presence of autoantibodies, the NETs are potent inducers of type I IFNs [247]. Once autoantibodies have been produced they can bind to the autoantigens and form large immune complexes. The immune complexes could form large aggregate and be trapped in tissue, activate the complement system, cause leukocyte infiltration and subsequent inflammation and tissue destruction. The immune complex-mediated complement consumption of the classical pathway will further reduce the possibility to clear apoptotic cells before turning necrotic. Thus there exists a vicious circle in SLE as illustrated in Figure 7.1 [248]. The immune complexes will also be phagocytosed by various immune cells and give rise to several pro-inflammatory cytokines and activate immune cells. There are profound differences in the immune cells in SLE patients as compared to healthy individuals with decreased lymphocytes and dendritic cells and increased frequency of pro-inflammatory monocytes [12].

<table>
<thead>
<tr>
<th>#</th>
<th>ACR criteria</th>
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<tbody>
<tr>
<td>1</td>
<td>Malar rash</td>
</tr>
<tr>
<td>2</td>
<td>Discoid rash</td>
</tr>
<tr>
<td>3</td>
<td>Photosensitivity</td>
</tr>
<tr>
<td>4</td>
<td>Oral ulcers</td>
</tr>
<tr>
<td>5</td>
<td>Arthritis</td>
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<tr>
<td>6</td>
<td>Serositis</td>
</tr>
<tr>
<td>7</td>
<td>Renal disorder</td>
</tr>
<tr>
<td>8</td>
<td>Neurological disorder</td>
</tr>
<tr>
<td>9</td>
<td>Hematological disorder</td>
</tr>
<tr>
<td>10</td>
<td>Immunological disorder</td>
</tr>
<tr>
<td>11</td>
<td>Antinuclear antibodies</td>
</tr>
</tbody>
</table>
Table 7.2. The SLE disease activity index 2000.

<table>
<thead>
<tr>
<th>Manifestation</th>
<th>SLEDAI 2K score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Seizure</td>
<td>8</td>
</tr>
<tr>
<td>Psychosis</td>
<td>8</td>
</tr>
<tr>
<td>Organic brain syndrome</td>
<td>8</td>
</tr>
<tr>
<td>Visual disturbance</td>
<td>8</td>
</tr>
<tr>
<td>Cranial nerve disorder</td>
<td>8</td>
</tr>
<tr>
<td>Lupus headache</td>
<td>8</td>
</tr>
<tr>
<td>Cerebrovascular accident</td>
<td>8</td>
</tr>
<tr>
<td>Vasculitis</td>
<td>8</td>
</tr>
<tr>
<td>Arthritis</td>
<td>4</td>
</tr>
<tr>
<td>Myositis</td>
<td>4</td>
</tr>
<tr>
<td>Urinary casts</td>
<td>4</td>
</tr>
<tr>
<td>Hematuria</td>
<td>4</td>
</tr>
<tr>
<td>Proteinuria</td>
<td>4</td>
</tr>
<tr>
<td>Pyuria</td>
<td>4</td>
</tr>
<tr>
<td>Rash</td>
<td>2</td>
</tr>
<tr>
<td>Alopecia</td>
<td>2</td>
</tr>
<tr>
<td>Mucosal ulcers</td>
<td>2</td>
</tr>
<tr>
<td>Pleurisy</td>
<td>2</td>
</tr>
<tr>
<td>Pericarditis</td>
<td>2</td>
</tr>
<tr>
<td>Low complement (C3/C4)</td>
<td>2</td>
</tr>
<tr>
<td>Anti-dsDNA antibodies</td>
<td>2</td>
</tr>
<tr>
<td>Fever</td>
<td>1</td>
</tr>
<tr>
<td>Thrombocytopenia</td>
<td>1</td>
</tr>
<tr>
<td>Leukopenia</td>
<td>1</td>
</tr>
</tbody>
</table>

One potent immunomodulatory cytokine is IFNα which is produced by immune complex-stimulated pDCs (Figure 7.1). IFNα has several effects on the immune system as described previously in Chapter 5. SLE patients have increased levels of serum IFNα, especially at active disease [249]. The type I IFN profile, increased type I IFN-regulated genes, is present in both PBMCs and platelets from SLE patients [214, 250-253]. In platelets, the type I IFN protein signature is highly associated with cardiovascular disease and venous thrombosis [214]. Another marker of disease activity in SLE is the elevated levels of the heterodimeric complex of the calcium-binding protein S100A8 and S100A9 (S100A8/A9 or calprotectin) [21]. The heterodimer is produced by monocytes, macrophages, PMNs and pDCs [12, 254] and exerts several pro-inflammatory properties such as activation of monocytes, regulation of migration of myeloid derived suppressor cells, amplification of cytokines and signals through the receptor for advanced glycation end products (RAGE) and TLR4 [13, 27, 28, 34].

7.5 Cardiovascular disease and venous thrombosis in SLE

Patients with SLE have an increased mortality rate, mainly due to infections and cardiovascular diseases [255]. The increased mortality rate in severe infections might depend on the use of immunosuppressive treatments and the subsequent decreased ability of the immune system to defend us against pathogens. The increased mortality rate in cardiovascular disease, however, is not as easily explained. SLE patients have an increased risk to develop cardiovascular disease and venous thrombosis and this is especially pronounced in younger women. Female SLE patients in the age of 35-44 years have a 50-fold increased risk to develop a myocardial infarction (MI) compared to healthy controls [210]. In a cohort of Swedish SLE patients, a 9-fold increased risk to develop a MI could be seen [256]. In all, SLE patients have a five to six fold increased risk to develop a cardiovascular
disease [210]. Traditional cardiovascular risk factors including hypertension, diabetes, smoking and hypercholesterolaemia [257, 258], can only partly explain the increased cardiovascular morbidity in SLE patients [210]. Thus, the disease itself seems to be an independent risk factor for development of cardiovascular disease and venous thrombosis.

Autoantibodies directed against phospholipids (cardiolipin) and beta-2-glycoprotein I as well as the functional lupus anticoagulant test have all been implicated in the increased risk to develop cardiovascular disease and venous thrombosis in SLE [259, 260]. The exact mechanisms of how these autoantibodies are involved in the increased risk of cardiovascular disease are still unknown. However, activation of the complement system seems to be crucial since mice deficient in C3 or C5 are protected against anti-phospholipid (aPL) antibody-mediated thrombosis [261]. Complement deficiency of C2 in humans also seems to render protection against aPL-mediated venous thrombosis [262], and treatment with anti-C5 antibodies can prevent aPL-mediated thrombosis in mice [263]. aPL antibodies might also interact with the endothelial cells and prevent binding of Annexin V [264, 265], a potent anti-coagulant that can inhibit the formation of the prothrombinase complex [266]. Thus, a reduced binding of Annexin V might lead to the prothrombinase complex formation and progression of the coagulation cascade.

One important part in the development of cardiovascular disease and venous thrombosis is endothelial dysfunction and subsequent atherosclerosis. Several studies have demonstrated subclinical atherosclerosis with increased arterial calcification in SLE, especially in younger patients. Another sign of endothelial dysfunction in SLE is the increased number of circulating endothelial cells, derived from damaged blood vessels. Importantly, there are strong associations between circulating endothelial cells and ongoing disease activity, complement consumption and anti-dsDNA antibodies which indicates that the inflammatory disease itself is a contributing risk factor in the development of cardiovascular diseases [267]. One specific marker of inflammation, the type I IFNs, have recently been suggested to play an important role in the development of atherosclerosis in SLE due to the ability to reduce the number of endothelial progenitor cells and their ability to differentiate into mature cells [268, 269].

Besides the inflamed calcified endothelium, platelets are important in the development of cardiovascular disease and venous thrombosis. We and others have demonstrated that SLE patients have more activated platelets [213, 214, 270-272]. One potent activator of platelets, frequently seen in SLE patients, is immune complexes [273]. Once activated, the platelets express several activation markers including P-selectin. P-selectin will mediate binding to monocytes and other immune cells. Complexes between monocytes and platelets are increased in SLE patients [213, 214] and such monocytes produce increased amounts of tissue factor, an important molecule in the initiation of coagulation [216].
Figure 7.1. An overview of some underlying mechanisms in the SLE pathogenesis. SLE patients have an increased rate of cell death. Due to an impaired complement function the apoptotic cells are not cleared properly and progress into secondary necrosis and expose lots of intracellular and nuclear antigens. In an inflammatory environment immune cells can become autoreactive and produce autoantibodies which form immune complexes with corresponding autoantigen. The immune complexes can activate and consume the components of the complement system and thus propagate the vicious circle by reducing the capability of clearing apoptotic cells further. Immune complexes are also phagocytosed by pDCs and induce high amounts of type I IFNs which might drive the development of SLE through production of autoantibodies by B cells. The recently described NETosis, release of nuclear material by activated polymorphonuclear neutrophils might also be a potent source of autoantigens in SLE. Upon PMN activation, the pro-inflammatory molecule S100A8/A9 is also released.
Chondroitin sulphate is another activation marker which is released upon platelet activation and mediates binding of C1q to the platelet surface [220, 221]. If the complement activation cascade is not inhibited properly by the complement regulators CD55 and CD59, complement activation might proceed to form the MAC which will cause release of platelet microparticles [223, 224]. Those microparticles are increased in SLE patients and could generate thrombin, a key component in the coagulation cascade [223]. Patients with SLE have increased levels of C4d on their platelets [222] and we have described that this is increased in patients with a cardiovascular disease or venous thrombosis (Paper IV). Thus, endothelial inflammation, aPL antibodies, type I IFNs, the complement system and platelets all seem to contribute to the increased risk to develop cardiovascular disease and venous thrombosis in SLE.

### 7.6 Therapies of today and tomorrow

SLE is a systemic disease and is thus mainly treated with systemic immunosuppressive drugs. However, as new pathogenetic pathways are discovered, more specific treatments could be developed to block certain parts of the disease rather than the immune system in general. Today, most patients are treated with low-dose corticosteroids which efficiently inhibit many of the immune pathways through inhibition of NF-κB [274]. Hydroxychloroquine, a common treatment in SLE, was not initially developed against SLE, but against malaria. However, the antimalarial drugs are effective in treating mild SLE manifestations. One possible mechanism of action is through the inhibition of endosomal acidification, thus inhibiting TLR-signaling and the downstream IFNα production [275, 276]. During a flare, or to prevent a flare, the patients can receive immunosuppressive drugs including mycophenolate mofetil, azathioprine and methotrexate. They all have a similar mode of action by inhibiting DNA synthesis and thus preventing expansion of activated immune cells [277]. There also exist immune cell-specific treatments and many of them target the B cells. Some therapies (Rituximab: anti-CD20 antibody, and Epratuzamab: anti-CD22 antibody) aim to deplete the B cells from the circulation thus decreasing the autoantibodies. Other B cell-targeting therapies include drugs affecting the B-lymphocyte stimulator (BLyS) such as Belimumab.

Rather than depleting all the B cells, immune complex-modulatory enzymes could be used. We have performed several studies with EndoS, a *Streptococcus pyogenes* derived endoglycosidase. The enzyme has shown very good results in animal models of chronic inflammatory disease as well as in human *in vitro* experiments and has the potential to be developed as a novel therapy in SLE [278-286]. Not all of the above mentioned therapies are in clinical use yet but further clinical trials will evaluate if they are beneficial for SLE patients. Finally, in many of the ongoing clinical trials, pro-inflammatory cytokines, including IFNα, are targeted by antibodies [277]. Thus, SLE patients are generally treated with common immunosuppressive treat-
ments. However, there are many new target-specific drugs under development that might be useful to treat SLE patients in the future.

7.7 Conclusions

SLE is an autoimmune disease characterized by inflammation in several organ systems including joints, skin, kidney and central nervous system. Immunologically, the disease is characterized by an increased apoptosis, complement consumption, the presence of autoantibodies, immune complexes and an ongoing production of type I IFNs. The cause of the disease is not known but several genetic and environmental factors are suggested to be important for the development of SLE. Today, the disease is treated with general immunosuppressive treatments including corticosteroids. However, new biological therapies are emerging which might provide new opportunities to target specific parts of the immune system involved in the SLE pathogenesis.
8 Present Investigation

8.1 Introduction

The overall aim of the thesis was to explore the pathogenesis of SLE with an emphasis on immune complexes, type I interferon system, complement system and platelets. The common denominator in all papers is the immune complex and its pro-inflammatory properties as depicted in Figure 8.1. In this section I will go through our aims and main results for each and every paper and put them in a wider perspective.

8.2 Paper I

Type I interferons (IFNs) and especially IFNα has been implicated as key cytokines in the SLE pathogenesis [287]. The type I IFNs are primarily produced by plasmacytoid dendritic cells (pDCs), but almost all cells are able to produce small amount of type I IFNs upon viral infections [113]. In SLE, there is an ongoing type I IFN production due to phagocytosis of RNA and DNA-containing immune complexes by pDCs. The increased type I IFN production could be detected in serum [249], and at the mRNA level in PBMCs [252] and platelets [214]. Type I IFNs have many immunomodulatory functions including decreasing the threshold for B cell activation [133] as well as increasing the maturation and ability to present antigens in myeloid dendritic cells [288]. Furthermore, treatment with recombinant IFNα could lead to loss of self-tolerance and development of autoimmune diseases such as SLE [201-203].

Complement components of the classical pathway are important in the SLE pathogenesis and genetic deficiencies of those components predispose for development of SLE [105]. Almost all individuals with a genetic C1q deficiency will develop SLE or a SLE-like disease. There exists a hierarchical association between the missing complement component and development of SLE. Thus, a deficiency in C1r, C1s, C4 or C2 is also associated with development of SLE but at a lower frequency [289]. The strong association between deficiencies in the classical pathway of the complement system and development of SLE has been addressed to their important role in the clearance of apoptotic cells. Apoptotic cells that are not cleared properly are believed to be the main source of autoantigens in SLE. Our group has previously described a vicious circle with an impaired complement function, accelerated apoptosis, release of nuclear antigens, loss of self tolerance and formation of pro-inflammatory immune complexes [248]. The increased risk to develop SLE in C1q deficient individuals as compared to other hereditary deficiencies of the classical pathway might not solely be explained by differences in the clearance of apoptotic cells. In an in vitro model to evaluate the individual contribution of the different complement components of the classical pathway in clearance of apoptotic cells, C1q, C2 and C4 were equally important [76]. Thus, C1q deficiency might operate in other parts of the SLE pathogenesis than only
clearance of apoptotic cells. C1q has previously been demonstrated to regulate TLR-mediated cytokine production both in mice and man [71, 72] and normal human serum could inhibit immune complex induced IFNα production [290]. Finally, we have previously described an inverse relation between serum levels of C1q and IFNα [249]. Thus, we wanted to investigate whether C1q could inhibit immune complex-mediated IFNα production by pDCs.

Figure 8.1. A schematic summary of the content of the thesis. All papers focus on the importance of immune complexes in the SLE pathogenesis and how these complexes, through interaction with blood cells and the complement system cause the disease. In paper I we discuss the importance of C1q as a regulator of IFN alpha production by plasmacytoid dendritic cells (pDCs). In paper II we further characterize the pDCs and show that these cells produce a pro-inflammatory protein complex (S100A8/A9) which is bound on the cell surface upon activation. In paper III and IV we address the question if activated platelets could be involved in the highly increased risk to develop cardiovascular disease in SLE patients, and finally in paper V, we demonstrate profound effects of EndoS, a bacterial endoglycosidase on the pro-inflammatory properties of immune complexes from SLE patients.
To investigate if C1q could have an interferon-regulatory effect we stimulated immune cells with three known interferogenic stimuli (CpG DNA, RNA-containing ICs and herpes simplex virus (HSV)) in the presence of physiological concentrations of human C1q. The IFNα levels were measured by an ELISA.

When using PBMCs, C1q dose-dependently inhibited all of the three stimuli used. However, PBMCs contain several different cell populations why we next isolated pDCs to examine if C1q exerted its IFNα inhibitory function directly through this cell population. Using highly purified pDCs we observed that C1q inhibited both CpG DNA and IC-induced IFNα production (Figure 8.2).

![Figure 8.2. C1q dose-dependently inhibits IC-mediated IFNα production by pDCs.](image)

Surprisingly, HSV-induced IFNα production by pDCs was amplified in the presence of C1q. Thus, in the presence of PBMCs, C1q could exert a general inhibitory effect on HSV-induced IFNα production which was reversed once using purified pDCs. Amongst the PBMCs, monocytes are the most frequent phagocyte and we speculated if this cell population could have mediated the general IFNα suppression. Once the monocytes were removed from the PBMCs C1q had lost its general IFNα inhibitory effect and the HSV-induced IFNα production was instead amplified (unpublished data). However, C1q could still dose-dependently inhibit both CpG DNA and IC-mediated IFNα production. Thus, so far, our data concluded that C1q exerted a general IFNα suppressing effect through monocytes but could also regulate the IFNα production by pDCs directly [74]. After we had published our paper, Santer and colleagues suggested that C1q did not exert any of its IFNα-inhibitory effects through pDCs but only through monocytes [73]. Even though we used highly purified pDCs it is difficult to confirm that there were no contaminating monocytes left which might have influenced our results. However, if there were any contaminating monocytes, they were not able to inhibit the HSV-induced IFNα production when using purified pDCs. Thus, our data still strongly suggest that C1q could exert its cytokine-regulatory functions directly through pDCs as well as through monocytes.

So far we had only used purified human C1q. Even though C1q is produced by macrophages locally, it is mainly found as a complex with C1r and C1s in the circulation. Thus, we had to investigate whether serum, containing the C1qr2s2 complex, could mediate the same IFNα
inhibition. As a control we used serum from an individual with a genetic C1q deficiency. Our results clearly demonstrated that normal human serum, if sufficient in C1q, efficiently inhibited the IFN\(\alpha\) production, but once deficient in C1q, it lost all its ability to inhibit the IFN\(\alpha\) production. The different capacities of these sera to inhibit IFN\(\alpha\) lead us to one of our main conclusions; a C1q deficient individual will not be able to suppress the IFN\(\alpha\) production and will thus be at risk to develop autoimmune diseases. Thus, the strong association between C1q deficiency and development of SLE might depend on the lost ability to inhibit IFN\(\alpha\), a cytokine well-known to induce autoimmune disease if not regulated properly [201, 203].

To identify the mechanism of the C1q-mediated IFN\(\alpha\)-regulation, we performed several experiments. Since the production of IFN\(\alpha\) demands receptor-mediated uptake and presentation to TLR7 in the endosome, we investigated whether C1q could affect the uptake of ICs. However, the addition of C1q to ICs did not decrease the phagocytosis. However, whether C1q was able to localize the ICs to other intracellular compartments than TRL7-containing endosomes is not known. During this experiment we observed that C1q bound to the surface of the pDCs, as well as to some other cell populations including the monocytes. Several C1q binding molecules/receptors have been described, but we could not find any of those on the cell surface of pDCs. However, a new candidate C1q receptor, CD91, was described after the publication of our paper [82]. CD91 and cC1qR have been suggested to be important in the removal of C1q-opsonized apoptotic cells [291]. Furthermore, CD91 was recently described to be found on the cell surface of pDCs, and exhibit anti-inflammatory signaling properties [292]. If C1q could regulate the IC-mediated IFN\(\alpha\) production through CD91 on pDCs remains to be elucidated.

Altogether, we have demonstrated that C1q could inhibit the IC-induced IFN\(\alpha\) production by purified pDCs (Figure 8.3). This might explain the strong association between C1q deficiency and development of SLE, since dysregulated IFN\(\alpha\) production might lead to autoimmunity through a decreased B cell activation threshold [133, 201]. Even though the mechanism of the C1q-mediated inhibition is yet unknown, we suggest that C1q binding molecules, including CD91, might be potential candidates. Further studies will aim to identify the C1q-binding molecule to find novel targets for development of therapies in SLE.

8.3 Paper II

As described in the previous paper, ICs and pDCs are central in the SLE pathogenesis. The ICs activate the complement system, recruit immune cells and cause inflammation and tissue destruction. At these inflammatory sites phagocytic cells release several pro-inflammatory substances, one of which is S100A8/A9 and this protein complex is increased in serum from SLE patients [21]. So far, the production of S100A8/A9 has only been described in monocytes and PMNs [254]. However, it is not known if pDCs are able to produce this pro-
inflammatory protein. Thus, we wanted to identify the cellular origin of S100A8/A9 in SLE patients, and whether this pro-inflammatory protein complex could be produced by pDCs.

By flow cytometry we could detect cell surface staining of S100A8/A9 on all cell populations investigated (monocytes, PMNs, mDCs, pDCs, B cells and NK cells) except for T cells. Furthermore, the cell surface S100A8/A9 was increased in patients with active disease as compared to inactive disease. Even though most cell populations had cell surface S100A8/A9 expression, only monocytes, PMNs and pDCs had a detectable mRNA expression of S100A8 and S100A9. Thus, for the other cell populations, the S100A8/A9 expression could not be explained by protein synthesis, but clearly the S100A8/A9 had to be deposited from an external source such as serum or NETs. Since we did not see any correlation between serum and cell surface S100A8/A9 and could not increase the cell surface S100A8/A9 expression by addition of serum, either serum S100A8/A9 was not responsible for the S100A8/A9 on the cell surface, or the S100A8/A9 ligand was already saturated. It is also possible that during isolation of the cells, or in the circulation, PMNs became activated and produced NETs which coated the surface of the cells with S100A8/A9.

**Figure 8.3.** RNA-containing immune complexes (IC) are phagocytosed by a plasmacytoid dendritic cell through FcγRIIA and internalized into TLR7-expressing endosomes. Through activation of TLR7, the pDC is able to produce high amounts of IFNα. Depicted in the figure is also the hypothetical interaction between IC-bound C1q and a putative C1q binding molecule (C1qRx) on the surface of pDCs. The exact mechanism of how C1q interferes with the IFNα production in pDCs is still to be elucidated.
Previously, only monocytes and PMNs were reported to be able to produce S100A8/A9, but we could demonstrate that also pDC were able to produce S100A8/A9. Upon activation the cell surface staining of S100A8/A9 increased on pDCs, suggesting that it might have biological functions. To this date, two receptors have been described for S100A8/A9; TLR4 and RAGE [28]. TLR4 is not expressed by the pDCs but there are reports demonstrating cell surface expression of RAGE [184]. It could be speculated that S100A8/A9 in pDCs acts as an amplification pathway through RAGE to induce high amounts of IFNα, but further experiments are needed to support this hypothesis.

In summary, we have demonstrated that pDCs produce the pro-inflammatory protein S100A8/A9 and this is increased in patients with active disease, both on the cell surface and in serum. The biological function of S100A8/A9 in pDCs is not known, but it could be hypothesized that it could amplify the cytokine production as has been demonstrated in other cell populations [34] (Figure 8.4).

![Figure 8.4](image.png)

**Figure 8.4.** pDCs are able to produce the pro-inflammatory S100A8/A9 protein complex. Upon immune complex (IC)-stimulation, S100A8/A9 is translocated to the cell surface and this is increased in active disease. The function of S100A8/A9 in pDCs is still unknown. However, it could be speculated that S100A8/A9 might interact with RAGE on the cell surface and amplify the type I IFN production.
Immune complexes have several important functions in the SLE pathogenesis through interactions with immune cells (discussed in previous papers) and may also bind to and activate platelets [273, 293]. SLE is characterized by an increased risk to develop cardiovascular disease and venous thrombosis [256]. In young women there is a 50-fold increased risk to develop myocardial infarction. The increased risk to develop cardiovascular disease and venous thrombosis can only partly be explained by traditional Framingham risk factors [210] which suggest that the disease itself is an independent risk factor. The role of platelets in the development of cardiovascular disease and venous thrombosis in SLE has gained recent attention and several reports have demonstrated that SLE patients have increased platelet activation [213, 214, 270, 271]. Thus, we wanted to investigate whether platelet activation could be involved in the increased risk to develop cardiovascular disease in SLE. To find differences in SLE patients’ platelets as compared to platelets from healthy controls we performed a microarray analysis on purified platelets. Since the platelet mRNA content is 10,000-fold less than the mRNA content in leukocytes [294], the platelets have to be extremely pure. This is also one of the main reasons for the low number of microarray studies on platelets [295-297]. When analyzing the microarray data it became obvious that platelets from SLE patients had a type I IFN signature with a high expression of many type I IFN-regulated genes. The type I IFN signature has previously been demonstrated in PBMCs, but never before in platelets [250-253].

We were able to verify the platelet type I IFN signature by real-time PCR on selected genes. Thus platelets from SLE patients seemed to have an increased expression of type I IFN-regulated genes. However, platelets are only cellular fragments derived from the bone marrow-residing megakaryocyte and they do not contain DNA. Thus, the increase of type I IFN-regulated genes had to happen in the megakaryocyte. To support this hypothesis we were able to demonstrate that IFNα could up-regulate the expression of several type I IFN-regulated proteins in a megakaryocytic cell line.

In all, we have verified the type I IFN signature in platelets from SLE patients, but whether or not this was associated with development of cardiovascular disease was not known. To investigate this we recruited SLE patients and healthy controls and analyzed platelet expression of type I IFN-regulated proteins by flow cytometry. Platelets isolated from SLE patients had increased levels of all type I IFN-regulated protein measured (Figure 8.5).

To investigate whether the platelet type I IFN signature might be associated with the increased risk of cardiovascular disease (CVD), we divided the patient material into patients with a previous CVD (stroke, myocardial infarction and venous thrombosis) and compared them to SLE patients without any CVD. Three of the four investigated type I IFN-regulated proteins were highly increased in platelets from SLE patients with CVD. PRKRA, one of the type I IFN-regulated proteins, could distinguish between...
SLE patients with or without CVD very efficiently (Figure 8.6). We could also demonstrate that SLE patients had more activated platelets (Figure 8.6), especially patients with CVD.

In summary, we found that SLE patients had a platelet type I IFN signature which was associated with platelet activation and CVD. However, it is difficult to know whether or not the type I IFN signature in platelets contributes to CVD or whether it is only a biomarker of other type I IFN-mediated effects. There are several observations linking development of cardiovascular diseases and venous thrombosis with type I IFNs. First of all, IFN-α may decrease the number and function of endothelial progenitor cells, which could promote atherosclerosis [268, 269]. Furthermore, risk alleles in STAT4, a type I IFN-regulated gene, have been associated with ischemic cerebrovascular events in SLE and primary anti-phospholipid antibody syndrome [298, 299]. Thus, further studies are needed to clarify whether type I IFNs activate platelets and make them more prone to participate in pro-thrombotic events or whether the platelet type I IFN signature only might be a novel biomarker of CVD. A figure illustrating IC-induced platelet activation and type I IFN signature is presented in Figure 8.7.

![Figure 8.5](image)

**Figure 8.5.** Increased type I IFN-regulated proteins in platelets from SLE patients. The expression of four type I IFN-regulated proteins (CD58, CD69, IFTM1 and PRKRA) was measured by flow cytometry in platelets from healthy individuals and SLE patients. The line represents the median value.
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Upon platelet activation the platelets expose phosphatidylserine and chondroitin sulphate which are recognized by C1q and activate the classical pathway of the complement system [220, 221]. Increased deposition of C4d has been detected on platelets from SLE patients and patients with stroke [222, 300], but the expression of other markers of the classical pathway (C1q and C3) has not been investigated. Furthermore, it is not known whether C4d on platelets is a marker of platelet activation or if platelet complement activation is associated with cardiovascular disease and venous thrombosis in SLE. Thus, we measured the deposition of C1q, C4d and C3d on the platelet surface by flow cytometry in a well-characterized SLE cohort. The patients had highly increased levels of all complement components as compared to healthy controls (Figure 8.8).

Furthermore, almost 50% of the SLE patients had increased C4d deposition as compared to 4% of the healthy controls. Thus, complement binding to platelets seemed to be a common event in the SLE pathogenesis. In the previous paper we described that SLE patients had more activated platelets [214]. Upon platelet activation complement activating components; chondroitin sulphate and phosphatidylserine, are present on the platelet surface [220, 221]. We also found a correlation between platelet activation markers and complement binding to platelets in vivo suggesting that platelet activation was a prerequisite for complement activation. The next question that had to be addressed was if complement activation on platelets in SLE patients was merely due to increased platelet activation or if other factors also could be involved.

![Figure 8.6](image)

**Figure 8.6.** A) Increased PRKRA platelet expression in SLE patients with CVD as compared to healthy controls (HC) and SLE patients without CVD. B) Increased platelet activation, measured as platelet-monocyte complexes, in SLE patients as compared to HC.
Figure 8.7. A schematic figure demonstrating our hypothesis of a central role of immune complexes (IC) in the type I IFN-, complement- and platelet-dependent development of cardiovascular diseases and venous thrombosis in SLE. Immune complexes induce high amounts of type I IFNs by plasmacytoid dendritic cells which in turn affects the type I IFN signature in megakaryocytes in the bone marrow and subsequently the megakaryocyte-derived platelets. Immune complexes activate platelets and allow complement binding and activation. Activated platelets will bind to monocytes and plasmacytoid dendritic cells (pDCs) to increase the production of tissue factor and IFNα, respectively. If the complement activation cascade is not regulated properly, it might progress and form the membrane attack complex and cause microparticle formation. Microparticles are potent inducers of thrombin generation. Finally, type I IFNs could also directly affect the endothelial progenitor cells and induce atherosclerosis. Altogether, immune complexes have several potent ways to participate in the development of cardiovascular disease and venous thrombosis in SLE.
By *in vitro* studies measuring serum-mediated complement activation on heterologous platelets we could demonstrate that serum from healthy controls supported equal complement activation on platelets as serum from SLE patients. However, a subgroup of SLE patients with the aPL antibody syndrome or venous thrombosis supported increased deposition of C4d (Figure 8.9). Importantly, most of the patients with venous thrombosis also had the aPL antibody syndrome. This indicates that there also exist other factors in SLE, possibly aPL antibodies, which might amplify the complement activation on platelets further. Thus, upon platelet activation, the complement system is activated as well, and we suggest that deposition of C4 on the platelet surface may be a novel marker of platelet activation in SLE patients. Since serum from healthy controls also supported complement deposition on platelets it ought to be a physiological event upon platelet activation. However, complement activation on platelets needs to be strictly controlled by complement regulating proteins. Both CD55 and CD59 are expressed on the platelet surface inhibiting the convertases and MAC formation, respectively. Whether or not these proteins are decreased on platelets in SLE patients is not known, but there are reports of decreased CD55 and CD59 expression on other cell populations in SLE [301]. If complement activation on platelets would proceed, the formation of the MAC will lead to a subsequent production of microparticles which are potent inducers of thrombin [223, 224]. Thus, dysregulated complement activation on the platelet surface may increase the thrombin generation and increase the risk to develop cardiovascular disease in SLE patients. Furthermore, activated platelets will bind to monocytes and increase the expression of tissue factor, an important initiator of coagulation [216].

**Figure 8.8.** Increased levels of complement components on platelets in SLE patients. The deposition of C1q, C3d and C4d on platelets was measured by flow cytometry.
The role of complement deposition on platelets was further emphasized once we divided our patient cohort into patients with or without CVD. Even though we had a small SLE cohort, we observed that SLE patients with CVD had increased complement deposition on platelets as compared to patients without CVD, supporting the hypothesis of the importance of dysregulated complement activation on platelets in development of CVD in SLE patients. Further studies aim to confirm these data in a larger SLE cohort as well as to investigate if patients with other autoimmune diseases also have increased complement activation on platelets.

In summary, we have demonstrated that activated platelets support complement activation and this is increased in SLE patients. Furthermore, SLE patients might have other factors, including aPL antibodies, which could amplify the complement activation further. These findings could help to explain the increased risk to develop CVD in SLE.

Figure 8.9. *In vitro* deposition of C4d on heterologous platelets. Heterologous activated platelets were incubated with serum from SLE patients or healthy controls (HC) and the C4d deposition measured by flow cytometry. The values are expressed as the median fluorescence ratio as compared to a negative control antibody. No difference could be seen between healthy controls and SLE patients, or the presence or absence of anti-cardiolipin (aCL) antibodies. However, patients with the aPL-antibody syndrome (APS) or deep venous thrombosis (DVT) supported increased C4d deposition.
As demonstrated in our papers as well as by others, SLE is characterized by an ongoing inflammation in several organ systems mainly due to the presence of autoantibodies and the subsequent formation of immune complexes (ICs). Tissue-deposited immune complexes will activate complement and cause leukocyte recruitment and tissue destruction. Furthermore, the immune complexes will be phagocytosed by immune cells, including plasmacytoid dendritic cells, and induce high amounts of numerous pro-inflammatory cytokines such as IFN-α. The present therapies used in SLE give a general immune suppression while novel therapies in development most often aim to inhibit specific parts of the immune complexes by affecting B cells or inhibit specific cytokines such as IFN-α [302, 303]. In the present study we have investigated whether EndoS, a bacterial endoglycosidase, could reduce the pro-inflammatory properties of immune complexes from SLE patients and thus have the potential to be developed into a new therapeutic. Since the discovery of the IgG hydrolyzing effect in 2001 several studies have been performed demonstrating good results of EndoS treatment in animal models as well as in in vitro experiments [278-286]. However, how EndoS would operate in human in vitro systems was not known.

First of all we could demonstrate that EndoS had the capability to reduce the molecular size and glycosylation patterns of IC isolated from SLE patients. Thus, EndoS could cleave the N-linked oligosaccharide even when the antibody was part of an IC. Previous studies have demonstrated that the removal of the N-linked oligosaccharide decreases the affinity to the Fc-γ-Rs [281], but it is not known if EndoS affects the interaction between ICs and Fc-γ-Rs on immune cells. In our experimental setting, the phagocytosis of ICs by pDCs was completely abolished after EndoS treatment. Furthermore, the pDCs did no longer become stimulated and did not produce any IFN-α. Thus, EndoS treatment would act upstream of type I IFN production. However, pDCs only express Fc-γ-RIIA [304] and other immune cells might handle EndoS-treated IC differently. Uptake of ICs to PMNs is one of the first laboratory measurements of SLE and was described already in 1948 [305]. Serum from SLE patients supported an antibody-mediated uptake of necrotic cells, but this was inhibited after EndoS treatment.

Also, the PMNs had a reduced IC-mediated oxidative burst after EndoS treatment. Once PMNs are activated they might release oxygen radicals as well as NETs, of which the latter has been recognized as an important source of autoantigens in SLE [306]. Furthermore, in the presence of autoantibodies, NETs are highly interferogenic [247]. Even though we did not measure the actual formation of NETs, it could be suggested that EndoS treatment hypothetically would target the release of autoantigens during NETosis and thus act even further upstream than type I IFN production. Altogether, we have shown profound effects of EndoS on the interactions between ICs and immune cells.

Besides the Fc-γ-R-mediated effects and interactions with immune cells, ICs can activate the classical pathway of the complement system.
Even though complement activation is needed for an efficient clearance of apoptotic cells [76], IC-mediated complement activation is an important part of the establishment of nephritis in SLE [307, 308]. Thus, the complement system has dual roles in the SLE pathogenesis. We were able to verify previous results demonstrating that EndoS-treated IgG antibodies did not support classical pathway activation [284]. Furthermore, by a "chemotaxis under agar" assay we could demonstrate that, after EndoS treatment, the IC-induced C5a-mediated recruitment of PMNs was decreased, which is an important part in the pathogenesis of lupus nephritis [307, 308]. Thus, treatment with EndoS could also inhibit complement activation and recruitment of PMNs which are two important events in nephritis but also in the initiation of inflammation in other tissue. A summarizing figure of our results is presented below (Figure 8.10).

Figure 8.10. EndoS, a bacterial endoglycosidase, hydrolyzes an N-linked oligosaccharide on the heavy chain of the IgG molecule which abolishes all of the investigated immune complex-mediated pro-inflammatory properties; phagocytosis by immune cells, IFNo production, oxidative burst, complement activation and chemotaxis of PMNs.
Even though we observed profound effects of EndoS treatment on many of the desired immunological pathways involved in the SLE pathogenesis there are still some concerns before this enzyme may be developed into a novel therapy. Since it affects all immunostimulatory effects of the IgG antibody, EndoS treatment might result in increased susceptibility to infections, but the same is also true for other immunosuppressive treatments. Another major concern is the bacterial origin of this enzyme and potential immunogenicity. Experiments in rabbits have indicated that they will develop an immune response against EndoS. However, the anti-EndoS antibodies will also be neutralized by EndoS and not affect the treatment or cause inflammation [283]. EndoS has a short half-life of less than 12 hours, and normal levels of functional IgG is present within two weeks after termination of EndoS treatment in mice [278].

Altogether, EndoS show prominent effects on several important immunological pathways involved in the SLE pathogenesis and it has the potential to be developed as a novel therapy in SLE. However, it could be suggested that it should only be given during flares when a rapid inhibition of all IgG-mediated effects is needed, such as in catastrophic APS, and not as a daily treatment.

8.7 Conclusions

- C1q inhibits immune complex-mediated IFNα production by plasmacytoid dendritic cells.
- Plasmacytoid dendritic cells produce the pro-inflammatory S100A8/A9 protein.
- SLE patients have more activated platelets and a type I IFN signature.
- SLE patients with cardiovascular disease have an increased expression of type I IFN-regulated proteins in platelets.
- Platelets from SLE patients have increased amount of complement components on platelets.
- SLE patients with venous thrombosis have an increased complement deposition on platelets.
- EndoS, a bacterial enzyme, abolish all pro-inflammatory properties of immune complexes, and has the potential to be developed into a new therapy in SLE.
9 References


78. mNI-11 antigen but does not bind C1q


209. 213.


