C-reactive protein (CRP) and anti-CRP autoantibodies in systemic lupus erythematosus

– a study on the occurrence and clinical implications of anti-CRP antibodies and CRP-mediated complement activation

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Linköping 2006
Till Johanna
"...a man who is right every time is not likely to do very much"

Francis Crick 1916-2004
## Contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abstract</td>
<td>11</td>
</tr>
<tr>
<td>Abbreviations</td>
<td>12</td>
</tr>
<tr>
<td>List of original publications</td>
<td>13</td>
</tr>
<tr>
<td><strong>Introduction</strong></td>
<td>15</td>
</tr>
<tr>
<td>Systemic lupus erythematosus</td>
<td>15</td>
</tr>
<tr>
<td>C-reactive protein</td>
<td>24</td>
</tr>
<tr>
<td><strong>Aims</strong></td>
<td>31</td>
</tr>
<tr>
<td><strong>Materials &amp; Methods</strong></td>
<td>33</td>
</tr>
<tr>
<td>Patients</td>
<td>33</td>
</tr>
<tr>
<td>Null ellipsometry</td>
<td>34</td>
</tr>
<tr>
<td>Detection of anti-CRP and anti-cytokine antibodies</td>
<td>35</td>
</tr>
<tr>
<td>Isolation of ICs and PBMCs, and IC-induced cytokine induction</td>
<td>37</td>
</tr>
<tr>
<td>Methodological considerations</td>
<td>38</td>
</tr>
<tr>
<td><strong>Results &amp; Discussion</strong></td>
<td>41</td>
</tr>
<tr>
<td>CRP-mediated complement activation (I)</td>
<td>41</td>
</tr>
<tr>
<td>Anti-CRP antibodies in SLE (II-III)</td>
<td>43</td>
</tr>
<tr>
<td>Anti-cytokine antibodies in SLE (IV)</td>
<td>45</td>
</tr>
<tr>
<td>IC-mediated cytokine induction in SLE (V)</td>
<td>47</td>
</tr>
<tr>
<td>CRP-mediated effects on neutrophil F-actin content</td>
<td>49</td>
</tr>
<tr>
<td><strong>Concluding remarks</strong></td>
<td>51</td>
</tr>
<tr>
<td><strong>Acknowledgements</strong></td>
<td>55</td>
</tr>
<tr>
<td><strong>Summary in Swedish – Sammanfattning på svenska</strong></td>
<td>57</td>
</tr>
<tr>
<td><strong>References</strong></td>
<td>59</td>
</tr>
<tr>
<td>Paper I–V</td>
<td></td>
</tr>
</tbody>
</table>
Abstract

Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by production of a wide range of autoantibodies, multiple organ involvement and by local formation or tissue deposition of immune complexes (ICs) in the inflamed organs. In contrast to most systemic inflammatory conditions, and despite raised levels of pro-inflammatory cytokines, SLE flares are rarely reflected by elevated C-reactive protein (CRP), an important acute-phase reactant in man with homologs in vertebrates and several invertebrates. As a part of the innate immune system, CRP binds certain molecules exposed on the surface of dying cells/apoptotic bodies and on the surface of pathogens and mediates their elimination by uptake in the reticuloendothelial system. CRP also interacts with IgG-containing immune complexes, binds Fc receptors and activates the complement system via C1q.

The aims of this thesis were to investigate the complement activation properties of CRP; to elucidate if anti-CRP antibodies occur in SLE and, if so, whether anti-CRP antibody levels correlate with disease activity in SLE; to test the hypothesis that autoantibodies to pro-inflammatory cytokines prevent rise of CRP; and to survey if autoantibodies to certain nuclear antigens or to CRP correlate with cytokine-inducing properties of ICs from SLE sera. We have demonstrated that CRP bound to phosphorylcholine is a powerful activator of the classical complement pathway already in the CRP concentration range 4 to 10 mg/L, but with a marked inhibition at CRP levels above 150 mg/L. Autoantibodies to the monomeric form of CRP were found in approximately 40 percent of SLE patients and in a few sera from patients with primary Sjögren’s syndrome, but not in rheumatoid arthritis or in inflammatory bowel disease. The anti-CRP antibody levels showed significant correlations to several laboratory and clinical measurements, and anti-CRP positivity was associated with renal involvement in SLE. Native CRP levels were not correlated with anti-CRP or anti-cytokine antibody levels. Hence, the presence of antibodies to monomeric CRP or to CRP-inducing cytokines is an unlikely explanation to the relative failure of CRP response in patients with active lupus. However, antibodies to TNFα were found in subnormal levels at disease flares, whereas antibodies to TGFβ were found in supranormal levels as compared to healthy subjects. In contrast to antibodies against CRP and DNA, anti-SSA and anti-SSB antibodies may regulate the inflammatory process in SLE by enhancing IC formation and subsequent production of cytokines such as IL-6, IL-10 and IL-12p40. Hypothetically, anti-CRP autoantibodies may be of pathogenic importance, for instance by binding to monomeric CRP on cell and tissue surfaces and thereby increasing the risk of extrahepatic deposition of apoptotic material and in situ formation of ICs.

Keywords: autoantibodies, complement activation, C-reactive protein, cytokines, immune complex, immunoregulation, opsonization, systemic lupus erythematosus

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Abbreviations

ACR  American college of rheumatology
ANA  antinuclear antibody
anti-CRP anti-CRP antibody
APR  acute-phase reaction
BFP-STS biological false positive serological test for syphilis
C    complement factor
CD   cluster of differentiation
CIC  circulating immune complex
CRP  C-reactive protein
DC   dendritic cell
dsDNA double-stranded DNA
ELISA enzyme-linked immunosorbent assay
ESR  erythrocyte sedimentation rate
FcγR Fc gamma receptor
FH   factor H
HAG  heat-aggregated gammaglobulin
HSA  human serum albumin
IC   immune complex
IF   immunofluorescence
IFN  interferon
Ig   immunoglobulin
IL   interleukin
IL-1ra interleukin 1 receptor antagonist
KLH  keyhole limpet hemocyanin
LDL  low-density lipoprotein
MBL  mannose-binding lectin
mCRP monomeric CRP
NHS  normal human serum
OD   optical density
PBMC peripheral blood mononuclear cell
PC   phosphorylcholine
PEG  polyethylene glycol
PTX3 pentraxin 3
RA   rheumatoid arthritis
SAA  serum amyloid A
SAP  serum amyloid P component
SLE  systemic lupus erythematosus
SLEDAI systemic lupus erythematosus disease activity index
Sm   Smith antigen
snRNP small nuclear ribonucleoprotein
SS   Sjögren’s syndrome
TGF  transforming growth factor
TNF  tumor necrosis factor
Å    Ångström
List of original publications

This thesis is based upon the following papers, which will be referred to in the text by their Roman numerals (I–V):

I  Sjöwall C, Wetterö J, Bengtsson T, Askendal A, Skogh T, Tengvall P.
C-reactive protein activates or inhibits the classical complement pathway in a concentration dependent manner.
Submitted

II  Sjöwall C, Eriksson P, Almer S, Skogh T.
Autoantibodies to C-reactive protein is a common finding in SLE, but not in primary Sjögren's syndrome, rheumatoid arthritis or inflammatory bowel disease.

III  Sjöwall C, Bengtsson AA, Sturfelt G, Skogh T.
Serum levels of autoantibodies against monomeric C-reactive protein are correlated with disease activity in systemic lupus erythematosus.
*Arthritis Res Ther* 2004;6:R87-94

IV  Sjöwall C, Ernerudh J, Bengtsson AA, Sturfelt G, Skogh T.
Reduced anti-TNFα autoantibody levels coincide with flare in systemic lupus erythematosus.

V  Åhlin E, Mathsson L, Sjöwall C, Skogh T, Rönnelid J.
Autoantibody profile and complement activation regulate immune complex-mediated cytokine induction in SLE. Pathogenic implications for anti-SSA/SSB antibodies.
Submitted
Introduction

Systemic lupus erythematosus

Historical background
The history of systemic lupus erythematosus (SLE) dates back at least to the Middle Ages when the term ‘lupus’ (Latin for wolf) was used to designate a variety of skin conditions, for instance acute cutaneous tuberculosis (lupus vulgaris) [1]. However, the first historical account of lupus erythematosus was made by Biett in 1833 [2]. Four decades later, Kaposi described the systemic nature of lupus erythematosus and also noted that the disease was more frequent in women [3]. Sir William Osler coined the term systemic lupus erythematosus with his own recognition of cardiac, pulmonary and renal involvement [4] in addition to the cutaneous manifestations previously described by Cazenave in 1851 [1] and by Kaposi in 1872 [3].

Our current understanding of SLE has evolved from the clinical descriptions that followed and was enhanced by the diagnostic breakthrough in the discovery of the ‘LE cell’ by Hargraves, Richmond and Morton in 1948 [5]. In 1909 and 1910, the German physicians Reinhart and Hauck separately presented the first cases of ‘biological false positive serological test for syphilis’ (BFP-STS) in disseminated lupus erythematosus [6, 7], based upon the ‘Wassermann reaction’ (WR) described already in 1906. The WR reflects occurrence of autoantibodies to phospholipid-related antigens, still recognized as a feature among the commonly used classification criteria for SLE (Table 1). In 1952, Conley and Hartmann noticed that a considerable proportion of SLE patients had a coagulation abnormality, which they called ‘lupus anticoagulant’ [8]. Five years later Laurell and Nilsson found that this coagulation inhibitor was frequently associated with a chronic BFP-STS, and that the factor responsible for its activity was an immunoglobulin (Ig) that could be absorbed by cardiolipin [9].

Holman and Kunkel made the first identification of autoantibodies directed against nuclear constituents in 1957 by use of immunofluorescence (IF) microscopy [10]. The subsequent recognition of the ‘antinuclear factor’ by Friou formed the basis of modern antinuclear antibody (ANA) diagnostics by indirect IF microscopy [11].

Epidemiology
SLE is a chronic disease that can affect people of all ages. The prevalence in Sweden has been estimated to about 60 per 100.000 individuals [12]. The results of a recent study from Italy indicated an overall SLE prevalence of 71 per 100.000 individuals, similar to most European countries [13]. In the USA, reports on prevalence rates vary between 15 to 124 per 100.000 in populations with different genetic, ethnic and socioeconomic background [14, 15]. It is often stated that SLE is mainly a disease of young fertile women. However, a Swedish cohort showed that the age-adjusted
incidence peaked between 65 to 74 years of age in both women and men [12]. More than 85 percent of SLE patients are women both in Europe and in the USA [16].

**Etiological aspects**

Genetic factors have long been known to have a prominent role in SLE based upon high concordance rates (24 to 58 percent) in monozygotic twins compared to less than 10 percent in non-identical twins [17]. However, the genetic component in lupus is complex and does not follow Mendelian inheritance. It is estimated that 5 to 12 percent of first-grade relatives of patients with SLE develop the condition [18]. Many genes have been indicated to be important regarding disease susceptibility, as well as different clinical manifestations [17, 19]. These genes include those encoding the major histocompatibility region, especially the class II HLA genes (HLA-B8, HLA-DR2, HLA-DR3, DQW1), complement components (e.g. C2 and C4 deficiency), Fc gamma receptors (FcγRs), the inhibitory immune receptors PDCD1 and CTLA4, type I interferon (IFN) regulating genes [20-25], and acute phase reactants such as mannose-binding lectin (MBL) and C-reactive protein (CRP) [26-29].

The marked female predominance implies that hormonal factors may be important for the development of SLE. In contrast to rheumatoid arthritis (RA), where estrogens probably are protective [30], there is some support for an unfavorable role in SLE, especially from experimental animal models [31]. Differences between male and female patients in the relative balance of estrogens to androgens do not, however, fully explain the sex and age distribution in SLE or in other autoimmune diseases [32, 33]. It has been reported that disease flares diminish after menopause [34]. Further, without adjustment for the age at menopause, it has been found that postmenopausal hormone replacement therapy may increase the risk of developing SLE [35] and slightly increase the flare rate [36]. However, there is little evidence that estrogen-containing contraceptives increase the risk of SLE development [37].

Exposure to sunlight can cause pathological cutaneous reactions (photosensitivity) as well as generalized exacerbation of the disease [38]. Ultraviolet radiation induces apoptosis in keratinocytes *in vivo*, requiring increased clearance and might thereby contribute to an additional autoantigen load [39].

Occupational and environmental factors have also been associated with the development of lupus. Exposure to heavy metals, such as mercury, may induce autoimmune kidney disease or lupus-like syndrome in some experimental animal models, but evidence from studies in humans is limited [40]. Occupational exposure to silica dust may be associated with the induction of autoantibodies, including ANA, and renal disease [41]. However, most occupations with high exposure to silica dust are traditionally male dominated. Smoking is associated with an increased risk of developing SLE, whereas the opposite has been seen for alcohol [42, 43]. The link between smoking and atherosclerotic disease, and the possible protective effect of moderate doses of alcohol in the general population, has been subject to extensive debate [44, 45]. Since atherosclerosis is a prominent feature of SLE [46], it is intriguing to speculate that the influence of cigarette smoke and alcohol on the
A potential role of aromatic amines and hydrazines has received attention because of their structural similarity to certain medications associated with drug-induced lupus [41].

An early clinical observation was that the onset of SLE often followed an infection, but a causal role of infectious agents has been difficult to conclude. Microbial superantigens, for instance several staphylococcal enterotoxins, enterotoxin of group A streptococci and a soluble product of *Mycoplasma arthritidis* have been suggested in the development of autoimmunity [48]. Viruses, and especially retroviruses, have been postulated in the pathogenesis of SLE [49]. Symptoms, such as chronic arthritis, rash, vasculitis and features of Sjögren’s syndrome (SS) as well as a variety of autoantibodies are commonly found in humans with retroviral infections, e.g. human immunodeficiency virus (HIV). Other viruses (Epstein-Barr, cytomegalovirus and herpes zoster) can trigger an immunostimulatory microenvironment that contributes to a generalized immune activation, with an antigenic preference for proteins and nucleic acids associated with the virus [50]. Another mechanism supported by experimental data is ‘molecular mimicry’, in which a microbial peptide sequence similar to an autoantigen can induce autoantibody formation and in some cases lupus manifestations [51].

**Classification criteria**

SLE is the prototype systemic inflammatory autoimmune disease with a wide variety of manifestations and an unpredictable disease course. The diagnosis of SLE is based on clinical manifestations together with immunological abnormalities combined with relevant anamnesis, including drug history and familial occurrence of autoimmune disease. However, the dynamic nature of lupus with changeable signs and symptoms may render difficulties in determining the diagnosis. In addition, there are other diseases with multi-organ involvement that may mimic SLE. The most common lupus symptoms at disease onset comprise arthritis and arthralgia, cutaneous manifestations (photosensitivity, discoid lesions, malar rash), hematological disorders (cytopenias), serositis (pleurisy, pericarditis) and constitutional symptoms such as fatigue, malaise, weight loss and fever in the absence of infection [52].

According to Fries and Holman, presence of multi-organ disease in at least two of seven specified organ systems and a positive ANA test can be used as a diagnostic principle [53]. For case definition in clinical research, the American college of rheumatology (ACR) has established classification criteria for SLE (Table 1), intending to ensure comparability of patients from different geographical locations [54]. Fulfillment of at least four of the 11 criteria allows classification of a patient as having SLE. Although the ACR criteria were established primarily for research purposes, they serve as helpful tools in everyday clinical routine and as reminders of those features that distinguish SLE from other connective tissue diseases.
<table>
<thead>
<tr>
<th>ACR criteria</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Malar rash</td>
<td>Fixed erythema over malar eminences</td>
</tr>
<tr>
<td>2 Discoid rash</td>
<td>Erythematous raised patches with keratotic scaling and follicular plugging; atrophic scarring in older lesions</td>
</tr>
<tr>
<td>3 Photosensitivity</td>
<td>Skin rash as a result of unusual reaction to sunlight</td>
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<td>4 Oral ulcers</td>
<td>Oral or nasopharyngeal ulceration</td>
</tr>
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<td>5 Arthritis</td>
<td>Non-erosive arthritis involving ≥2 peripheral joints</td>
</tr>
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<td>6 Serositis</td>
<td>Pleuritis or pericarditis</td>
</tr>
<tr>
<td>7 Renal disorder</td>
<td>Persistent proteinuria (≥0.5 g/day) or cellular casts</td>
</tr>
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<td>8 Neurological disorder</td>
<td>Seizures or psychosis</td>
</tr>
<tr>
<td>9 Hematological disorder</td>
<td>Hemolytic anemia with reticulocytosis or leukopenia or lymphopenia or thrombocytopenia</td>
</tr>
<tr>
<td>10 Immunological disorder</td>
<td>Positive LE cell preparation, presence of antibodies to Sm antigen, or to native DNA in abnormal titer, or BFP-STS</td>
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<tr>
<td>11 Antinuclear antibody</td>
<td>Abnormal titer of ANA by IF or equivalent assay</td>
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Table 1: The 1982 revised ACR criteria for SLE [54].

Clinical features
SLE is clinically characterized by a plethora of symptoms and involvement of multiple organs. The manifestations may be diverse, calling for attention in most disciplines in clinical medicine. Thus, few if any of the clinical findings are unique to SLE. Patients can present with unexplained fever, fatigue, weight loss, anemia, photosensitivity, rash, arthritis/arthralgia, Raynaud’s phenomenon, serositis, seizures, alopecia, phlebitis, recurrent abortion, psychosis or nephritis. On the other hand, symptoms and signs may develop sequentially over many years [52-54].

Survival in SLE has improved significantly over the past five decades as a consequence of improved management, but lupus patients still have a three- to fivefold increased standardized mortality rate compared to the general population [55]. Early mortality usually occurs due to severe active disease with involvement of the central nervous system or kidneys. Later deaths are frequently the result of complications to SLE or its treatment and generally comprise cardio- or cerebrovascular disease [56]. Another leading cause of death in SLE patients of all ages is infections [57].

The most critical predictor of mortality in SLE is renal disease [58]. On light microscopy examination, the kidney appears to be involved in 60 to 70 percent of all SLE cases, but if immunofluorescence (IF) and electron microscopy are included in the examination of biopsy specimens, almost all patients show some degree of renal abnormality [59]. However, renal abnormality does not necessarily mean that the patients fulfill the ACR criteria for renal involvement. Pathogenic mechanisms in lupus nephritis are not fully understood, but are apparently dependent on glomerular deposition of circulating immune complexes (ICs) or local IC formation causing cell proliferation and/or glomerular inflammation due to complement (C) deposition/activation and recruitment of inflammatory cells [59, 60].
Cardiac involvement in SLE, such as pan- or pericarditis and Libman-Sacks endocarditis, has been described since the early 20th century [61]. However, it was not until the 1970s that the co-morbidity of cardiovascular disease in SLE was highlighted. The increased risk in some lupus patients may obviously be very high and is essentially unaffected by anti-rheumatic treatment [62, 63]. The cause underlying premature atherosclerosis and vascular pathology in SLE is unclear. Presence of activated immunocompetent cells is typical of atherosclerotic lesions and suggests the involvement of T lymphocytes, at least in the remodeling process of the plaque’s fibrous cap [64]. Another theory is that IC deposition causes the initial damage [65], followed by accelerated development of atherosclerosis in patients with traditional risk factors (hypertension, smoking, dyslipidemia) [66] and/or lupus-related risk factors such as presence of autoantibodies to oxidized low-density lipoprotein (LDL), lipoprotein lipase and cardiolipin [66-69]. SLE patients with enhanced LDL oxidation and/or raised levels of interleukin (IL-) 10 and tumor necrosis factor alpha (TNFα) also seem to be at increased risk [70, 71]. The effect of corticosteroids on atherosclerosis in relation to SLE remains controversial. Corticosteroids could be expected to be atherogenic due to their effects on plasma lipoproteins, but may on the other hand decrease the vascular inflammation and thereby limit the atherosclerotic process [62, 63, 71].

Assessment of disease activity and clinical outcome
Estimating disease activity in a systematic fashion is a prerequisite for optimal clinical care. Routine laboratory measures such as blood cell count, CRP and erythrocyte sedimentation rate (ESR) are indispensable. Renal involvement can present silently at disease onset or following many years of lupus. Therefore, it is important with regular measurements of serum creatinine and urinalyses [59, 72]. The 1982 revised ACR criteria recognizes proteinuria of >0.5 g of albumin per 24 hours, presence of hematuria and/or cellular casts by urinalyses in the absence of infection [54]. A renal biopsy may provide a more accurate documentation of renal disease. However, it is important to keep in mind that most patients with lupus manifest some abnormality on biopsy specimen [59, 72]. Sequential measurements of antibodies to double-stranded (ds) DNA as well as C1q, C3, C4 and anti-C1q autoantibodies are used to reflect activity [72-74]. However, there is need for new biochemical markers indicating renal involvement in SLE [75].

Standardized methods to assess disease activity have been devised, e.g. the British Isles Lupus Assessment Group (BILAG) index, the European Consensus Lupus Activity Measurement (ECLAM), the Systemic Lupus Activity Measure (SLAM), the SLE disease activity index (SLEDAI) and the Lupus Activity Index (LAI) [76]. These instruments are valid, reliable, fairly sensitive to changes in disease activity and they can be used in children [76, 77]. Many physicians find SLEDAI the easiest to use. The SLEDAI covers 24 different features and gives a weighted score of 0 to 105 [78]. A raise of ≥3 in SLEDAI score is commonly considered as a flare. The index may also be modified (mSLEDAI) by exclusion of certain laboratory items (i.e. complement and anti-dsDNA) [79]. The Systemic Lupus International Collaborating Clinics/ACR
damage index (SLICC/ACR DI) is reliable and useful to estimate disease severity and damage due to side-effects of medication [80].

**Medical treatment**

Glucocorticoids are often used to treat disease exacerbations. Disease-modifying drugs such as anti-malarials (hydroxychloroquine), azathioprine, mycophenolate mofetil (MMF), cyclosporin A and methotrexate are used as maintenance therapies [81, 82]. The most severe forms of active glomerulonephritis and other serious disease manifestations call for treatment with cyclophosphamide or MMF [72, 82]. Although some have found it challenging to use TNF inhibitors in lupus [83], they are not included in the arsenal, since they in some instances may aggravate the disease, e.g. by reducing clearance of nucleosomes, leading to the induction of anti-nucleosomal antibodies and thereby worsening the disease [84]. B cell depletion by treatment with anti-CD20 antibodies (rituximab) is a promising therapeutic modality, i.e. for lupus nephritis [85]. Cytokine-targeted therapies are also interesting future possibilities (see below).

**Cytokines of importance in SLE**

Cytokines play essential roles in shaping an immune response to foreign or self-antigens. These mediators have been classified according to their cellular source and effector functions often referring to the T helper (Th) type 1/Th2 paradigm, or simply as pro- or anti-inflammatory. Cytokines such as TNFα, IL-6, IL-10, IL-12, IL-18, transforming growth factor (TGF) β and IFNs have attracted special interest in SLE [86-92]. Although many have suggested that SLE is predominated by Th2 responses [93, 94], this has been contradicted by others [95]. In recent years, treatments aiming specifically at blocking pro-inflammatory cytokines such as TNFα have resulted in significant clinical improvement in patients with conditions such as RA and Crohn’s disease [96].

**IFNα** is frequently raised in SLE patients, and the circulating levels are correlated with both disease activity and severity [90, 97]. Interestingly, apoptotic cells in combination with IgG from SLE sera induce the production of IFNα in purified plasmacytoid dendritic cells (DCs), suggesting a lupus model for enhanced production of IFNα in context with apoptotic material and IgG autoantibodies [98].

**TNFα** is a pleiotropic pro-inflammatory and pro-apoptotic cytokine, which also displays properties as a B cell growth factor, has numerous effects on T lymphocytes and inhibits IFNα production in plasmacytoid DCs [87, 99]. In mice, TNFα may enhance physiologic clearance of circulating immune complexes (CIC) and reduce the severity of IC-induced nephritis in murine lupus [100, 101].

**TGFβ** has marked inhibitory effects on the immune system but also serves as a co-stimulatory factor in the development of T cells with down-regulatory activities (Th3) and capability to induce mucosal B cells to produce secretory IgA antibodies [93].
TGFβ production from SLE lymphocytes has been reported to be decreased [94], while TGFβ is raised and recognized as a key cytokine in systemic sclerosis [102].

**IL-10** usually displays suppressive effects on the immune system, but seems to have dual actions in SLE [103]. Raised serum levels of IL-10 correlating with disease activity have been reported in SLE [104]. IL-10 is also suggested to be disease-promoting in SLE and responsible for the polyclonal activation of B cells [88, 105, 106]. Anti-IL-10 antibody treatment was reported successful in arthritic and cutaneous manifestations of lupus by Llorente and coworkers [107].

**IL-6** is a pleiotropic cytokine and a B cell differentiation factor, which has received interest owing to its ability to promote B cell dependent secretion of IgG autoantibodies [108]. IL-6 is increased in SLE as compared to healthy blood donors [109]. In addition, IL-6 has an important role in the acute-phase response (see below).

**IL-12** is considered to be a Th1 cytokine and a major inducer of IFNγ, but it has inhibitory effects on the production of most other cytokines [94]. IL-12 has been reported to be decreased in SLE compared to healthy subjects due to IL-10-mediated suppression, but the exact relation between IL-10 and IL-12 in lupus is under debate [110, 111].

### Soluble receptors and anti-cytokine antibodies
Growth factors and cytokines can be targeted by the immune system, resulting in the production of autoantibodies, which may occur in patients with inflammatory diseases as well as in apparently healthy individuals [112]. Several soluble cytokine receptors have been identified and are continuously generated along with the formation of its respective cytokine to achieve a balance in the immune responses.

Anti-IL-1α autoantibodies are found in sera from healthy individuals [112]; these antibodies have been suggested to prevent bone erosions [113] and to predict a favorable outcome value in RA [114]. Decreased serum levels of IL-1 receptor antagonist (IL-1ra) have been observed in relation to renal involvement in SLE [79].

Autoantibodies against granulocyte colony stimulating factor (G-CSF) can be found in neutropenic lupus patients [115], while a low prevalence of anti-IL-3 antibodies has been reported in Felty’s syndrome [116]. Neutralizing IL-6 autoantibodies have been described in systemic sclerosis, healthy blood donors and in relation to mortality of patients with alcoholic cirrhosis [117-119]. The presence of anti-IL-6 in lupus has been observed in a frequency comparable to blood donors [120].

TNFα receptors (p55TNFR and p75TNFR), which neutralize or modulate bioactive TNFα, are present in the circulation and on cell surfaces. Circulating levels of these receptors are increased in SLE, as compared to RA, and the levels correlate with disease activity [121]. Anti-TNFα autoantibodies have been described in SLE and other rheumatic diseases as well as in sepsis and in chronic infections [122, 123].
Additionally, autoantibodies against the soluble TNFα receptor p75TNFR have been noted in SLE [124].

Anti-IFNγ autoantibodies have been observed in parallel to disease remission in Guillain-Barré syndrome [125]. Neutralizing autoantibodies to IFNα2, IFNω and IL-12 have been reported in patients with late-onset myasthenia gravis (MG) and in patients with thymoma with or without concomitant MG [126]. Raised levels of autoantibodies to INFγ, TNFα, IL-4 and IL-10 have previously been detected both in cerebrospinal fluid and plasma from patients with multiple sclerosis and aseptic meningitis [127]. Antibodies against type I as well as type II IFNs have also been described in lupus [128, 129].

Autoantibodies in SLE
Autoantibodies are Igs that bind via their combining sites to antigens originating from the same individual or species. IgG class autoantibodies are most common in SLE, but IgM class autoantibodies also occur [130]. ANA is a central feature of SLE and targets nucleic acids, DNA-protein complexes, and ribonucleoprotein complexes. Apart from ANA, antibodies against cytoplasmic and extracellular antigens, including plasma proteins, are commonplace in SLE [131]. Interestingly, and in favor for those who believe that autoantibodies have a central role in the pathogenesis of lupus, the appearance of autoantibodies in lupus patients tends to follow a predictable course with a progressive accumulation of certain autoantibodies before clinical disease onset [132].

Within the cell nucleus, DNA is condensed under the influence of cationic proteins, designated histones. The repeated DNA–histone octamer packages are referred to as nucleosomes or chromatin. Anti-DNA autoantibodies were discovered almost 50 years ago and are still a hallmark in the diagnosis of SLE [10, 11, 73]. At least three forms of DNA are targeted by autoantibodies in SLE; single-stranded, double-stranded (ds) and left-handed DNA. Many lupus sera contain antibodies to more than one form of DNA. Presence of anti-dsDNA antibodies by indirect IF microscopy using the *Crithidia luciliae* as source of antigen has a very high specificity for SLE (≥95 percent), but is detected only in a minority of the patients, predominately in patients with nephritis [73, 131].

Antibodies against ribonucleoproteins such as the Smith (Sm) antigen, U1-small nuclear ribonucleoproteins (snRNP), Ro (SSA) and La (SSB) are frequently found in SLE [131]. Anti-Sm is of particular interest because of its high specificity for SLE, but it is only detected in a small fraction of Caucasian lupus patients [133]. Anti-SSA is associated with photosensitivity, interstitial lung disease, homozygous deficiency of C2 or C4, subacute cutaneous LE, neonatal lupus as well as being a marker of primary SS [133]. Anti-SSA/SSB levels have not been associated with disease activity in SLE [73, 133, 134].

Phospholipids have important roles in the coagulation system. BFP-STS and lupus anticoagulant were the first serological abnormalities identified in SLE [6-9].
Autoantibodies to cardiolipin alone and/or β2-glycoprotein I are seen in up to a third of all SLE patients and are often associated with thrombosis, recurrent abortion and autoimmune cytopenia [67, 131].

**The waste disposal theory**

Apoptosis, or programmed cell death, is essential in the normal function of multicellular organisms, and is implicated in developmental and homeostatic mechanisms. It is a complex and firmly regulated process aiding to prevent intracellular material from being recognized by the immune system. During the late phase of apoptosis, nuclear autoantigens (e.g. nucleosomes, SSA, SSB and Sm) are sequestered in surface blebs and may in turn become available to professional antigen-presenting cells, for instance DCs [135, 136]. Interestingly, DCs but not macrophages efficiently present antigens derived from apoptotic cells to cytotoxic T cells [137]. In addition, during apoptosis the autoantigens that are composed of complex particles often become modified, leading to increased immunogenicity [138].

Rapid removal of apoptotic cells or cellular debris by phagocytosis is critical to ensure safe elimination of potentially pro-inflammatory or immunogenic material from the circulation [135-137]. Under normal circumstances, any material that escapes clearance by phagocytosis can be rapidly cleared from the circulation by a number of additional mechanisms, including opsonization by proteins such as CRP, MBL or C1q and/or by IC-forming antibodies. These antigen–protein complexes are then actively removed from the circulation via the reticuloendothelial system [139, 140]. There is considerable evidence for dysfunction in several of these key events in human SLE. Supported also by results from lupus animal models, apoptosis of lymphocytes, monocytes as well as neutrophils have been reported to be accelerated in SLE [141-143]. In addition, several groups have reported defective processing, and Fc dependent clearance, of ICs and apoptotic cells in lupus patients [144-146]. Further indications of deficient removal of apoptotic material in SLE patients include the associations between relative or absolute deficiencies of certain components of the classical complement pathway (C1q, C1r, C1s, C4 or C2) and the occurrence of SLE [147].

Taking it all together, it is conceivable to regard SLE as a disease with dysregulated apoptosis and/or defective clearance, leading to increased levels of circulating autoantigens and a ‘mission impossible’ for the body’s waste disposal system. Structurally altered autoantigens on apoptotic blebs have potentials to be presented to professional antigen presenting cells. Subsequently, DCs may, via presentation of autoantigens to T lymphocytes, activate B cells, which in turn mature into autoantibody producing plasma cells. Eventually, the binding of autoantibodies to their target antigen results in the formation of ICs in situ and/or in the circulation, constituting a pathogenic vicious circle [148].
C-reactive protein

Historical background
CRP was discovered 75 years ago at the Rockefeller University. Investigating blood from patients with acute febrile illness, William S Tillett and Thomas Francis Jr demonstrated the precipitation of a non-antibody serum component with a soluble extract of *Streptococcus pneumoniae*. This serum reaction was present during the acute phase of the disease and diminished as the patients recovered. Identification of C-polysaccharide as the bacterial constituent gave rise to its designation as C-reactive protein [149]. In parallel with the discovery of minor CRP elevation being a useful risk marker in cardiovascular disease, substantial progress has been made over the last decade concerning the biological properties and physiological importance of CRP in health and disease.

The acute-phase response
The acute-phase reaction (APR) is an early set of inflammatory reactions composed of non-specific biochemical and biophysical responses of endothermic animals and is initiated by microbes/microbial constituents and tissue degradation. In humans, the APR comprises increased hepatic production and release of acute-phase proteins, such as CRP, serum amyloid A (SAA), haptoglobin, \( \alpha_1 \)-acid glycoprotein, ferritin and \( \alpha_1 \)-antitrypsin; while other proteins are reduced, for instance albumin and transferrin [150]. Elevated ESR is also used as a measure of the APR, although the increase is slower compared to CRP, and is also affected by non-APR factors such as sex, hypergammaglobulinemia, red blood cell shape and size and the hematocrit.

The pentraxin family
Pentraxins are phylogenetically conserved pentameric acute-phase proteins, which are expressed during infection, systemic inflammation or tissue damage. The family includes long pentraxins, *e.g.* pentraxin 3 (PTX3) produced by mononuclear cells in response to lipopolysaccharide, and liver-derived short pentraxins, *i.e.* CRP and serum amyloid P component (SAP). Pentraxins have been found in all vertebrate species as well as in some invertebrates. For instance, CRP was found in the phylogenetically ancient horseshoe crab, *Limulus polyphemus* [151].

Molecular structure
The pentameric structure of CRP is composed of five identical non-covalently bound subunits with 206 amino acids (~23-kDa) arranged in cyclic symmetry around a central pore [151, 152]. The subunits are synthesized as non-glycosylated monomers
consisting of two anti-parallel β-sheets with flattened jellyroll topology (Figure 1). Each subunit has a single phosphorylcholine (PC) binding site and two bound calcium ions adjacent to a hydrophobic pocket. All five binding sites are located on the same face of the pentamer [153]. The binding of complement factor C1q occurs at the opposite face of the pentamer where binding to FcγRs is also presumed to take place [152, 154].

**Gene expression**
Circulating CRP is essentially derived from hepatocytes, but small amounts may also be produced locally (see below). The synthesis is mainly regulated at the transcriptional level through IL-6 and IL-1β directed induction of the CRP gene, located on the short arm of chromosome 1 by activation of NF-κB and transcriptional factor NF-IL-6/CAAT-enhancer binding protein (C/EBP) family members C/EBPβ and C/EBPδ [152, 155, 156]. TNFα may also indirectly enhance the production, while TGFβ displays an inhibitory influence [151, 157]. Single nucleotide polymorphisms in the CRP promoter gene are associated with differences in baseline levels of CRP [158, 159]. Recently, the impact of stress and hormones on the regulation of CRP production has been discussed [160, 161].

**Protein synthesis**
When intracellular transport of newly synthesized rabbit CRP was examined by radiolabeled protein, the intact CRP pentamer was transported more rapidly across the secretory pathway from the endoplasmic reticulum to the extracellular space in rabbits undergoing an APR compared to uninflamed animals [162]. This implies that secretion may provide another level of CRP regulation. Apart from hepatocytes, CRP synthesis has been reported to occur in neurons [163], lymphocytes [164], smooth muscle cells [165], alveolar macrophages [166] and tubular epithelial cells in the kidney [167]. The mechanisms by which synthesis is regulated at these sites are not known. Although extrahepatic CRP may mediate local effects, it is unlikely that it substantially affects the plasma levels.

**Monomeric CRP**
Under conditions of altered pH, high urea or low calcium concentration, the native CRP dissociates irreversibly into monomers [168]. These subunits, designated monomeric CRP (also recognized as modified CRP or neo-CRP in the literature), undergo conformational rearrangement resulting in expression of a distinct isomer with unique antigenic and physiochemical characteristics [169]. There is now strong evidence that such dissociation occurs under physiological conditions after binding to plasma membrane [170]. Monomeric CRP (mCRP) has a lower isoelectric point than the pentameric form and is considered to be a tissue and/or cell based form of the acute phase protein [170-173].
Cellular effects

The biological functions of native CRP are fairly well recognized whereas the properties and biological effects of mCRP have attracted less interest. Like many cytokines, native CRP has pleiotropic actions, having for instance pro- as well as anti-inflammatory effects. It inhibits many functions of neutrophil granulocytes, including the chemotactic response to IL-8 [174] and the production of reactive oxygen species and degranulation [175], possibly via alteration of actin polymerization by increasing F-actin and decreasing G-actin [176] (see also 'Results & Discussion'). By contrast, mCRP up-regulates complement receptor 3 (CD11b/CD18) [177] and activates neutrophils, monocytes and platelets [168]. While mCRP enhances attachment of neutrophils to endothelial cells and thereby promotes transmigration [177], native CRP exerts modulatory effects on monocytes both by activating and limiting the early stages of diapedesis [178].

In peripheral blood mononuclear cells, native CRP was shown to stimulate IL-1ra production to a greater extent than it stimulates the generation of IL-1β [179]. In mice with experimental allergic encephalomyelitis (EAE), native CRP increases the release of IL-10 while decreasing the secretion of TNFα and IFNγ [180]. CRP has also been linked to enhanced expression and activity of plasminogen activator inhibitor-1 (PAI-1) in human monocytes [181] and decreased release of prostacyclin by endothelial cells [182]. On the contrary, megakaryocyte proliferation and platelet generation is stimulated by mCRP [183], which also exerts anti-apoptotic actions on human neutrophils [184] and inhibitory effects on the growth of mammary adenocarcinoma in mice [185]. In apolipoprotein E knockout mice, mCRP reduced atherosclerosis whereas the opposite was seen for native CRP [186].

CRP receptors

Although previously questioned [187, 188], there is now compelling evidence that CRP interacts with IgG receptors in man and mouse, eliciting a response from phagocytic cells [189-191]. The binding of CRP to cellular FcγRs is believed to account for its direct opsonizing properties; pentameric CRP primarily binds to the low-affinity FcγRIIa (CD32) and to some extent to the high-affinity FcγRI (CD64), whereas mCRP binds to the low-affinity FcγRIII (CD16) [152, 177, 189-194]. Ligand recognition and binding by CRP may thus contribute to a range of metabolic, scavenging and host-defense functions.

CRP ligands

Native CRP plays several important roles by calcium-dependent binding to specific ligands, such as PC in oxidized phospholipids on damaged cell membranes [151] or LDL [195], fibronectin [196, 197] and protein A [198]. However, mCRP does not have affinity for PC [199]. The ability to bind nuclear structures at physiological ionic strength, both nucleosome core particles and extrachromosomal constituents such as snRNPs, is also well documented for native CRP [152, 200]. Interestingly, many of the nuclear antigens to which CRP binds are the same as those targeted by ANA seen in patients with SLE and other systemic inflammatory rheumatic diseases [73, 131]. It
is conceivable that CRP, by FcγR-mediated uptake in phagocytes, facilitates the clearance of circulating nucleosomes and apoptotic blebs on which nuclear antigens are exposed [136, 200, 201], thereby limiting the contact of these autoantigens with the adaptive immune system. In an inflammatory microenvironment with acidic conditions, native CRP dissociates into CRP monomers, which bind to IgG-containing ICs [202] and to phagocytic FcγRIII [192].

**CRP interaction with complement**
Activation of the complement cascade is regarded as one of the main physiological functions of CRP. When a ligand binds to a CRP subunit, it induces a conformational change revealing a cleft on the opposite side of the subunit. The collagensous portion of C1q binds avidly in this cleft, thereby inducing complement activation via formation of the classical C3 convertase, which in turn leads to decoration of the ligand surface with opsonizing complement fragments [154, 203]. The ability to induce the complement cascade has been reported unique to the pentameric CRP form [168, 204] and the activation is progressively increased by the presence of apoptotic cells with immobilized cell surfaces [205].

In contrast to IgM- and IgG-mediated complement activation, CRP-mediated activation appears to be essentially limited to the initial stage involving C1-C4 with less formation of the membrane attack complex (MAC) [206]. This is presumably due to a direct interaction between CRP and factor H (FH), leading to inhibition of the alternative complement pathway C3 and C5 convertases [203, 207-209]. Another maneuver by which CRP may regulate complement activation is by increasing the expression of complement-inhibitory proteins, such as decay-accelerating factor (DAF; CD55), membrane cofactor protein (MCP; CD46) and protectin (CD59) [210]. Thus, CRP participates in host defense at the same time as it restricts potentially harmful side-effects of inflammation (Figure 2).
CRP in health and disease
Since hepatic CRP can rise rapidly with the plasma concentration increasing from less than 1.0 to more than 500 mg/L within 24 to 72 hours, it has been extensively used in the clinics as a measurement of the APR, to evaluate the response to antibiotic therapy and to distinguish bacterial from viral infections [151, 152]. In healthy volunteer blood donors, the median CRP concentration is 0.8 mg/L. The median baseline value in the ostensibly healthy population is slightly higher and tends to increase with age. Females have negligibly higher CRP levels than men. The plasma half-life of CRP is about 19 hours, surprisingly unaffected of simultaneous disease [211].

Atherosclerosis. Minor CRP elevations have been shown to reflect a low-grade vascular inflammation. Numerous studies have established the high-sensitivity CRP test with levels ≥3.0 mg/L as the most powerful independent biochemical marker in the prediction of future coronary vascular events and survival in patients with angina pectoris as well as in apparently healthy subjects [212-214]. The molecular mechanisms that link CRP to atherogenesis are incompletely understood, but recent investigations have revealed that CRP directly interacts with several major components in the process of atherosclerosis [215, 216].

Atherogenesis is associated with several changes in the vascular endothelium, including loss of vasoreactivity, expression of adhesion molecules and secretion of chemoattractants. Interestingly, CRP causes these changes when exposed to endothelial monolayers. CRP inhibits the expression of endothelial nitric oxide synthase (eNOS), the production of nitric oxide (NO) and cGMP in endothelial cells [217]. In patients with cardiovascular disease, minor elevations of CRP correlate with the loss of NO-mediated vasodilation [218]. Recruitment of monocytes to the arterial wall during atherogenesis is central. Treatment of endothelial cells with native CRP increases the expression of the adhesion molecules E-selectin, ICAM-1 and VCAM-1 and stimulates the production of monocyte chemoattractant protein-1 (MCP-1) [219], whereas mCRP seems to potentiate these actions [220]. Furthermore, CRP increases endothelial secretion of IL-6, which amplifies hepatic CRP production and systemic inflammation [150, 214]. The widespread atherosclerosis in lupus patients may thus reflect this pathogenic role of CRP [46, 63]. In this context, an interesting finding was made in murine lupus where CRP-inducing cytokines were overexpressed in myocytes of the heart muscle [221].

CRP directly affects monocytes in each stage of atherogenesis by inducing adhesion to endothelium, chemotaxis, cytokine and tissue factor expression and the release of reactive oxygen species [174, 175, 191, 219, 220, 222, 223]. While enhancing phagocytosis in macrophages, CRP mainly inhibits cellular functions of neutrophils, which suggests that it may be crucial in the conversion of acute inflammation to a chronic inflammatory state in the vessel wall during atherogenesis [214]. After vascular injury, CRP has been shown to increase the migration and proliferation of vascular smooth muscle cells (VSMCs) and their production of matrix proteins within the neointima. Increased expression of inducible nitric oxide synthase (iNOS) in VSMCs, as a response to CRP, has been observed [224].
Oxidized and enzymatically modified LDL is present at high concentrations in atherosclerotic plaques. Both these forms of LDL have the ability to bind to CRP, which probably facilitates the uptake by macrophages and the formation of foam cells [195]. The LDL-CRP complexes have an increased ability to convert C3 and activate complement. Hence, CRP has been found to colocalize with MAC in early atherosclerotic lesions of coronary arteries [225].

**SLE.** Most studies show that the CRP response is limited or absent at lupus flares despite high disease activity [226-232] and this may be of etiopathogenic importance concerning both immunoregulation and induction of autoimmunity [152, 214]. Regarding the capacity of CRP to bind nuclear antigens/apoptotic cells and to interact with FcγRs, it has been proposed that the modest CRP response in SLE contributes to the deficient handling of apoptotic material, thereby increasing the risk of abnormal immunization to autoantigens [205].

In mice, deletion of the SAP gene leads to the development of a lupus resembling illness [233]. In analogy, CRP supplementation to lupus-prone (NZB x NZW) F₁ mice delays the onset of nephritis, decreases autoantibody levels, leads to less autoimmune manifestations and prolongs the survival through an FcγR and IL-10 dependent mechanism [234, 235]. When the same lupus prone mouse strain was conducted to express human CRP, similar results were achieved, with the exception that anti-DNA antibody levels were not lowered [236].

In humans, it has recently been shown that a polymorphism at the CRP locus influences the basal CRP expression and predispose to SLE [28]. Modest CRP reactions during active disease are also common in ulcerative colitis. Patients with either of these conditions share the intact capability of a ‘normal’ CRP response in intercurrent infections [227, 237-240]. This is in agreement with the findings of a normal CRP turnover in SLE and resembles other inflammatory disorders [241]. However, the remarkably low CRP levels seen in patients with hypocomplementemic disease involving skin and kidneys, often in contrast to raised levels of other acute-phase reactants, could hypothetically be due to CRP consumption by ICs [242-247]. Deviating cytokine patterns, as a consequence of aberrant immunoregulation, have been proposed as a reason for the pronounced discrepancies between CRP in flare and infection in lupus. Although raised circulating levels of many cytokines are seen, in some instances paralleling disease activity [96, 104], IL-6 levels do not correlate with circulating CRP in SLE [248]. Genetic polymorphisms of CRP inducing cytokines and their concomitant receptors have been found in association with SLE and might predispose to distinct clinical and immunological features [249-252].

**Alzheimer’s disease (AD).** The role of pentraxins, and particularly SAP, in AD has been extensively investigated. From immunohistological studies on AD brain, there is strong evidence that complement activation plays a central part in the neurodegeneration, typical of the condition. Colocalized with complement in the temporal cortex, expression of both SAP and CRP mRNA, supporting de novo synthesis, has been reported in AD [163, 253]. Serum levels of CRP in AD patients are normal. Given the facts that complement is important in AD pathogenesis and that CRP is a strong activator of the classical complement cascade, it is not surprising that
drugs aiming at blocking complement activation have been suggested in AD [253, 254].

**Autoantibodies to CRP and other acute phase proteins**

A characteristic feature of SLE is the multitude of autoantibodies to nuclear antigens, such as dsDNA, histones, DNA–histone complexes (nucleosomes) and extrachromosomal antigens such as Ro/SSA, La/SSB, Sm and snRNP [73]. In addition, autoantibodies in SLE are frequently directed against cytoplasmic constituents (e.g. ribosomal phosphoprotein and phospholipids) and extracellular antigens, for instance plasma proteins such as β₂-glycoprotein I, C₁q, IgG (rheumatoid factor), MBL and SAA [74, 131, 255-258].

Already in 1985, Frank A Robey and coworkers described autoantibodies against CRP in one out of eight SLE patients and reported a depressed ability of CRP to solubilize chromatin in some SLE individuals [259]. Later, Susanne A Bell demonstrated a high frequency of IgG antibodies to cryptic epitopes of CRP in patients suffering from the ‘autoimmune-like’ toxic oil syndrome [260]. Bell and colleagues also reported high frequencies of autoantibodies to mCRP in SLE (78 percent) and lower prevalences in subacute cutaneous lupus erythematosus and primary biliary cirrhosis [261]. Recently, Rosenau and Schur demonstrated antibodies against CRP in sera from patients with different rheumatologic conditions, including SLE, where they observed a frequency of 23 percent [262].
Aims

The work of this thesis aimed to elucidate the biological properties of CRP in relation to systemic inflammatory diseases, particularly SLE. General objectives were to explore the interaction between CRP and complement, to investigate if the occurrence of autoantibodies against CRP could explain the modest CRP levels often seen in SLE, and furthermore, to determine the role of certain autoantibodies in IC-mediated cytokine production in SLE. Specific goals were to:

- Disclose complement activation properties of CRP on defined surfaces in vitro (Paper I)
- Elucidate if anti-CRP antibodies occur in SLE and if this may explain low serum CRP in SLE despite high disease activity (Paper II and III)
- Explore whether anti-CRP antibody levels correlate with disease activity in SLE (Paper III)
- Test the hypothesis that autoantibodies to pro-inflammatory cytokines prevent rise of CRP in SLE and/or if levels of anti-cytokine antibodies correlate with disease activity (Paper IV)
- Survey if autoantibodies to certain nuclear antigens or to CRP correlate with cytokine-inducing properties of ICs from SLE sera (Paper V)
Materials & Methods

Patients

**Paper I**
Blood samples were drawn from two healthy individuals with CRP concentrations below 5 mg/L, and from 4 patients with acute infection and one patient with thrombosis, all with CRP levels above 100 mg/L.

**Paper II**
56 sera from 31 patients were included on the basis of a known content of IgG anti-dsDNA antibodies, as assessed by indirect IF microscopy (*Crithidia luciliae* test). Of the 31 patients, 27 had a clinical diagnosis of SLE and 4 had autoimmune hepatitis. One serum sample each was also taken from 16 patients with primary SS, 15 patients with RA, 31 patients with Crohn’s disease and 37 patients with ulcerative colitis. 100 normal human sera (NHS) served as reference.

**Paper III**
10 patients fulfilling the ACR criteria for SLE were followed with serial blood sampling at 5 occasions over a period during which disease exacerbation occurred. Sera from 100 healthy blood donors (different from paper II) served as controls.

**Paper IV**
In addition to the 50 serum samples from paper III, another 28 SLE patients were included and from each of these patients blood samples were taken at two occasions, *i.e.* at active and inactive disease. The same controls as in paper III were used.

**Paper V**
147 serum samples from 64 patients with a clinical diagnosis of SLE were studied. For the anti-CRP analysis, a new material of 100 serum samples from healthy blood donors served as reference. For the cell culture experiments, peripheral blood mononuclear cells (PBMCs) were isolated from healthy blood donors.
Null ellipsometry

The principle of ellipsometry was described at the end of the 19th century and the concept was developed further for protein adsorption studies by Leo Vroman [263]. Null ellipsometry is an optical method based on monochromatic light reflection and refraction. Due to changes in the reflected light intensity, polarization state and electrical field composant phase shifts after reflection, the thickness of the thin organic film can be iterated under the assumption of a known refractive index (Figure 3) [264]. The method is excellent for the analysis of a thickness up to ~1000 Ångströms (Å; 1 Å=1x10⁻¹⁰ m) and has a practical sensitivity of ~1 Å or less [265, 266]. Compared to methods such as enzyme-linked immunosorbent assay (ELISA) and radioimmunoassay (RIA), ellipsometry has advantages in that it is direct, non-destructive and quantitative. Furthermore, it is excellent for real-time analyses of surface phenomena, and it is robust and sensitive. When combined with polyclonal antibodies, the method allows convenient identification of adsorbed proteins without labeling procedures. Disadvantages may be that a flat and optically reflective surface is required. The method has a sensitivity comparable to quantitative RIAs [266].

![Image of null ellipsometry set-up](image)

**Figure 3:** The null ellipsometry set-up. The method measures the light beam polarization state and intensity before and after reflection at a surface. The analyzer (λ/4-plate) is rotatable and moves to find the calculated ellipsometric angles where the intensity into the detector is zero, hence 'null'. Based on changes in the reflected light after adsorption, the thickness of the thin organic film can be iterated under the assumption of a known refractive index.
In paper I, we chose to use silicon as model substrate due to its even optical properties. Three layers of fibrinogen were covalently attached and cross-linked to silicon and PC conjugated with keyhole limpet hemocyanin (PC-KLH) was immobilized on top. A normocomplementemic low CRP serum with and without added CRP or acute phase sera were subsequently exposed to the PC surfaces. The total serum protein deposition was quantified and deposition of IgG, C1q, C3c, FH and CRP was detected with polyclonal antibodies.

Detection of anti-CRP and anti-cytokine antibodies

Anti-CRP assay
The assay used in paper II is based on the description by Bell et al [261]. Briefly, 96-well microtiter plates were coated overnight at room temperature with native human CRP in carbonate/bicarbonate buffer. Patient sera were diluted in PBS-Tween, added in triplicates and incubated for 60 minutes. An alkaline phosphatase-conjugated rabbit anti-human IgG, specific for $\gamma$-chains diluted in PBS-Tween, was added to each well and plates were incubated for 60 minutes. The substrate, $p$-nitrophenyl phosphate diluted in deionized water, was added to each well and the plates were incubated for 60 minutes at room temperature.

Optical densities (OD) were measured at 405 nm and the results were expressed as percentage of a positive reference sample from an SLE patient at flare (‘SLE reference’) or as the OD value. All results refer to the net OD values after subtraction of the background OD obtained on uncoated plates. In paper III and V, the assay was slightly modified. Instead of diluting the patient sera 1:20, each sample was diluted to a standard concentration of 0.3 mg IgG/mL.

Figure 4: A dilution series of anti-CRP antibody reactivity in the ‘SLE reference’.
Anti-cytokine assays
The assays used in paper IV are based on the description by Elkarim et al [125]. Briefly, 96-well microtiter plates precoated with human serum albumin (HSA) were incubated with recombinant human cytokines (IL-1β, IL-6, IL-10, TNFα or TGFβ1, respectively). Diluted patient sera were added in triplicate and incubated for 120 minutes. A γ-chain specific biotinylated rabbit anti-human IgG was applied for 60 minutes followed by an avidin-biotin-alkaline phosphatase complex (ABC-AP) for 30 minutes. Prior to the addition of p-nitrophenyl phosphate, unbound ABC-AP was removed by washing. ODs were measured at 405 nm. Each serum was also tested on HSA-coated plates without adsorbed cytokine, but otherwise treated as described above. All results refer to net OD values after subtraction of the background obtained on HSA-coated plates without cytokine. For all anti-cytokine assays, a strongly positive control serum was used as reference.

Figure 5: Schematic illustration of the anti-cytokine antibody assay. ABC-AP; Avidin-biotin-alkaline phosphatase complex.
Isolation of ICs and PBMCs, and IC-induced cytokine induction

**Precipitation of ICs**
ICs were isolated from patient sera by polyethylene glycol (PEG 6000) precipitation and single-step centrifugation essentially as described by Pontes-de-Carvalho et al [267]. Briefly, each serum was mixed with an equal volume of PEG 6000 containing 0.1 M EDTA and left at 4° C overnight. One mL of PBS with 5 percent HSA and 2.5 percent PEG 6000 (PBS-HSA-PEG) was added. The PEG-treated sera were diluted 1:3 in culture medium containing 2.5 percent PEG 6000 and added on top of the PBS-HSA-PEG forming a two-phase system. The IC precipitates were pelleted by centrifugation at 2100 g, 4° C for 20 minutes and at the same time washed in the PBS-HSA-PEG bottom phase. The two-phase supernatants were discarded and the pellet immediately resolved in ice cold PBS to the original serum volume. The dissolved PEG precipitates were then placed on ice until used in the cytokine induction experiments.

**Preparation of PBMCs and cell cultures**
Buffy coats from healthy blood donors were diluted in PBS at room temperature and separated using a density gradient (Ficoll-Paque Plus, GE Health Care, Uppsala, Sweden). Following two washes in PBS, the cells were diluted to 1x10⁶ cells/mL in culture medium. Freshly prepared PEG-precipitated ICs were added to the cells within two hours of preparation and cultured for 20 hours.

**Cytokine ELISAs**
The levels of IL-10, IL-6 and IL-12p40 in the cell culture supernatants were measured by ELISA, following a recently described protocol [268].
Methodological considerations

**Does the purchased CRP contain pentameric or monomeric CRP?**
According to the manufacturer (Sigma-Aldrich, St. Louis, MO, USA), human plasma was the source of CRP, which had been isolated by gel chromatography and had a purity of \( \geq 99 \) percent as measured by SDS-PAGE. The manufacturer also ensured that the CRP had a pentameric form. To test this, we made two ‘model antigen surfaces’ for the detection of mCRP and pentameric (p) CRP, respectively.

One ELISA plate was coated with acid-treated CRP according to the description by Potempa *et al.* [168]. The other ELISA plate was coated with PC-KLH in a calcium-containing buffer (veronal buffer). Untreated CRP was then coated onto the layer of PC-KLH. Both plates were eventually incubated with two monoclonal antibodies, specific for either mCRP or pCRP (generously provided by Dr Larry Potempa) [269] and treated in accordance with the previously described anti-CRP antibody assay (see above). The results are shown in Table 2.

<table>
<thead>
<tr>
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<th>3H12 (anti-mCRP)</th>
<th>2C10 (anti-pCRP)</th>
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</thead>
<tbody>
<tr>
<td>Acid-treated CRP (mCRP)</td>
<td>100%</td>
<td>58%</td>
</tr>
<tr>
<td>PC-KLH+CRP (pCRP)</td>
<td>11%</td>
<td>100%</td>
</tr>
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**Table 2:** Binding reactivity of the two different mouse monoclonal antibodies, 3H12 and 2C10, to each model antigen surface, respectively. The antibody binding to their corresponding antigen was defined as 100 percent.

**Does the purchased CRP contain DNA?**
This question was raised early and checked for. In paper II, we made an ‘in-house’ anti-nucleosome antibody ELISA and noticed that the levels of anti-CRP antibodies in the samples did not correlate with anti-nucleosome antibody reactivity. Furthermore, the same procedure was done in paper III, where the results of the ‘in-house’ anti-nucleosome ELISA were also confirmed using a commercially available antibody assay (Anti-nucleo, GA Generic Assays GmbH, Dahlewitz, Germany). In addition, we had the CRP preparation checked by polymerase chain reaction (PCR) technique for potential DNA contamination. No DNA was detected. However, the CRP preparation was found to reduce the PCR detection limit for DNA to 0.2 percent.
Against which form of CRP is the anti-CRP autoantibody directed?
In paper II, we showed that anti-CRP autoantibodies, in contrast to a commercial rabbit anti-CRP antibody, were not capable of reducing the CRP levels in a test tube with a known CRP concentration upon sedimentation with protein G sepharose.

In paper III, we prepared mCRP by urea-treatment [270]. After dialysis against Tris-HCl buffer, the urea-modified CRP in solution was found to inhibit anti-CRP antibody binding dose-dependently (Figure 6).

We also checked the ‘SLE reference’ on the two model antigen surfaces (described above). Compared to the native model antigen, there was an evidently higher antibody binding to the mCRP model antigen (Figure 7).
**Do ICs interfere with the detection of anti-CRP antibodies?**

The question of whether ICs could interfere with the detection of anti-CRP was raised. If this is true, also sera from patients with diseases such as RA and inflammatory bowel disease could be expected to produce positive results in the anti-CRP ELISA, but this was not the case. Neither did heat-aggregated rabbit IgG block the anti-CRP reactivity of SLE sera. Finally, in paper V, we demonstrated that PEG-precipitated and re-solubilized ICs from SLE sera did not induce positive anti-CRP tests (Figure 8). In our view, these findings strongly contradict the possibility of interference with ICs in the anti-CRP assay.

![Figure 8](image)

**Figure 8:** Sera and PEG-precipitated ICs from eight patients were applied to a CRP-coated ELISA plate and analyzed in parallel with regard to anti-CRP reactivity. This graph demonstrates that sera from SLE patients (empty bars), but not their corresponding precipitated ICs (filled bars), gave rise to positive anti-CRP antibody tests.

**How accurate are the methods of anti-cytokine antibody detection?**

The methods to analyze anti-cytokine antibodies in paper IV were based on the description by Elkarim *et al* [125]. We successfully performed specificity analyses according to the directions of Klaus Bendtzen in order to exclude the possibility of cytokine–Fc part interactions or non-specific IgG binding to the ELISA plate [271]. After our study was published it was revealed that all cytokines used, except for IFNγ, contained considerable amounts of bovine serum albumin (BSA), whereas the IFNγ used for specificity analysis, contained HSA. In a subsequent setting, analyzing anti-cytokine antibodies in patients with RA, it was revealed that anti-TNF antibody reactivity was decreased by preincubation with BSA. Thus, anti-BSA antibodies, which commonly occur in healthy individuals [272], may to some extent produce falsely positive results in anti-cytokine antibody assays utilizing antigens containing BSA as a carrier molecule. This issue will be subjected to further investigation.
Results & Discussion

CRP-mediated complement activation (I)

In paper I, the capacity to activate the complement system was compared between a normocomplementemic low CRP serum with or without purified CRP and acute phase sera, originated from patients with different inflammatory conditions. We demonstrated that purified CRP and CRP in patient sera activate the classical pathway on immobilized PC similarly in a dose-dependent manner. Even at concentrations below 10 mg/L, CRP proved to be a powerful complement activator (Figure 9, insert). Control experiments showed that plasma and sera from same individuals displayed the same capacity to activate complement, i.e. fibrinogen seemed not to be involved in the process. The findings have implications both for the normal innate immune homeostasis and for cardiovascular disease where high sensitivity analysis of CRP is nowadays a widely used risk marker [212].

![Figure 9: Deposition of anti-C3c and anti-CRP onto PC-KLH-surfaces after addition of 0 to 500 mg/L CRP to a serum containing 0.4 mg CRP per liter.](image)

FH, which is a serum alternative complement pathway inhibitor protein with affinity for immobilized C3b, binds CRP in vitro, whereby it possibly limits the generation of MACs [206]. In contrast to several previous studies [207-209], which used purified proteins, we used complete serum. However, our findings support the notion that FH
may be important, also under (patho-) physiological conditions, for the control of the alternative pathway during CRP-mediated complement activation.

Regardless of whether acute phase sera or CRP-supplemented sera were used, a dramatic decrease in C1q-mediated complement activation at CRP levels above 300 mg/L was seen. This phenomenon was unexpected and has not been described before. We hypothesize that down-regulation of complement activation, when approaching harmful intensity, might reflect an attempt to restrict potential tissue damage. A lack of such CRP-mediated inhibition could possibly contribute to IC-mediated tissue damage in SLE, where the CRP response often appears to be lacking or in disharmony with other acute-phase reactants [205].

Previous reports have not found that mCRP activates complement [168, 204, 273]. However, in the present study we demonstrate that immobilized mCRP may activate the complement system with kinetics similar to that of pentameric CRP (Figure 10). We believe that the binding strength between the immobilized mCRP and serum C3 is relatively weak, since the binding of the polymeric anti-C3c onto the serum layer varied from one experiment to another.

![Figure 10: Anti-C3c binding on top of the total serum deposition during 60 minutes of incubation. A significant difference between serum deposition and anti-C3c binding was seen at 10 minutes of incubation. * p<0.05](image-url)
Anti-CRP antibodies in SLE (II–III)

The selection of patient sera was different in these two studies. In paper II, we selected anti-dsDNA antibody positive samples for the anti-CRP analyses. The majority of these sera were derived from patients with a diagnosis of SLE whereas, in paper III, the serum samples and clinical data were limited to 10 well-characterized lupus patients.

In paper II, we found IgG reactive with human CRP in 20 of the 56 anti-dsDNA sera (36 percent). Of these 20 samples, 18 originated from patients with a diagnosis of SLE. Altogether, 13 of the 27 SLE patients (48 percent) were anti-CRP antibody positive on at least one occasion, four of which were positive and negative in different sera. Two of the 16 SS sera were positive, while none of the sera from RA and inflammatory bowel disease patients were positive. All positive sera were derived from female patients. No correlations between anti-CRP levels and anti-dsDNA titers were found. Since anti-CRP did not reduce the CRP levels in solution upon sedimentation with protein G sepharose, it is unlikely that the occurrence of anti-CRP autoantibodies explains the modest CRP reactions often seen at SLE flares. However, we hypothesize that anti-CRP may have other pathogenic implications, for instance by reacting with surface-bound CRP on cells and tissue surfaces. Furthermore, considering the extensive number of nuclear structures to which CRP possibly can bind, it cannot be excluded that that anti-CRP antibodies could result in falsely positive ANA tests.

Figure 11: Results of the anti-CRP analyses in paper II. 5/100 NHS, 20/56 anti-dsDNA antibody positive sera (dsDNA+), 2/16 sera from patients with SS, 0/15 RA, 0/31 Crohn’s disease (CD) and 0/37 ulcerative colitis (UC) were anti-CRP positive. Positive samples are indicated by (●) and negative by (○). The OD value obtained for the serum containing the highest level of anti-CRP reactivity was defined as 100 percent.

Paper II did not answer the question whether or not anti-CRP antibody levels correlate with disease activity, although individuals were sometimes positive on one occasion and negative on another. In paper III, we demonstrated that the serum
levels of anti-CRP antibody paralleled the clinical disease activity, usually with high levels at the time point of flare (Figure 12). Anti-CRP antibodies were detected in 20 of 50 serum samples (40 percent). Seven of 10 patients were positive on at least one occasion, while three patients were consistently anti-CRP negative. Six of ten patients were positive at flare. In three cases (HG, AM and DD), elevated levels of anti-CRP preceded the flares whereas preflare samples were not available for three patients.

All patients with active kidney involvement were anti-CRP positive at flare, while four of six patients without ongoing kidney involvement were anti-CRP negative at flare. None of the three consistently anti-CRP negative patients (IS, AR, BB) showed signs of nephritis during the study period. Contrasting to paper II, we found correlations between anti-CRP and anti-dsDNA levels. The reason hereto may be sought both in methodological differences for anti-DNA antibody detection and the different selection of patient sera. In agreement with observations by others [260, 262], the detected anti-CRP antibodies did not correlate with circulating levels of CRP in either of the two studies.

Renal involvement often results in more pronounced discrepancies between CRP and disease activity [79]. The low CRP levels seen in SLE patients with nephritis, often contrasting to levels of other acute-phase reactants, could possibly be due to CRP consumption by ICs [242-247]. With this in mind, we find the high frequency (4/4) of anti-CRP antibodies in patients with ongoing kidney involvement interesting considering the pathogenic implications of ICs in lupus nephritis, but extended
studies on larger materials are needed. Prospective studies are also needed to evaluate if changes in anti-CRP antibody levels can predict disease flares.

The binding of CRP to cellular FcγRs is believed to account for its opsonizing properties [152]. It is conceivable that mCRP exposed on cellular surfaces may be a target for anti-CRP autoantibodies. In this connection, and regarding earlier findings of mCRP expression on human peripheral blood lymphocytes [274, 275] and accelerated apoptosis of lymphocytes from SLE patients [141], the inverse relation between high anti-CRP antibody levels and lymphopenia is thought-provoking. Hypothetically, this correlation may result from an opsonization of lymphocytes expressing mCRP on their cell surface, leading to increased elimination via the reticuloendothelial system. CRP facilitates the clearance of apoptotic debris by FcγR-mediated uptake in phagocytes [276] and when the tissue microenvironment becomes acidic due to inflammation, CRP is dissociated to mCRP, which further enhances the binding of ICs to FcγRs [202]. In addition, via C1q-binding CRP has complement activating properties, which also promote IC clearance [139, 203, 273]. Speculatively, anti-CRP autoantibodies could interfere with the physiological mCRP-mediated removal of ICs and/or nuclear constituents [136, 139, 202, 276].

Anti-cytokine antibodies in SLE (IV)

In paper IV, the possibility of anti-cytokine antibody-mediated cytokine regulation/dysregulation in SLE was evaluated. Despite the fact that a variety of autoantibodies is a prominent feature, the presence and possible roles of anti-cytokine autoantibody mediated effects in SLE has attracted surprisingly little interest. The present study was undertaken to investigate the presence of anti-cytokine antibodies and to elucidate if they could be related to the poor CRP response in SLE. We chose to analyze antibodies against IL-6, IL-1β and TNFα, since all of these cytokines can increase hepatic CRP production [151, 152]. TGFβ1 has been reported to decrease CRP production [157], whereas IL-10 has anti-inflammatory properties and is implicated as a disease promoting cytokine in SLE [88, 103, 106].

Anti-cytokine (IL-1β, IL-6, IL-10, TNFα and TGFβ1) autoantibody levels at active and inactive disease and in NHS were compared, respectively. No significant differences were found between SLE patients and the controls concerning the occurrence of anti-IL-1β, anti-IL-6 or anti-IL-10 antibodies, using either flare or remission samples. However, anti-TGFβ antibodies were found at supranormal levels (p<0.01), regardless of whether flare or remission samples were used; no aberrance was shown between active and inactive disease. As shown in Figure 13, significantly lower levels of anti-TNFα antibodies were found in SLE patients with active disease compared to NHS (p<0.001) and to remission (p<0.001).

The levels of autoantibodies to IL-1β, IL-6, IL-10 and TNFα were not significantly correlated with circulating levels of their corresponding cytokines in serum.
Correlations between anti-cytokine antibody levels and disease activity measures were calculated on accumulated data from all sampling occasions \((n=106)\). Significant inverse correlations were found between anti-TNF\(\alpha\) vs SLEDAI \((p<0.01)\) as well as mSLEDAI \((p<0.01)\). A positive correlation was also found between anti-TGF\(\beta\) and C1q \((p<0.005)\). None of the anti-cytokine antibodies were significantly associated with medication, kidney involvement or any particular symptom/ACR criteria.

![Figure 13: Anti-TNF\(\alpha\) autoantibody levels at active and inactive disease in the 38 SLE patients. *** p<0.001](image)

Unexpectedly, we found no evidence of raised autoantibody levels to CRP-inducing cytokines in SLE. Neither did we find any correlations between CRP levels and any of the tested anti-cytokine autoantibodies. Thus, we suggest that neutralizing antibodies against cytokines is an unlikely explanation to the moderate CRP reactions commonly seen in SLE. Altogether 106 sera from 38 SLE patients were analyzed and significantly lower levels of anti-TNF\(\alpha\) antibodies were found at disease exacerbation, compared to remission and to healthy blood donors. The depressed levels of anti-TNF\(\alpha\) in active disease could possibly indicate lack of physiological cytokine network control or antibody consumption by inflamed tissues. Notably, in the SLE sera, we also found supranormal levels of antibodies to TGF\(\beta\), a cytokine with clear-cut antagonistic effects on TNF\(\alpha\) release from macrophages [277].
IC-mediated cytokine induction in SLE (V)

The disease process in SLE includes interaction between autoantibodies and autoantigens leading to the formation of ICs, which induce classical complement activation and the production of certain cytokines. Several studies have indicated that anti-dsDNA antibodies are present in ICs of SLE patients [60, 135], and that raised levels of these antibodies may reflect active disease [72, 73]. In contrast, levels of other SLE-related autoantibodies, such as anti-SSA/SSB, have not been shown to vary in relation to disease activity [134]. Paper V was undertaken to investigate the relation between certain autoantibodies, levels of CIC, complement activation and IC-mediated cytokine production.

The functional part of the study comprised paired sera from SLE patients obtained at occasions with distinct degrees of complement activation. Samples with increased complement activation were associated with enhanced IC-induced IL-10 production (Figure 14). Decreased levels of C3 were associated with increased IL-12p40 production ($p=0.033$). We also found a strong correlation between changes in IC-induced levels of IL-10 and IL-6 ($p=0.0015$), but not with IL-12p40 levels. The same association was determined when the samples were analyzed by cross-section ($p<0.0001$).

In addition, we found that PEG precipitates from patients with both anti-SSA and anti-SSB antibodies induced higher levels of IL-10 ($p=0.021$) compared to those lacking these antibodies. Furthermore, IC induced levels of IL-6 were highest among anti-SSA/SSB antibody-positive SLE patients ($p=0.011$). We found a correlation between the amount of IC-induced IL-10 and CIC measured in serum ($p=0.012$). To analyze the impact of antibody status and complement function in parallel, we examined levels of CIC- instead of IC-induced cytokine production. Employing ANOVA analysis with CIC as the dependent variable, we determined a strong interaction between decreased complement function and the occurrence of anti-SSA antibodies (complement $p<0.0001$, anti-SSA $p=0.0009$, interaction $p=0.0072$). No
corresponding association was seen for anti-dsDNA, anti-U1-snRNP or for anti-CRP antibodies. Thus, complement activation was associated with increased levels of CIC and with the capacity of serum derived ICs to induce cytokine secretion by PBMCs from healthy donors. This feature was especially prominent if the patient had detectable, precipitating anti-SSA auto antibodies at the time of sampling. Hypothetically, in active SLE with ongoing complement activation, anti-SSA and anti-SSB antibodies together with antigens derived from apoptotic cells may participate directly in the inflammatory process by enhancing IC formation and subsequent production of cytokines like IL-6, IL-10 and IL-12. In quiescent disease on the other hand, autoantigens are not released from cells and the corresponding autoantibodies may circulate as monomers without being incorporated into ICs.

The cross-sectional part of the study consisted of 78 samples with a normal complement profile, which showed increased IL-12p40 levels in PBMC cultures stimulated with PEG-ICs from anti-SSA antibody-containing samples ($p=0.0105$). This effect was even stronger in samples containing both anti-SSA and anti-SSB antibodies ($p<0.0001$).

The prevalence rate of anti-CRP antibodies in this study (50/125; 40 percent) corroborated our previous observations (Paper II and III). No differences were detected concerning anti-CRP antibody levels when comparing patients with and without IF-ANA, anti-dsDNA, anti-SSA, anti-SSB or anti-U1-snRNP antibodies. Anti-CRP antibodies do not produce immune precipitates in immunodiffusion, a fact that may explain why the occurrence of anti-CRP antibodies had no association with elevated levels of CIC or PEG precipitate-induced cytokine secretion. Increasing anti-CRP antibody levels in the two functional experiments (including paired samples from 42 patients) were associated with decreased C3 levels ($p=0.0082$) and increased C3d/C3 ratios ($p=0.048$). This is in line with our previous findings (Paper III) and strengthens the notion that anti-CRP antibody analysis is a useful tool in assessing disease activity.
CRP-mediated effects on neutrophil F-actin content

Native CRP has been reported to inhibit several functions of neutrophil granulocytes, including the chemotactic response to IL-8 [174] and the production of reactive oxygen species and degranulation [175]. The exact mechanisms underlying these inhibitory effects are yet incompletely understood. In 2004, Yates-Siilata and coworkers published a study demonstrating that CRP increased the cortical distribution of F-actin with a resultant loss of lamellipod formation in neutrophils from healthy blood donors [176]. However, in 2003 we studied the effects of native CRP on F-actin content in neutrophils stimulated with IgG-opsonized yeast. By evaluation with confocal microscopy, we found enhanced polymerization of F-actin in cells exposed to CRP compared to neutrophils treated with buffer (Figure 15). Hence, we propose that increased F-actin may stabilize the cell membrane of neutrophils and, once the cells are stimulated, limit the release of reactive oxygen species [278].

![Figure 15: Effects of native CRP on the F-actin content in neutrophils stimulated with IgG-opsonized yeast [278].](image-url)
The current view on lupus pathogenesis is that autoantigens from dying cells are abnormally exposed to the immune system as a consequence of dysregulated apoptosis and/or deficient elimination of apoptotic material via the reticulo-endothelial system [135, 279]. Hence, successful removal of apoptotic cells or cellular debris is critical to avoid undesired immune reactions. The players of the innate immune system have central roles in this process. CRP, MBL and C1q have opsonic properties, but they also form a bridge with the adaptive immune system by interactions with Igs and FcγRs [152]. On the other hand, imbalances in these systems may promote the induction of autoimmunity [26-28, 201, 255, 280].

We have confirmed that the presence of autoantibodies against mCRP is common in SLE, and we are the first to describe correlations between disease activity measures (i.e. SLEDAI scores, C1q, anti-DNA antibodies) and serum levels of anti-CRP antibodies (Paper III). So far, in all patients with active lupus nephritis, we have found anti-CRP antibody-positivity at flare, which raises the question of a pathogenic connection. Control sera from patients with RA and inflammatory bowel disease respectively, have consistently turned out negative in the anti-CRP assay, whereas a few patients with primary SS have tested positive (Paper II).

It is not likely that the presence of anti-CRP antibodies explains the relative failure of CRP response in patients with active SLE. Instead, the possibility of post-translational modification of the CRP molecule by glycosylation could be relevant both with regard to clearance of circulating CRP and the induction of anti-CRP autoantibodies. In fact, it was recently demonstrated that CRP molecules in different disease states, including SLE, differ both in their carbohydrate content and their amino acid sequences [281, 282]. Interestingly, CRP purified from pooled sera of SLE patients showed a single band by SDS/PAGE, suggesting an identical abnormal glycosylated variant [281].

As with most autoantibodies, the origin of anti-CRP antibodies is unknown. The presence of IgG class autoantibodies to C1q in lupus was first reported in 1984 [283]. Further investigations revealed that the majority of IgG binding to C1q in solid phase assays was attributable to autoantibodies reacting with an epitope only exposed in structurally modified C1q [284]. Such a change in structure, to reveal a ‘neo-epitope’, may follow proteolytic cleavage, a conformational change following activation or following binding to another protein. Thus, it seems likely that anti-C1q antibodies develop as a part of an autoantibody response to structurally altered forms of C1q, possibly evolving from binding to cells, proteins or ICs. Exposure of hidden epitopes on conformationally changed antigens or the appearance of neo-epitopes on post-translationally modified autoantigens (e.g. glycosylation or citrullination) may result in the production of various autoantibodies [147, 255, 285-287]. Increased immunogenicity of modified autoantigens is also supported by data from mice [138].
Two groups have recently reported autoantibodies to MBL in SLE [256, 257]. One of them, Seelen and coworkers, also present data which indicate that reduced functional activity of MBL leads to enhanced production of autoantibodies against cardiolipin and C1q [288]. When CRP dissociates into its neo-CRP subunits and deposits on tissue surfaces, it is conceivable that it may result in immunization in a way similar to that of C1q and MBL. Hypothetically, mCRP exposed on cell surfaces, for instance in the renal glomeruli [289-291], could also constitute a target for circulating anti-CRP antibodies, which may subsequently initiate or amplify inflammation in the target organs [205]. In addition, anti-CRP autoantibodies could possibly interfere with the physiological mCRP-mediated removal of ICs [136, 201, 202], thereby increasing the risk of extrahepatic IC deposition with a subsequent complement-mediated inflammation in the affected tissues (Figure 16).
Figure 16: Schematic illustration of the physiological roles of CRP and complement for the removal of apoptotic material ICs by the reticuloendothelial system, and the consequences when such CRP/complement-mediated clearance is insufficient. A prevailing hypothesis is that this may result in abnormal exposure of autoantigens (e.g. nucleosomes) to the adaptive immune system, which (together with other activating signals) may result in excessive autoantibody formation. Deficient CRP and/or complement-mediated removal of ICs and nucleosomes/apoptotic bodies may also increase the risk of extrahepatic deposition of apoptotic material and in situ formation of immune complexes [278].
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Systemisk lupus erytematosus (SLE) är en autoimmun sjukdom som karakteriseras av produktion av ett brett spektrum autoantikroppar, multipelt organengagemang och av lokal bildning och vävnadsdeposition av immunkomplex i de drabbade organen. Till skillnad från många andra systemiska inflammatoriska tillstånd, och trots höga nivåer av pro-inflammatoriska cytokiner, avspeglar SLE-skov genom förhöjt C-reactivt protein (CRP), vilket är en viktig akut-fas reaktant hos människa med motsvarighet hos både ryggrads- och ryggradslösa djur. Som en del av det medfödda immunystemet binder CRP till särskilda strukturer som exponeras på döende celler, apoptotiskt cellmaterial, och på ytan av vissa patogener för att därigenom medierar eliminering via det retikuloendoteliala systemet. CRP binder även till IgG-innehållande immunkomplex, liksom till Fc-receptorer samt aktiverar komplementsystemet genom att fixera C1q.

Syftet med denna avhandling var att undersöka komplementaktiverande egenskaper hos CRP; klarlägga om anti-CRP antikroppar förekommer vid SLE och, om så är fallet, undersöka om anti-CRP antikroppsnivåer samvarierar med sjukdomsaktivitet vid SLE; pröva hypotesen att autoantikroppar mot pro-inflammatoriska cytokiner förhindrar CRP-stegring; samt att belysa nukleära respektive CRP-antikroppars inverkan på cytokin-inducerande egenskaper hos immunkomplex i SLE-sera.

Vi har lyckats visa att CRP bundet till fosforylkolin är en kraftfull aktiverare av komplementsystemets klassiska väg redan i koncentrationsintervallet 4 till 10 mg CRP per liter, men med en tydlig hämning vid nivåer över 150 mg/L. Antikroppar mot den monomera formen av CRP fann vi hos cirka 40 procent av alla SLE-patienter samt hos enstaka patienter med primärt Sjögrens syndrom, men inte vid reumatoid artrit eller inflammatorisk tarmsjukdom. Anti-CRP antikroppsnivåer uppvisade signifikanta samband med många kliniska respektive laboratorie-parametrar avseende sjukdomsaktivitet vid SLE, och anti-CRP positivitet visade sig även vara associerad till njurengagemang. Nivåer av nativt CRP korrelerade varken till anti-CRP eller till anti-cytokin antikroppsivåer. Det är därför högst osannolikt att förekomsten av antikroppar mot monomert CRP/CRP-inducerande cytokiner förklarar den relativa bristen på CRP-svar hos patienter med aktiv SLE. Antikroppar mot TNFα var emellertid lägre vid hög sjukdomsaktivitet, medan antikroppar mot TGFβ var högre hos SLE-patienter jämfört med friska. I motsats till antikroppar mot CRP och DNA kan anti-SSA och anti-SSB reglera den inflammatoriska processen vid SLE genom att underlätta bildning av immunkomplex, resulterande i produktion av cytokiner som IL-6, IL-10 och IL-12p40. Hypotetiskt skulle anti-CRP autoantikroppar kunna vara av patogenetisk vikt; till exempel genom att binda monomert CRP på cell- och vävnadsytter och därigenom öka risken för extrahepatisk deposition av apoptotiskt material och in situ-bildning av immunkomplex.

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59


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