Immune Complex Regulated Cytokine Production in Rheumatic and Lymphoproliferative Diseases

LINDA MATHSSON
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Abstract

Immune complexes (ICs) are produced during normal immune responses and facilitate clearance of foreign antigens. ICs not efficiently cleared from the circulation can cause tissue damage. This might happen if ICs are formed with autoantibodies and autoantigens. Well described effects of ICs are neutralization of antigen, classical complement activation or FcR-mediated phagocytosis, whereas cytokine inducing effects of ICs in human clinical settings are less well described. I have investigated cytokine-inducing properties in vitro of ICs from patients with rheumatoid arthritis (RA), systemic lupus erythematosus (SLE) and cryoglobulinemia in association with lymphoproliferative diseases.

Cryoglobulin (CG)-induced cytokine production varied with changes in temperature and ionic strength in parallel to CG precipitation. IgG CG-induced cytokine production was also mediated via FcγRⅡa on monocytes. Blockade of the complement system, resembling the in vivo situation of complement consumption in CG-associated diseases, increased IgG CG induced IL-10 and decreased TNF-α production. This represents hitherto not described mechanisms for CG-associated inflammation.

ICs from SLE patients induced IL-10 and IL-6 production from PBMC cultures via FcγRⅡa. Occurrence of anti-SSA autoantibodies and signs of in vivo complement activation contributed to increased levels of circulating ICs in SLE patients, corresponding to increased amounts of IC-induced IL-10 in vitro. This represents a possible vicious cycle that might perpetuate antibody dependent pathology in SLE, and put anti-SSA in a new pathological context.

RF-associated ICs from RA joints and ICs formed with antibodies against collagen type II from RA serum induced pro-inflammatory cytokine production from monocytes via FcγRⅡa, showing how specific autoantibodies might induce or perpetuate joint inflammation in RA.

I have described how ICs can induce significant amounts of pathophysiologically important monocyte-derived cytokines in three major IC-dependent diseases. Blockade of FcγRⅡa and suppression of monocytes/macrophages might be a means of reducing pathogenic IC-induced cytokine production in these diseases.

Keywords: Immune complex, Cytokines, Rheumatic diseases, Lymphoproliferative diseases

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List of papers

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* The authors contributed equally

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### Abbreviations

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<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>aa</td>
<td>amino acid</td>
</tr>
<tr>
<td>ACR</td>
<td>American College of Rheumatology</td>
</tr>
<tr>
<td>ADCC</td>
<td>Antibody-dependent cell-mediated cytotoxicity</td>
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<tr>
<td>BCR</td>
<td>B cell receptor</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CII</td>
<td>Collagen type II</td>
</tr>
<tr>
<td>CCP</td>
<td>Cyclic citrullinated peptide</td>
</tr>
<tr>
<td>CG</td>
<td>Cryoglobulin</td>
</tr>
<tr>
<td>CIA</td>
<td>Collagen-induced arthritis</td>
</tr>
<tr>
<td>CR1</td>
<td>Complement receptor 1</td>
</tr>
<tr>
<td>CR2</td>
<td>Complement receptor 2</td>
</tr>
<tr>
<td>CRP</td>
<td>C-reactive protein</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic cells</td>
</tr>
<tr>
<td>dsDNA</td>
<td>Double stranded deoxyribonucleic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme linked immunosorbent assay</td>
</tr>
<tr>
<td>ELISPOT</td>
<td>Enzyme linked immunospot</td>
</tr>
<tr>
<td>ESR</td>
<td>Erythrocyte sedimentation rate</td>
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<tr>
<td>FcR</td>
<td>Fc receptor</td>
</tr>
<tr>
<td>FcγR</td>
<td>Fc gamma receptor</td>
</tr>
<tr>
<td>FcγRIIa</td>
<td>Fc gamma receptor Ila</td>
</tr>
<tr>
<td>FcγRIIb</td>
<td>Fc gamma receptor Ilb</td>
</tr>
<tr>
<td>FcγRIIIa</td>
<td>Fc gamma receptor IIIa</td>
</tr>
<tr>
<td>FcγRIIIb</td>
<td>Fc gamma receptor IIIb</td>
</tr>
<tr>
<td>HAGG</td>
<td>Heat-aggregated gammaglobulin</td>
</tr>
<tr>
<td>HSA</td>
<td>Human serum albumin</td>
</tr>
<tr>
<td>ICs</td>
<td>Immune complexes</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>ITAM</td>
<td>Immunoreceptor tyrosine-based activation motif</td>
</tr>
<tr>
<td>ITIM</td>
<td>Immunoreceptor tyrosine-based inhibitory motif</td>
</tr>
<tr>
<td>IVIg</td>
<td>Intravenous gammaglobulin</td>
</tr>
<tr>
<td>mAb</td>
<td>Monoclonal antibody</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>MCP</td>
<td>Metacarpophalangeal (joints)</td>
</tr>
<tr>
<td>MBL</td>
<td>Mannose-binding lectin</td>
</tr>
<tr>
<td>MM</td>
<td>Multiple myeloma</td>
</tr>
<tr>
<td>PBMC</td>
<td>Peripheral blood mononuclear cells</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>PDC</td>
<td>Plasmacytoid dendritic cells</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethylene glycol</td>
</tr>
<tr>
<td>PIP</td>
<td>Proximal interphalangeal (joints)</td>
</tr>
<tr>
<td>RA</td>
<td>Rheumatoid arthritis</td>
</tr>
<tr>
<td>RF</td>
<td>Rheumatoid factor</td>
</tr>
<tr>
<td>SE</td>
<td>Shared epitope</td>
</tr>
<tr>
<td>SF</td>
<td>Synovial fluid</td>
</tr>
<tr>
<td>SLE</td>
<td>Systemic lupus erythematosus</td>
</tr>
<tr>
<td>Sm</td>
<td>Smith antigen</td>
</tr>
<tr>
<td>SSA</td>
<td>Sjögren’s syndrome antigen A</td>
</tr>
<tr>
<td>SSB</td>
<td>Sjögren’s syndrome antigen B</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll like receptor</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor-alpha</td>
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<tr>
<td>WM</td>
<td>Waldenström’s macroglobulinemia</td>
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</table>
Introduction

The immune system

Our body is exposed every day to numerous foreign substances e.g. bacteria and viruses. An effective defense system, the immune system, has evolved to protect us from infections by foreign substances. The immune system is divided into innate and adaptive immunity. Innate, or natural, immunity is the first line of defense and consists of barriers and proteins that are present in active form even before the infection. The skin and the mucosal membranes with antibacterial substances are the first defense line in innate immunity. Phagocytic cells such as neutrophils and macrophages can by phagocytosis eliminate foreign substances. Several blood proteins, including the complement system and other inflammatory proteins such as cytokines are also involved in innate immunity. The innate immune system recognizes a few highly conserved structures present on most pathogenic microorganisms.

The adaptive immune response develops in response to an antigen and is specifically activated by antigen. It “adapts” to the foreign substance and also has “memory” and can therefore respond more strongly to repeated exposure of the same microbe. The adaptive immune system can distinguish between different, even closely, related microbes and molecules. The main cells of adaptive immunity are B- and T-lymphocytes. Substances that stimulate an adaptive immune response are called antigens. Innate and adaptive immunity are closely regulated systems, which cooperate in the defense against foreign substances.

One of the most amazing functions of the immune system is its ability to distinguish between self and foreign substances. This is strictly regulated by several mechanisms. Components of innate immunity recognize structures that are characteristic of microbial pathogens and that are not present on mammalian cells. The host cells might also express molecules that prevent innate immune reactions. B- and T-lymphocytes that recognize self-antigens during their maturation in the bone marrow and thymus are eliminated by apoptosis. Self-antigens not presented in the thymus or bone marrow can induce tolerance in peripheral lymphoid organs such as the spleen and lymph nodes. Tolerance to self is achieved through several mechanisms including anergy, by which T and B cells are unable to respond to antigens because of lack of adequate co-stimulatory molecules. Tolerance can also be induced if lymphocytes are repeatedly activated by the same antigen. This can then
trigger activation-induced cell death. Failure in tolerance to self-antigens can lead to autoimmune diseases with subsequent damage of cells or organs of the body. In this thesis I have studied the autoimmune diseases systemic lupus erythematosus (SLE), rheumatoid arthritis (RA) and cryoglobulinemia (CG) associated with lymphoproliferative diseases, which all have in common that the B cells produce antibodies against self-antigens (auto-antibodies) leading to formation of pathogenic immune complexes.

Basic characteristics of antibodies

Antibodies, or immunoglobulins, are glycoproteins produced in membrane-bound or secreted form by B lymphocytes and they are able to bind specifically and with high affinity to foreign or autologous substances (antigens). Membrane-bound antibodies work as receptors, which activate B cells when antigens are bound to them. Secreted antibodies can through binding of antigen activate different effector mechanisms such as neutralization of foreign substances, classical complement activation, Fc receptor (FcR)-mediated phagocytosis and antibody-dependent cell-mediated cytotoxicity (ADCC). Antibodies bound to their specific antigen form so-called immune complexes (ICs). Antibodies are composed of two identical heavy and light protein chains. The N-terminals of the light and heavy chains have high degrees of variability and bind specifically to antigens while the C-terminal of the heavy chain is highly conserved and mediates contact with other molecules of the immune system (figure 1).

Figure 1. Antibody structure.
There are five different classes of antibodies, IgA, IgD, IgE, IgG and IgM, which differ in their constituent heavy chain constant parts. In humans IgA and IgG can be further divided into the subtypes IgA1, IgA2 and IgG1-4 respectively. The different antibody classes are specialized for different effector functions and have different distributions in the body. IgA is the major antibody class found in mucosal secretions and is important in mucosal immunity. Most of the antibodies in the serum are of the IgG type and they play an important role in phagocytosis of bacteria and viruses, but can also activate complement when bound to antigen. The IgM antibodies are also present in serum and when bound to antigen they are very good activators of the complement system. IgE antibodies are specialized in defense against parasites but they are also the main players in allergy. IgD antibodies can be found in small amounts in serum and on naive B cells, but their function is not clear. The molecular weight of soluble antibodies varies between 150 kDa for monomeric IgG and IgA up to 900 kDa for pentameric IgM.

**Immune complex properties**

ICs are produced during normal immune responses and is a means of eliminating foreign antigens. Normally ICs are transported by erythrocytes to the liver and spleen where they are removed from the erythrocytes and are phagocytosed. However, ICs can cause tissue damage when present in large amounts and if not efficiently cleared from the circulation, or when antibodies are directed against autoantigens. In the case of autoantibodies there is a never-ending source of antigen and ICs. Tissue deposition of ICs is dependent on the composition of the ICs. Small ICs are often not phagocytosed and tend to deposit in vessels more often than do large ICs, which are usually phagocytosed. Positively charged ICs bind more strongly to negatively charged surfaces on the basement membrane of blood vessels and kidney glomeruli, which can cause IC deposition. Furthermore, capillaries in the renal glomeruli and synovia are locations where plasma is ultrafiltered through the capillary wall to produce urine and synovial fluid respectively, and these locations are among the most common sites for IC deposition. The ICs can then activate complement- and FcR-mediated recruitment and activation of inflammatory cells, leading to tissue damage.

Intravenous gammaglobulin (IVIg) was first introduced for treatment of antibody deficiencies, but was later also demonstrated to be effective in treatment of autoimmune disorders involving autoantibodies, such as immune thrombocytopenia and myasthenia gravis (reviewed in [1]). RA and SLE patients have also benefited from IVIg treatment but this has not been investigated in large-scale clinical trails [2]. Despite its extensive use during recent years the mechanisms underlying the therapeutic effect of IVIg are not fully known, but modulation of FcR expression, interference with the
complement system and neutralization of autoantibodies by anti-idiotypic antibodies are a few potential mechanisms thought to be involved [3]. Recent findings have now indicated that the small ICs formed by IVIg can up-regulate expression of the inhibitory FcγRIIb, but also block or down-regulate activating FcγRs in murine models of IC-associated diseases [4, 5].

The complement system

The complement system consists of numerous plasma proteins, which are important in early innate immunity against invading microorganisms and foreign substances. The complement system has three pathways of activation: the classical, mannose-binding lectin (MBL) and the alternative pathways (figure 2). The classical and MBL pathways only differ in the initiation step where C1 (C1q, C1r and C1s) bound to ICs initiates the classical pathway and MBL bound to mannose residues on bacterial surfaces initiates the MBL pathway. The following steps in the classical and MBL pathways include cleavage of C4 and C2, resulting in formation of the C3 convertase C4bC2a. Covalent binding of small amounts of C3b to foreign surfaces activates the alternative pathway. Factor B then binds to C3b and is cleaved by factor D which forms the alternative pathway C3 convertase C3bBb. The C3 convertases can then cleave C3 to produce more C3b that can bind to the foreign surfaces, ICs, pathogens or to the C3 convertase and form the C5 convertase, which cleaves C5 and starts the formation of the membrane-attack complex, causing membrane lysis [6].

IgM, IgG1 and IgG3 are good activators of the classical complement pathway. Binding of IgM to antigen induces a conformational change that exposes C1q binding sites on the Fc region of IgM. IgM bound to antigen is capable of binding two C1q molecules while IgG bound to antigen can only bind one C1q molecule. IgM is therefore a more efficient complement-binding antibody than IgG is. Activation of complement is tightly regulated to limit deposition and complement activation on host cells. Regulatory proteins present on host cells and not on microbes inhibit autologous complement activation. Deficiency in this balance might cause tissue injury and result in disease.
Figure 2. The complement system.

The classical complement pathway is important in the clearance of ICs. ICs coated with C3b bind to complement receptor 1 (CR1) on erythrocytes, which transport the ICs to the liver and spleen. In these organs, ICs are stripped from the erythrocytes via FcR-mediated mechanisms and phagocytosed [7]. Phagocytosis can also be mediated via iC3b binding to complement receptors 3 or 4 on phagocytic cells. If the ICs cannot be eliminated then the complement system becomes chronically activated and can cause inflammation and tissue damage. Complement can act as a bridge between innate and adaptive immunity e.g. C3dg (fragment of C3) bound to antigen can via complement receptor 2 (CR2) activate B cells much more effectively than antigen without complement [8].

Immune complex-associated diseases

Systemic Lupus Erythematosus (SLE)

A prototype of IC-associated disease is SLE, in which several autoantibodies to nuclear antigens are found. The most common clinical manifestations are rashes, arthritis and glomerulonephritis caused by tissue deposition of ICs. SLE mostly affects women (female to male ratio 10:1) between 20 and 60
years of age with a prevalence of around 60/100,000. The American College of Rheumatology (ACR) classification criteria for SLE can be helpful in the diagnosis of SLE, but is mainly intended as inclusion criteria in clinical studies (table 1).

Table 1. The 1982 revised ACR criteria for SLE [9]. Four or more of the 11 criteria should be fulfilled in order to be classified as SLE in a clinical study.

<table>
<thead>
<tr>
<th>Criteria</th>
<th>Definition</th>
</tr>
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<tbody>
<tr>
<td>1. Malar rash</td>
<td>Fixed erythema over the malar eminences</td>
</tr>
<tr>
<td>2. Discoid rash</td>
<td>Erythematous raised patches with adherent keratotic scaling and follicular plugging; atrophic scarring may occur in older lesions</td>
</tr>
<tr>
<td>3. Photosensitivity</td>
<td>Skin rash as a result of unusual reaction to sunlight</td>
</tr>
<tr>
<td>4. Oral ulcers</td>
<td>Oral or nasopharyngeal ulceration</td>
</tr>
<tr>
<td>5. Arthritis</td>
<td>Nonerosive arthritis involving 2 or more peripheral joints</td>
</tr>
<tr>
<td>6. Serositis</td>
<td>Pleuritis or Pericarditis</td>
</tr>
<tr>
<td>7. Renal disorders</td>
<td>Persistent proteinuria greater than 0.5 g/day or cellular casts</td>
</tr>
<tr>
<td>8. Neurological disorders</td>
<td>Seizures or psychosis</td>
</tr>
<tr>
<td>9. Hematologic disorders</td>
<td>Hemolytic anemia, leukopenia, or thrombocytopenia</td>
</tr>
<tr>
<td>10. Immunologic disorders</td>
<td>Positive LE cell preparation, antibodies to Sm or antibodies to native DNA in abnormal titers, or false positive test for syphilis</td>
</tr>
<tr>
<td>11. Antinuclear antibody</td>
<td>Abnormal titer of ANA by IF or equivalent assay</td>
</tr>
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</table>

A revision to the criterion number 10 was added in 1997 suggesting that “positive LE cell preparation” should be deleted and instead “positive finding of antiphospholipid antibodies” should be added.

The complement system and especially the classical complement pathway is of great importance in SLE. The classical disease model for SLE is that autoantibodies form ICs with their autoantigens and that these ICs activate complement, which causes tissue injury and disease. However, C1, C4 and C2 deficiencies (components of the classical pathway of complement) are highly associated with the development of SLE. This might depend on ineffective clearance of ICs caused by the defects in the classical pathway [10].

Several factors are thought to play a role in the development of SLE, both genetic and environmental (reviewed in [11, 12]). Many different genes have been associated with disease, including HLA-DR2 and DR3, and FcγRIIa and FcγRIIIa polymorphisms [13]. However, the genetic background cannot alone explain the pathogenesis of SLE and environmental factors such as infections, diet (e.g. alfalfa sprouts), toxins and UV light have been linked to SLE [14]. Increased apoptosis and defective clearance of apoptotic material is evident in SLE patients, which might lead to release of nuclear antigens.
Together with increased B cell activation and production of autoantibodies directed against nuclear antigens, this is thought to play a central role in the pathogenesis of SLE [15].

Autoantibodies to nuclear antigens are frequently detected in SLE, e.g. autoantibodies against double stranded (ds) DNA and anti-Sm (antibodies against the Smith antigen) are highly specific markers for SLE, but other autoantibodies also occur such as; anti-Sjögren's Syndrome antigen A and B (anti-SSA and anti-SSB) and anti-ribonucleoprotein (U1RNP). These autoantibodies may form ICs with antigens released from apoptotic cells.

Certain B cell subsets are expanded in the peripheral blood of SLE patients, and these are probably responsible for production of the autoantibodies [16]. Depletion of B cells with the chimeric mouse/human anti-CD20 monoclonal antibody rituximab has shown significant improvement in SLE [17]. An alternative to targeting B cells could be to interfere with the cytokines that regulate them. IL-10 is overproduced by B cells and monocytes in SLE and has been successfully blocked in SLE [18]. The effect of TNF-α blockade in SLE has had controversial effects, e.g. blockade of TNF-α in a few RA patients has led to lupus-like syndromes including development of ANA and anti-DNA antibodies which disappeared when therapy was stopped. In a small clinical trial with TNF-α blockade in SLE, increased anti-DNA, unchanged complement levels and rapid improvement of organ and joint inflammation was reported [19]. Another cytokine of importance in SLE is IL-6, which is highly expressed in glomerulonephritis and is important in B cell activation and plasma cell differentiation [16]. Serum levels of IFN-α are increased in SLE and have been shown to correlate to both disease activity and severity and IFN-α can be induced by ICs containing IgG and DNA or RNA [20-22].

Rheumatoid Arthritis (RA)

RA is a chronic inflammatory disease mainly affecting the joints and it occurs in 0.5-1% of the adult population worldwide [23]. RA is 2-3 times more common in women than in men and the median age for disease onset is 55-60 years of age. RA is characterized by chronic inflammation in the small joints of the fingers and feet, but it can also include larger joints such as the shoulders, elbows and knees. Inflammation is often symmetric, meaning that both sides of the body are simultaneously involved. The ACR has established classification criteria for RA that are mainly used for clinical studies, but they can also be helpful in the diagnosis of RA (table 2).
Table 2. The 1987 revised ACR criteria for RA [24]. A patient is classified as having RA if at least 4 of the 7 criteria are fulfilled. Criteria 1-4 must have been present for at least 6 weeks.

<table>
<thead>
<tr>
<th>Criteria</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Morning stiffness</td>
<td>Morning stiffness in and around the joints, lasting at least 1 hour</td>
</tr>
<tr>
<td>2. Arthritis of 3 or more joints</td>
<td>At least 3 joint areas simultaneously have had soft tissue swelling and fluid observed by physician</td>
</tr>
<tr>
<td>3. Arthritis of hand joints</td>
<td>At least 1 area swollen in a wrist, MCP, or PIP joint</td>
</tr>
<tr>
<td>4. Symmetric arthritis</td>
<td>Simultaneous involvement of the same joint areas on both sides of the body</td>
</tr>
<tr>
<td>5. Rheumatoid nodules</td>
<td>Subcutaneous nodules, over bony prominences, or extensor surfaces, or in juxtaarticular regions observed by a physician</td>
</tr>
<tr>
<td>6. Rheumatoid factor</td>
<td>Demonstration of abnormal amounts of rheumatoid factor in serum by a method for which the results has been positive in &lt;5% of normal control subjects</td>
</tr>
<tr>
<td>7. Radiographic changes</td>
<td>Radiographic changes typical of RA, which must include erosions or unequivocal bony decalcification</td>
</tr>
</tbody>
</table>

MCP = metacarpophalangeal joints, PIP = proximal interphalangeal joints

The cause of RA is unknown, but both genetic and environmental factors are of importance in its development. Smoking is thought to be the major environmental risk factor for development of RA [25]. The strongest genetic association determined in RA is between certain HLA-DR alleles and RA. These include HLA-DRB1*0101, *0102 (HLA-DR1), HLA-DRB1*0401, *0404, *0405, *0408 (HLA-DR4), HLA-DRB1*1001 (HLA-DR10), which have a common amino acid (aa) sequence called the shared epitope (SE) [26, 27] and which is situated in the third hypervariable region of the β1 chain of the HLA-DR class II molecule [28, 29]. An increased prevalence of RA has been reported in several American Indian populations e.g. Pima and Yakima Indians and a higher frequency of HLA-DRB1*1402 have been found in these populations [30].

An early histopathological finding in RA joints is infiltration of lymphocytes and macrophages with later pannus formation and bone and cartilage destruction [28]. RA has long been thought to be a T cell-associated disease, especially because of the strong association to certain HLA-molecules and clinical benefit of T cell-directed therapies (reviewed in [28, 31]). However, the number of T cells in the inflamed RA joint is small and levels of T cell cytokines are also low in synovial fluid (SF)[28]. Conversely, increased amounts of the pro-inflammatory cytokines TNF-α and IL-1 have been reported in the SF and serum of RA patients with active disease [32]. These cytokines are mainly produced by monocytes/macrophages and can stimulate synovial fibroblasts, osteoclasts and chondrocytes to release matrix metalloproteinases that cause degradation of bone and cartilage. Recent thera-
Cytokine interventions like TNF-α and IL-1 inhibitors have shown to be of great benefit in treatment of RA [33, 34].

The role of T cells has been extensively studied in RA, but lately the importance of B cells in RA has been highlighted. B cells have many functions that might be of importance in RA pathogenesis, e.g. they can act as antigen-presenting cells, secrete pro-inflammatory cytokines, produce rheumatoid factor (RF) and other autoantibodies and also activate T cells (reviewed in [35-37]). B cell depletion has had a significant clinical effect in RA, which further supports a central role for B cells in RA (reviewed in [38]).

Several autoantibodies have been described in RA. The first to be identified was RF, which binds specifically to the Fc portion of IgG [39, 40] (reviewed in [41]). RF is at present the only serological test for RA included in the ACR criteria. Even though RF is detectable in about 75% of RA patients it can also be seen in other diseases and in healthy individuals in association with IC [41-43]. RF production can be induced by ICs in both humans [42] and mice [44]. Antibodies against proteins or peptides containing the aa citrulline have recently been identified as being a highly specific diagnostic marker for RA [45, 46]. These antibodies can be detected in serum several years before disease onset and are associated with worse disease outcome [47]. Other antibodies (e.g. directed against collagen type II (CII), which is the predominant collagen in joint cartilage), have also been found in serum of RA patients [48-50]. Autoantibodies against both citrulline and CII can be produced by synovial B cells [23, 51, 52], indicating that antibodies and consequently local production of ICs are important in the pathogenesis of RA.

Cryoglobulinemia in association with lymphoproliferative disease

Cryoglobulins (CGs) are immunoglobulins that reversibly agglutinate and form ICs in serum when cooled below normal body temperature, but also because of changes in pH and ionic strength [53]. Structural changes in the antibody molecule e.g. changes in the H and L chain, decreased concentrations of sialic acid residues and reduced amounts of galactose in the Fc portion of IgG have all been proposed to contribute to cold precipitation of cryoglobulins [54]. There are three forms of CGs: type I, type II and type III (table 3). Type I CGs consists of a single monoclonal immunoglobulin and occur in patients with lymphoproliferative diseases such as multiple myeloma (MM) and Waldenström’s macroglobulinemia (WM). Type II CGs are composed of a monoclonal IgM and a polyclonal IgG while type III CGs are composed of both polyclonal IgM and IgG. Type II and III CGs are present in autoimmune diseases like SLE and RA, but also in patients with chronic infections such as hepatitis C [55]. RF occurs in both type II and type III CGs.
Table 3. Classification of cryoglobulins.

<table>
<thead>
<tr>
<th>Cryoglobulins</th>
<th>Composition</th>
<th>Disease association</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type I</td>
<td>One monoclonal immunoglobulin, self-association through the Fc part</td>
<td>Lymphoproliferative diseases; e.g. multiple myeloma, Waldenström’s macroglobulinemia</td>
</tr>
<tr>
<td>Type II</td>
<td>One monoclonal immunoglobulin (usually IgM) and one polyclonal Ig (usually IgG)</td>
<td>Autoimmune diseases, chronic infections</td>
</tr>
<tr>
<td>Type III</td>
<td>Polyclonal immunoglobulin, one of which has RF activity</td>
<td>Autoimmune diseases, chronic infections</td>
</tr>
</tbody>
</table>

Typical clinical manifestations exhibited in patients with cryoglobulinemia are purpura, arthralgias and asthenia. CGs can cause systemic vasculitides, mainly affecting the small and medium arteries and veins in peripheral organs. The mechanism underlying tissue damage is thought to be deposition of ICs in vessel walls with subsequent activation of the complement system, leading to inflammation [56]. CGs are known to be strong activators of the complement system and complement proteins can be found in the CG precipitates.

A small amount of CGs can be detected in healthy individuals and they are thought to facilitate the clearance of ICs through their strong complement activating capacity [56]. The presence of CGs can easily be missed because normal blood collection procedures are not performed at 37°C. Few studies have investigated the presence of CGs in different diseases; however in one such study about 25% of SLE patients had detectable levels of CGs [57], 47% of RA patients, 10% of IgG myeloma and 18% of patients with WM had detectable CG levels [58]. Hepatitis C infection is evident in up to 90% of patients with type II and III CGs [59]. In this thesis I have studied two type I CGs, one from a patient with MM and the other from a patient with WM.

**Fc receptors**

ICs mediate cell-dependent effector functions via Fc receptors (FcR), which bind to the Fc part of immunoglobulins. There are specific Fc receptors for different antibody classes, i.e. FcγR bind IgG, FcεR bind IgE and FcαR bind IgA [60]. Recently an Fcα/μR was described that binds both IgM and IgA [61]. Today this is the only described IgM receptor on blood cells [62].

FcR are mainly membrane-bound receptors specialized in different effector functions e.g. FcγR on macrophages and neutrophils mediate phagocytosis of opsonized microorganisms and, as we have investigated, secretion of
cytokines. In most cases several FcRs have to be crosslinked with ICs in order to activate the receptors and induce signals in the effector cell. When IgE crosslinks FcεRs on mast cells or basophil granulocytes chemical mediators are released. These mediators are important in parasite defense but can also cause allergic reactions [60].

**Fcγ receptors**

In humans there are three groups of Fcγ receptors (FcγRs); FcγRI (CD64), FcγRII (CD32) and FcγRIII (CD16) (table 4). These receptors are further divided into activating and inhibitory receptors. The activating receptors are FcγRI, IIa and IIIa, which are associated with an intracellular immunoreceptor tyrosine-based activation motif (ITAM). FcγRIIib is the only receptor among the FcγRs that has an intracellular immunoreceptor tyrosine-based inhibitory motif (ITIM). FcγRIIIb is a lipid-anchored receptor that lacks an intracellular domain but which can via cross-linking with FcγRIIa induce cell activation [63]. FcγRI is a high-affinity receptor capable of binding monomeric IgG, while all other FcγRs are low-affinity receptors, which require binding of ICs to become activated.

Table 4. Human Fcγ receptors

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Structure</th>
<th>Affinity for IgG</th>
<th>Expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>FcγRI (CD64)</td>
<td><img src="image1.png" alt="Diagram" /></td>
<td>IgG3&gt;IgG1&gt;IgG4&gt;&gt;IgG2</td>
<td>Macrophages, monocytes, neutrophils, eosinophils, dendritic cells</td>
</tr>
<tr>
<td>FcγRIIa (CD32a)</td>
<td><img src="image2.png" alt="Diagram" /></td>
<td>IgG3&gt;IgG1&gt;&gt;IgG2, IgG4</td>
<td>Macrophages, monocytes, neutrophils, eosinophils, platelets, dendritic cells, Langerhans cells, T cells</td>
</tr>
<tr>
<td>FcγRIIb (CD32b)</td>
<td><img src="image3.png" alt="Diagram" /></td>
<td>IgG3=IgG1&gt;IgG4&gt;&gt;IgG2</td>
<td>B cells, mast cells, basophils, macrophages, eosinophils, neutrophils, dendritic cells, Langerhans cells</td>
</tr>
<tr>
<td>FcγRIIIa (CD16a)</td>
<td><img src="image4.png" alt="Diagram" /></td>
<td>IgG1=IgG3&gt;IgG4&gt;&gt;IgG2</td>
<td>Macrophages, NK cells, eosinophils, dendritic cells, Langerhans cells, T cells</td>
</tr>
<tr>
<td>FcγRIIIb (CD16b)</td>
<td><img src="image5.png" alt="Diagram" /></td>
<td>IgG1=IgG3&gt;&gt;IgG2, IgG4</td>
<td>Neutrophils</td>
</tr>
</tbody>
</table>
Fcγ receptors in autoimmune diseases

The importance of FcγRs in development of autoimmune diseases has been extensively investigated in animal models [64, 65]. Deletion or inactivation of the inhibitory FcγRIIb in several mouse models leads to the development of autoimmune diseases resembling those seen in humans. FcγRIIb-deficient C57BL/6 mice spontaneously develop a lupus-like disease with IC-associated glomerulonephritis and enhanced autoantibody production by B cells [66, 67]. Collagen-induced arthritis (CIA) is a commonly used murine model of RA. Deletion of FcγRIIb in mice that are normally not susceptible to arthritis leads to development of CIA [68, 69]. Conversely, when deleting the activating FcγRIII in several autoimmune models the animals were protected from disease. FcγRIII deletion protected susceptible mice from development of CIA [70, 71]. The importance of activating FcγRs has also been shown in several lupus-models [72, 73]. The activating FcγRIIa is primate-specific and has until recently not been possible to study in animal models. FcγRIIa transgenic mice are now available and they have been shown to spontaneously develop autoimmune syndromes with similarities to RA (e.g. erosive arthritis) and SLE (e.g. glomerulonephritis with IC deposition in the kidneys) [74]. In Man, FcγRIIa is the most widely distributed receptor of the FcγRs, being present on most inflammatory cells and being proposed as the physiological human counterpart of FcγRIII in rodents [75].

Macrophages from both RA serum and synovium express higher levels of FcγRII and FcγRIII compared to healthy controls [76]. A shift in the balance towards more activating receptors has also been reported on RA monocytes [77].

In SLE several FcγR polymorphisms have been linked to the disease e.g. FcγRIIa131R/H and FcγRIIIa158F/V. Homozygosity for R/R of FcγRIIa and F/F of FcγRIIIa lessens the ability to interact with ICs [78]. However, there have also been reports about no association between FcγR polymorphisms and SLE [79].

The C5a receptor in autoimmunity

The complement activation fragment C5a has a central role in several experimental models of IC diseases such as IC-induced lung disease [80], IC peritonitis [81] and autoimmune hemolytic anemia [82]. Binding of C5a to the C5a receptor (C5aR) on macrophages induces up-regulation of activating FcγRIII and down-regulation of inhibitory FcγRIIb.

C5aR mRNA has been found to be up-regulated in the glomeruli of patients with lupus nephritis [83]. Blockade of C5aR in the MRL/lpr mouse model of human SLE resulted in prolonged survival, decreased renal infiltration of neutrophils and macrophages and down-regulation of cytokines like...
IL-1β [84]. However, levels of circulating IC and anti-dsDNA autoantibody were not affected by C5aR blockade in that study.

Expression of C5aR has been reported in the RA synovium [85] and C5aR expression correlates with joint swelling, erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP) levels [86]. In the experimental arthritis model CIA, C5aR deficient mice were protected from development of arthritis [87]. In that study, collagen antibody deposition was nonetheless observed in the joints of C5aR-deficient in the same way as in the C5aR+/+ mice, but the C5aR-deficient mice had significantly less infiltration of inflammatory cells into the synovium compared to in C5aR+/+ mice.

The importance of C5aR in the induction of several experimentally IC-induced diseases and the finding of increased expression of this receptor in autoimmune diseases like SLE and RA points out a central role for this receptor in the pathogenesis of these diseases.

**Toll-like receptors in autoimmunity**

Innate immunity is activated by a few highly conserved structures present on most pathogenic organisms e.g. bacterial lipopolysaccaride (LPS), peptidoglycans, mannans, bacterial DNA and double- and single-stranded RNA. Specific receptors are specialized in recognition of these structures. Mannane-binding lectin activates the MBL pathway of complement and Toll-like receptors (TLRs) are capable of binding to both cell surface molecules and nuclear constituents of microbes [88]. Ten TLRs have been identified in humans (TLRs1-10). TLRs1, 2, 6 and 10 recognize peptidoglycan found in Gram-positive bacteria. TLR4 binds to LPS on Gram-negative bacteria and TLR5 is the receptor for flagellin on gram-negative bacteria. TLRs3, 7, 8 and 9 recognize DNA and RNA [89]. DNA containing ICs from SLE patients can stimulate plasmacytoid dendritic cells (PDC) to produce cytokines via cooperative interaction of FcγRIIa and TLR9. FcγRIIa on PDC binds the ICs and delivers them to intracellular lysosomes containing TLR9, which activates the cells upon binding to DNA in the ICs [90]. RNA containing SLE-ICs have also been shown to induce IFN-α from PDC via FcγRIIa and TLR-dependent mechanism. TLR7, which binds to RNA and is expressed in PDC, is probably involved in this cytokine induction [22]. DNA- and RNA-containing ICs can activate B cells via binding to B cells expressing RF-specific B cell receptors (BCR). After binding to the BCR the ICs can be transported to intracellular compartments where TLRs are expressed. It has further been shown that uncomplexed DNA can also activate anti-DNA producing B cells by direct binding of DNA to anti-DNA BCR followed by internalization of the DNA and activation of TLRs (reviewed in [91]).

A role for TLRs in RA has also been indicated (reviewed in [92]). Monocytes and dendritic cells (DC) from RA patients respond more strongly and
produce more pro-inflammatory cytokines following TLR stimulation than do cells from healthy controls. TLR2 was highly expressed on monocytes and synovial macrophages from patients with RA and stimulation of TLR2 on these cells resulted in high production of TNF-α. Bacterial DNA and peptidoglycans have been detected in the RA synovium, which might activate TLRs on inflammatory cells in the synovium. TLR4 is mainly involved in LPS recognition but it has also been shown to interact with endogenous ligands such as heat-shock proteins, fragments of hyaluronic acid and fibronectin released from cells under stress, damage or necrotic death which might occur in the inflamed RA synovium [93].

Autoimmunity, immune complexes and cytokines

Both naturally occurring ICs isolated from patients with juvenile arthritis [94] and artificially prepared ICs with varying antigen:antibody ratios [95, 96] have been demonstrated to stimulate peripheral blood mononuclear cells (PBMC) to produce cytokines. The area is however not extensively evaluated in clinical settings, this being the central area of my thesis work.

Cytokines play an important role in autoimmune diseases. Increased serum levels of several cytokines have been reported in SLE, e.g. IL-10 [97], IL-6 [98] and IFN-α [20] have been found in SLE. IL-10 and IL-6 are important cytokines in regulation of the production of autoantibodies against dsDNA. Correlation between SLE disease activity and serum levels of IL-10 [99] and IFN-α [100] has been proposed in SLE. SLE is a prototype IC-associated disease and recent findings reveal that ICs containing anti-nuclear antibodies from SLE patients are capable of stimulating PBMC and PDC to produce IFN-α [21, 101]. In this thesis I have chosen to focus on IL-10, IL-6 and IL-12 production in SLE.

The recent breakthrough with TNF-α- and IL-1β-blockade in RA treatment has greatly increased interest in cytokines in autoimmune diseases. TNF-α has been implicated as the main inducer of inflammation in RA joints while IL-1 by some researchers is thought to be more central in the induction of bone destruction [102]. The mechanisms underlying cytokine induction in rheumatic diseases are not fully understood. However, IC stimulation of monocytes/macrophages [76] and monocytoïd dendritic cells [103] has been suggested to be of importance in RA pathogenesis. IL-10 up-regulate FcγRI and FcγRIIa on monocytes from RA patients [104], while TNF-α down-regulates FcγRIIb and activating FcγRs [77].

To my knowledge there are no studies of cytokines in cryoglobulinemia. Nonetheless, cytokines have been shown to be of importance in B cell proliferation in both MM and WM, which are the sources of cryoglobulins in this thesis. IL-6 [105, 106] and IL-10 [107, 108] are important for prolifera-
tion of human MM cells. TNF-α has been shown to be important in myeloma growth in MM [109].
Present investigation

Aims

General aim
The aim of this thesis was to investigate the cytokine-inducing properties of ICs from patients with RA, SLE and cryoglobulins in association with lymphoproliferative diseases.

Specific aim

Paper I
The aim of paper I was to investigate if CGs could induce cytokine production from healthy PBMC. The effect of changes in temperature, pH, ionic strength and FcγR blockade on CG-induced cytokine production was also investigated as well as the effect of blockade of the classical complement pathway.

Paper II
In paper II we wanted to investigate if RF was linked to levels IC and IC-induced cytokine production. Cytokine inducing effects of polyethylene glycol (PEG) precipitated IC from SF and sera of RA patients were investigated. We also wanted to investigate if IC-induced cytokine production correlated to any clinical parameters as well as to the occurrence of RF. FcγR and monocyte involvement was additionally investigated in this paper.

Paper III
In paper III we studied the cytokine-inducing effects of ICs made in vitro containing known antibodies and antigens, namely collagen type II and anti-collagen type II antibodies from RA patients. In this paper we also investigated the role of FcγRs and monocytes on IC-induced cytokine production.

Paper IV
The aim of paper IV was to investigate the cytokine-inducing properties of PEG-precipitated SLE IC and also try to define the role of FcγRs in this process.
Paper V
In paper V we wanted to investigate how classical complement activation \textit{in vivo} and levels of autoantibodies \textit{in vivo} correlate with levels of circulating IC \textit{in vivo}, and also to IC-induced cytokine production \textit{in vitro}.

Materials and Methods

Patients and healthy controls
Sera and plasmapheresis plasma from one patient with multiple myeloma (MM) and from one patient with Waldenström’s macroglobulinemia (WM), both patients having cryoglobulinemia, were used as sources for CG in paper I. In paper II paired sera and SF from 47 patients fulfilling the ACR classification criteria for RA were included. In paper III sera from 65 arthritis patients of which 11 fulfilled the ACR criteria for RA and the other 54 were obtained from an early arthritis (disease duration of <12 months) cohort. All arthritis sera and SFs in papers II and III were obtained from the Department of Rheumatology at Karolinska University Hospital, Solna. Twenty patients fulfilling the ACR criteria for SLE were investigated in paper IV. In paper V 147 serum samples from 63 patients with a clinical SLE diagnosis were included. Sera from healthy blood donors were used as controls in papers II, III and IV.

All patients and controls had given informed consent to participate in the studies except for in paper V in which the serum samples had been anonymized prior to the study. The investigations were approved by the local ethical committees at the University Hospital in Uppsala and at the Karolinska University Hospital in Solna.

Preparation of immune complexes

Precipitation of cryoglobulins
Whole blood samples from patients with cryoglobulinemia were collected and separated at 37°C after which serum was left to stand at 4°C for 5 days. The CG precipitates were then washed twice in ice-cold PBS and thereafter reconstituted to the initial volume in sterile phosphate-buffered saline (PBS). In some cases CG were purified from plasmapheresis plasma and then the plasma was pre-treated with thrombin at 37°C for one hour to induce coagulation. The serum obtained thereafter was treated as above. CG levels as well as Ig subclass determination was performed using rate nephelometry.

Heat-aggregated gammaglobulin (HAGG)
Artificial ICs, HAGG, were prepared by heating human IgG (Gammagard, Baxter, Belgium) 50mg/mL, to 63°C for 30-60 minutes immediately before
each experiment and diluting it in PBS to the desired concentration. As negative control, the same monomeric IgG preparation without heat treatment was used.

**Polyethylene glycol (PEG) precipitation of immune complexes**

In paper IV SLE sera were PEG-precipitated by a multi-step procedure in which sera were mixed with 3% ice-cold PEG 6000 and left on ice for 30 minutes and thereafter centrifuged (2000g) and washed three times in sterile PBS, subsequently being diluted to the initial serum volume in sterile PBS. In papers III and V, PEG precipitates were purified and washed in a single-step centrifugation procedure. Briefly, sera or hyaluronidase-treated SF were mixed with equal volumes of 5% PEG 6000 with 0.1M EDTA and left to stand at 4°C overnight. Thereafter a mixture of 2.5% PEG 6000 with 5% human serum albumin (HSA) in PBS was added to 1.5 mL Eppendorf tubes. Plastic cylinders made out of 5 mL pipette tips, by cutting of about 1.5 cm of the tips, were introduced in the Eppendorf tubes. The precipitates from the previous day were diluted in 2.5% PEG in RPMI and carefully placed on top of the PEG-HSA solution in the Eppendorf tubes. An interface was formed between the less dense RPMI solution on top and the PEG-HSA solution in the bottom. The tubes were then centrifuged at 2100g, 4°C for 20 minutes whereby the precipitates in the upper phase were centrifuged down to the bottom of the tubes. All the liquid was removed from the tubes leaving only the PEG precipitated ICs. These ICs were redissolved in ice-cold sterile PBS to the original volume of SF or serum. All PEG precipitates were placed on ice until used in cell culture experiments.

**Solid-phase immune complexes with collagen type II and anti-collagen type II antibodies**

Collagen type II (10μg/mL; 50μL/well) was bound to MaxiSorp ELISA plates overnight at 4°C. Plates were then blocked with 1% HSA in sterile PBS for 1 hour at room temperature. After discarding the blocking solution, 50μL of diluted patient and control sera (10% dilution in sterile PBS) were added and incubated for 2 hours at room temperature. Thereafter the plates were washed and responder cells diluted to 1 x 10^6 in cell culture medium were added.

**Preparation of PBMC and cell cultures**

PBMC were obtained from heparinized blood or buffy coat preparations using standard Ficoll-Paque density gradient. PBMC were thereafter diluted to 10^6 cells/mL in complete medium consisting of RPMI-1640 supplemented with 1% glutamine, 1% penicillin-streptomycin, 1% HEPES and 10% fetal calf serum (FCS; paper IV), 10% normal human serum (NHS; paper I) or 1 or 4% of Ultroser G® (paper I-V). In paper III 12.5μg/mL of polymyxin B
was also added to the cell culture media. Ultroser G® is a serum-supplement that has been shown to sustain IC-induced cytokine production in otherwise serum-free cell culture systems. Ultroser G® is a serum derived product and optimal concentrations in cell cultures had to be determined for every batch. Some PBMC donors are poor IC responders and give either high cytokine production or generally low cytokine production without any effect of IC addition. At least two PBMC donors were therefore investigated in parallel in each experiment and the PBMC donor showing the strongest net response to IC stimulation is shown.

Rheumatoid factor measurements
In paper II levels of RF was measured in all serum samples using nephelometry. The analysis was standardized using the international standard NIBSC 64/002 and the cut-off was set to 20IU/mL. In a control group consisting of 100 healthy blood donors, only two showed marginally positive values (20.4 and 21.6IU/mL, respectively). Due to technical limitations, probably because of intrinsic light-dispersing properties of SF, we only obtained RF results from 59% of the RA SF samples using nephelometry, even after hyaluronidase treatment. Latex agglutination also proved unusable for SF RF measurements as the negative control beads without an Ig coat also agglutinated after addition of SF.

Enzyme linked Immunosorbent assay (ELISA)

Cytokine ELISA
After IC stimulation cytokine levels in the cell supernatants were measured by ELISA. ELISA plates were coated with whole primary antibodies directed against IL-1β, IL-6, IL-8, IL-10 and TNF-α, respectively, followed by blocking with 1% bovine serum albumin (BSA) in PBS. Thereafter cell supernatants and diluted recombinant standards were added to the plates. Biotinylated secondary antibodies against the cytokines mentioned above were then added followed by the enzyme horseradish peroxidase bound to streptavidin. The substrate 3,3’-5,5’-tetramethylbenzidinie was added after incubation with enzyme. In papers II and V F(ab’)2-fragmented primary and secondary antibodies against TNF-α and IL-10 respectively were used for ELISA.

Anti-Cyclic citrullinated peptide (CCP) ELISA
Serum anti-CCP was measured using the Immunoscan RA mark II assay (Eurodiagnostica, Malmö, Sweden) and the cut-off was set to 25IU/mL. In a control group consisting of 99 healthy individuals two showed borderline reactivity (30 and 42U/mL) and one showed high positive reactivity (1643 IU/mL).
IgG ELISA

In paper II IgG levels in PEG precipitates were measured using an IgG ELISA. The ELISA was constructed to not be influenced by RF or heterophilic antibodies. Rabbit F(ab’)$_2$ directed against the human IgG$\gamma$ chain was used as primary antibody. For detection an alkaline phosphatase conjugated goat F(ab’)$_2$ antibody directed against the human IgG$\gamma$ chain adsorbed against bovine immunoglobulins was used. A well-characterized normal human serum was used to construct a standard curve.

IgG anti-CII ELISA

Half of the wells in the ELISA plate were coated with human native CII and incubated overnight at 4°C. Thereafter the coating solution was discarded and the entire plate was blocked with PBS/1% HSA. Sera diluted 1:100 were added to the plate in duplicate, to both CII-coated and the only blocked parts of the plate, and left at room temperature for 2 hours. After washing with PBS/Tween the same detection antibody as used in the IgG ELISA above was added to the plate and incubated for 1 hour at room temperature. As substrate, 4-nitrophenyl phosphate disodium in diethanolamine buffer was used. The plate was read at 405 nm after 45 minutes. OD values were calculated as: (mean OD value in CII-coated wells) – (mean OD value in wells only blocked).

ELISPOT

ELISPOT was used in paper IV to measure cytokine induction from single cells. ELISPOT plates were coated with monoclonal antibodies against IL-6 and IL-10. Cell suspensions were added in duplicate to the plates followed by PEG precipitates or HAGG and negative IC controls (monomeric IgG). The plates were then incubated at 37°C for 20 hours. After washing the plates, secondary biotinylated antibodies against IL-6 and IL-10 were added and incubated overnight at 4°C. The enzyme alkaline phosphatase bound to avidin was then added. As substrate 5-bromo-4-chloro-3-indolyl phosphate was used with a development time of 5 hours. After final washing in deionised water the spot forming cells were counted using an inverted microscope.

Fc$\gamma$R blocking experiments

Blocking mAbs against Fc$\gamma$RII (IV.3; Fab-fragment) or anti-Fc$\gamma$RIII (3G8; F(ab’)$_2$-fragment) were added to the cells and incubated at 4°C for 30 minutes before addition of ICs. The antibody concentration used was 1.5µg/mL; preliminary experiments had shown equivalent blocking effect using either 1.5 or 4µg/mL. The IV.3 antibody had earlier been shown to react specifically with Fc$\gamma$RIIa [110, 111]. In paper I we also used the pan-Fc$\gamma$RII
(blocking FcγRIIa, b and c) blocking antibody AT.10 at a concentration of 1.5μg/mL.

Monocyte depletion/enrichment
To investigate the hypothesis that monocytes are important in IC-induced cytokine production, monocytes were either enriched or depleted prior to IC stimulation. Monocyte enrichment or depletion cocktails (RosetteSep™) were added to heparinized blood and purification performed according to the manufacturer’s instruction. This enrichment protocol yields totally untouched monocytes for subsequent functional studies. Depletion and enrichment were verified by staining with anti-CD14 FITC-conjugated antibodies followed by flow cytometric analysis. Cells depleted and enriched for monocytes were diluted in cell culture medium to the same total cell concentration as used for untreated PBMC, and thereafter stimulated with IC.

Functional complement test and complement blockade in cell culture experiments
Functional activity of the classical pathway was measured (papers I and V) according to Nilsson and Nilsson [112]. In paper V, 20μL from each patient serum and from a reference serum pool were diluted 1:5 before mixing with 100μL of a 40% solution of sheep erythrocytes coated with rabbit IgM and incubated for 20 minutes on a shaker at 37°C. The reaction was stopped by adding 3mL of 0.01 M EDTA, whereupon the samples were centrifuged at 650g for 10 minutes at 4°C. Two hundred and fifty μL from each supernatant were added to ELISA plates and the absorbance was measured at 541nm. Hemolytic activity of the classical pathway was defined as the absorbance in the patient samples divided by the absorbance of the reference serum pool.

In paper I 10% NHS in cell culture media (the same concentration of serum as used in the cell cultures) was pretreated with the cyclic complement blocking peptide Compstatin or the scrambled linear control peptide before addition of ICs (HAGG, CG or mIgG). Thereafter the activity of the classical complement pathway was measured in the same way as in paper V.

Statistics
Non-parametric statistics were mainly used. Mann-Whitney U test was used in unpaired design and Wilcoxon signed rank test was used for paired comparisons. Correlations were determined with the Spearman rank correlation test. Parametric statistics were partly used in papers III and V. Pearson’s product-moment correlation with Fisher’s r-to-z transformation was used in paper III and two-way ANOVA was employed in paper V.
Results and Discussion

Paper I. Cryoglobulins induce complement and FcγRIIa-dependent cytokine production from monocytes.

Before the initiation of this study not much was known about the mechanisms underlying inflammation and tissue destruction induced by CGs, except for the general IC theory of complement-mediated tissue damage. In paper I we investigated two type I CGs, one of IgG type and one of IgM type. We found that type I CGs could induce cytokine production from monocytes. This cytokine production was increased when the CGs were added to the cells at 4°C compared to at 37°C, i.e. varying in parallel with CG precipitation. Precipitation of cryoglobulins is also known to depend on variation in pH and ionic strength, which are possible causes for CG precipitation in the kidneys. For the IgM CG we determined increased cytokine production at low ionic strength in parallel with higher degree of precipitation at low ionic strength. IgG CG and artificial IC (HAGG) gave maximal cytokine induction and precipitation around physiological ionic strength. Parallel experiments were also performed to address the effect of pH on CG precipitation and cytokine induction. Although we observed CG precipitation at pH values corresponding to their isoelectric points (IgM CG precipitated at neutral-to-low pH, while IgG CG precipitated at higher pH), all cell cultures (including LPS-stimulated cultures) only exhibited cytokine production within a quite narrow range around neutral pH. No conclusions concerning the effects of pH on IC-induced cytokine production could therefore be drawn from these experiments.

We further demonstrated that IgG CG-induced cytokine production was reduced when blocking FcγRIIa compared to control cultures without FcγRIIa blockade. The cytokine production induced by the IgM CG might be mediated via the recently described Fcα/µ receptor, which has been shown to bind IgA and IgM in both humans and rodents and which at least in rodents is expressed on B cell and macrophages [61, 113]. Expression of the Fcα/µ receptor in humans has been reported on mesangial cells [114]. The IgM CG was later shown to be RF positive and might be able to bind IgG and possibly thereby also indirectly activate cytokine induction via FcγRIIa.

CGs are known to be potent activators of the classical complement pathway. When complement activation was blocked with Compstatin in cell cultures stimulated with the IgG CG, IL-10 production was increased and reciprocally correlated with decreased production of TNF-α. This negative correlation between IL-10 and TNF-α for IgG CG was strong and highly significant. We have earlier determined that complement blockade increases IL-10 production using artificial IC (HAGG) [115]. The finding of increased IgG CG-induced production of IL-10 when blocking the complement system might be of importance in relation to lymphoproliferative diseases associated
with IgG CG. CGs are often associated with profound activation and exhaustion of the classical complement pathway [116]. In such situations functional assays of the classical complement pathway yield very low hemolytic activity similar to the results obtained with the complement inhibitor Compstatin in vitro. During such circumstances when the classical complement pathway is constantly exhausted, IgG CG-induced IL-10 might be a growth factor for malignant B-lymphocytes [117].

In this paper we proposed a novel mechanism for IgG CG-induced inflammation through FcγRIIa-dependent induction of cytokines from monocytes. We have also demonstrated that IgG CG-induced cytokine production is dependent on the classical complement pathway.

Paper II. Immune complexes from synovial fluids of RA patients induce FcγRIIa dependent and RF correlated production of TNF-α from monocytes.

In paper II we investigated the cytokine-inducing properties of PEG precipitated ICs from healthy control sera and from RA serum and SF. We observed a non-significant trend toward higher IgG levels and greater induction of TNF-α by serum PEG precipitates from RA patients compared with from healthy controls. In two separate studies we recorded a positive correlation between IgG levels in SF precipitates and TNF-α production from PBMC stimulated with SF precipitates. There was also a positive correlation between RF measured in serum and IgG levels in SF precipitates. We also tried to measure RF in SF but because of technical limitations we only obtained measurable values in about 60% of the samples. However, for the samples with measurable SF RF levels there was a closer correlation to SF IC-induced TNF-α production than for RF measured in serum. The association was only valid for SF PEG precipitates as we did not find any correlation between IgG, RF and TNF-α induction for the serum precipitates. Furthermore, the amount of TNF-α produced from PBMC following stimulation with SF precipitates, but not serum precipitates, correlated with the number of swollen and tender joints. We also noted that IC-induced TNF-α levels were reduced after blockade of FcγRIIa, but not of FcγRIII. Depletion of monocytes from the cell cultures totally eliminated IC-induced TNF-α production.

A strong association between cytokine induction, IgG levels and RF was apparent for the SF precipitates but not for serum precipitates, which is in agreement with the general belief that RF levels in serum reflect inflammation in the joints. RF has been associated with IC in several other diseases than RA [41, 43] and has also be found after vaccination in healthy individuals during the time interval when antibodies and antigen form circulating ICs [42]. RF-producing B cells are present in the inflamed RA joint [118] and
RF measured in serum might therefore mirror the production of RF in the joints of RA patients. Our findings support the hypothesis that RF is produced in the joints in response to IgG-containing ICs with TNF-α inducing properties. We also observed stronger cytokine inducing properties of ICs obtained from joints of RF positive RA patients, which is in agreement with the fact that seropositive RA is associated with a more severe disease outcome [119, 120].

Monocytes/macrophages were the main or perhaps only responder cells in the induction of TNF-α in our experimental systems. The importance of monocytes in IC-driven joint inflammation is supported by earlier rodent experiments in which synovial macrophages were shown to play a central role in IC-induced arthritis models [121-123]. In addition, most disease-modifying drugs in RA are directed at suppressing monocytes and monocyte-derived cytokines [124-126]. Recent findings have also highlighted the importance of monocytes/macrophages [76] and monocyte-derived dendritic cells [103] in IC-induced cytokine production in RA joints.

Many studies have reported the importance of FcγR in experimental arthritis models. Knockout mice lacking the activating FcγRIII [70] are protected from arthritis development while knockout mice lacking the inhibitory FcγRIIb develop arthritis on a non-arthritis susceptible background [68]. Rodents lack the primate specific activating FcγRIIa, which is elevated on RA monocytes compared with in healthy control individuals [77]. FcγRIIa has been proposed as the functional counterpart of FcγRIII in rodents [75].

In paper II we demonstrated a clear correlation between RF, IgG levels in PEG-precipitated ICs from RA SF and TNF-α production induced by SF ICs. This supports the hypothesis that ICs are formed in the inflamed RA joint in parallel with RF production. Such ICs may then stimulate monocytes/macrophages in the joint to produce TNF-α via FcγRIIa.

Paper III. Collagen type II-containing ICs in RA induce production of pro-inflammatory cytokines from monocytes via FcγRIIa.

CII is the most abundant collagen type in joint cartilage and has been implicated as a possible autoantigen in RA. Antibodies to CII can be detected in sera and SF of a subpopulation of RA patients [48, 49, 127]. Susceptible rats and mice immunized with native CII develop collagen-induced arthritis (CIA), a disease partly dependent on anti-CII. CIA has immunological and pathological similarities to RA.

In paper III we investigated if antibodies from RA sera directed against CII can form solid-phase ICs with cytokine-inducing properties in a model theoretically resembling the situation in the inflamed joint. During joint inflammation CII epitopes are exposed to anti-CII due to disruption of the
proteinaceous covering that protects the intact cartilage surface [128]. Pre-
requisites therefore exist for the formation of cartilage surface-bound IC in
the inflamed RA joint.

We found that solid phase ICs containing antibodies to CII from RA pa-
tients induced production of TNF-α, IL-1β and IL-8 from monocytes. There
was a close correlation between the amount of ICs, measured as IgG anti-CII
values in ELISA, and the induction of the different pro-inflammatory cyto-
kines investigated. This indicates that anti-CII-containing IC can induce
cytokines of pathogenic importance in the RA joint. This concords with the
findings of elevated levels of TNF-α and IL-6 as well as increased ESR and
CRP levels in anti-CII positive compared to anti-CII negative RA patients
[129].

We have also shown that anti-CII containing ICs induced cytokine pro-
duction from monocytes via FcγRIIa. This cytokine production was totally
abolished when monocytes were depleted from the cell cultures. Blockade of
the activating FcγRIIa significantly reduced IC-induced cytokine production
in our solid-phase system. Earlier studies have also suggested monocytes as
the main cytokine producer upon IC stimulation [95].

FcγRIIa is not expressed in rodents and it has until recently not been pos-
sible to study this receptor in experimental arthritis models. CIA in rodents is
probably dependent on anti-CII antibodies [130, 131] and can only be elic-
ted in susceptible strains with a suitable genetic background. An otherwise
non-susceptible strain has recently been reported to be susceptible to CIA
after transfer of the human FcγRIIa gene [75], supporting an important role
for this receptor in arthritis development. In addition, passive transfer of
anti-CII induced more rapid and severe arthritis in FcγRIIa-transgenic mice
compared to non-transgenic mice, and IC-induced production of TNF-α
was predominantly mediated via the transgenic FcγRIIa [74].

The findings in this paper represent a possible mechanism for perpetua-
tion of joint inflammation in a subgroup of RA patients with high levels of
anti-CII by means of solid-phase IC formation with cytokine-inducing prop-
erties.

Paper IV. SLE ICs induce IL-10 and IL-6 production from
PBMC cultures via FcγRII, which might stimulate antibody
production by B cells.

In paper IV, which is chronologically the first paper, we investigated the
possibility that ICs from SLE patients could stimulate PBMC to produce B
cell-activating cytokines. We used the ELISPOT technique to investigate the
cytokine-inducing properties of SLE serum added to PBMC. When evaluat-
ing the technique with artificially prepared IC (HAGG) we determined that
the number of IL-10 producing cells was increased in a dose-dependent
manner when HAGG was added, while no increase in number of IL-6 producing cells was observed. We therefore investigated the effect of SLE serum on IL-10 producing cells using ELISPOT. However, supernatant levels of both IL-10 and IL-6 were increased after stimulation with HAGG. Therefore both IL-10 and IL-6 levels were measured by ELISA in cell cultures stimulated with PEG-precipitated SLE IC. Addition of 10% SLE sera to PBMC increased the number of IL-10 producing cells compared to healthy controls. PEG-precipitated ICs from SLE sera also induced increased production of IL-10 and IL-6 compared to control PEG precipitates. Increased IL-6 [98] and IL-10 [132, 133] levels have been reported in SLE. IL-10 has been shown to stimulate SLE B cells to produce anti-DNA antibodies [134]. Both levels of IC [135] and serum levels of IL-10 correlate with disease activity in SLE [97, 99]. IL-10 blockade in a small clinical trial gave rapid clinical improvement in the included patients [18].

We further demonstrated that the cytokine production was mediated via FcγRII. IL-10 and IL-6 levels induced by IC additionally correlated with the expression of FcγRII on monocytes. IgG-rich fractions of SLE sera have been shown to induce IFN-α production that can be blocked by FcγRII-antibodies [136]. When our study was performed we did not know that the IV.3 antibody that we used for blockade of FcγRII specifically blocked FcγRIIa [110, 111].

The findings in this paper provide a possible mechanism for the enhanced IL-10 and IL-6 levels evident in SLE by IC-induced cytokine production from monocytes via FcγRIIa. This might create a vicious cycle that might help in maintaining B cell hyperactivity and autoantibody production in SLE.

Paper V. Cytokine induction by circulating ICs in association with complement activation and occurrence of anti-SSA antibodies in SLE sera (Paper V).

SLE is a prototype IC-mediated disease associated with IC-induced classical complement activation. Autoantibodies directed against nuclear constituents are associated with SLE. In paper V we investigated if amount and functional effects of SLE IC correlated with complement activation and occurrence of specific autoantibodies. We concluded that increased levels of IC-induced cytokines were associated with both increased classical complement activation and the occurrence of anti-SSA and possibly also with anti-SSB antibodies, but not with other autoantibodies. The highest levels of circulating ICs were observed in serum samples with low classical complement function and the occurrence of anti-SSA. We did not find this association with any other autoantibody e.g. anti-dsDNA, anti-U1-snRNP or anti-CRP. The fact that we did not determine any association between anti-dsDNA, IC-
levels, IC-induced cytokine production or the degree of complement activation is surprising because of the strong association between anti-dsDNA and SLE (especially active SLE) reported in other studies. However, our results do not rule out the role of other autoantibodies e.g. anti-dsDNA in vivo. Anti-dsDNA levels in ICs are decreased during SLE flares, possibly because of tissue deposition of ICs [137]. Positively charged antibodies and especially anti-dsDNA antibodies also bind more strongly to glomeruli basement membranes compared to non-cationic antibodies in SLE. Anti-dsDNA can be trapped in the tissue and create ICs in situ without any association to circulating IC levels, and this might explain the lack of association between circulating IC levels on one hand and complement activation and anti-dsDNA and other antibodies on the other in this study.

We hypothesize that in active SLE, anti-SSA and anti-SSB antibodies form ICs with antigens released from apoptotic cells, resulting in complement activation and cytokine production from leukocytes. Conversely, in quiescent disease these autoantigens are not released, leaving circulating autoantibodies uncomplexed and consequently not leading to induction of cytokines. Båve et al have recently reported that anti-SSA/SSB positive sera from patients with Sjögren’s syndrome mixed with either apoptotic or necrotic cells can induce IFN-α production from PDC and PBMC [138].

Anti-SSA and anti-SSB antibodies are regarded as disease markers in SLE but have not previously been associated with disease activity in SLE. The results in this paper indicate that these antibodies might be of importance in formation of circulating ICs and might participate in the inflammatory process in SLE by enhancing IC formation and subsequent cytokine production.

**General conclusion**

In this thesis I have investigated the cytokine-inducing properties of ICs from patients with SLE, RA and cryoglobulinemia in association with lymphoproliferative diseases. IC formation during the normal immune response is a means of eliminating foreign antigens. However, if the ICs are not efficiently cleared from the circulation they could cause tissue damage. This might happen if ICs are formed with autoantibodies and autoantigens, causing a never-ending production of ICs.

Autoantibodies against nuclear antigens are common in SLE and these might form ICs with autoantibodies. The ICs can then activate the complement system, leading to tissue injury and disease. Defective clearance of apoptotic cells by macrophages has been reported in SLE patients [139, 140] and apoptotic cells might then be a source of nuclear autoantigens in SLE. However, defects in the classical complement pathway, which leads to ineffective clearance of ICs, is also highly associated with SLE [8]. Irrespective
of the source, ICs are very important in the pathogenesis of SLE. Cytokines are also important in SLE. Increased serum levels of several cytokines have been reported in SLE, e.g. IL-10 [97], IL6 [98] and IFN-α [20]. In this thesis I have shown that ICs from SLE patients can induce IL-10 and IL-6 production from PBMC cultures via FcγRIIa. Furthermore, I showed that occurrence of anti-SSA autoantibodies and signs of in vivo complement activation contribute to increased levels of circulating ICs in SLE patients, corresponding to increased amounts of IC-induced IL-10 in vitro. IL-10 and IL-6 are two cytokines of importance in B cell activation and antibody production. IC-induced production of these two cytokines might therefore contribute to increased autoantibody production in SLE, and further IC production, thereby contributing to a vicious pathogenic cycle.

Several autoantibodies, e.g. RF, anti-CII and anti-CCP, are produced by B cell in the RA synovium [51, 118, 141]. These can form pathogenic ICs, which might cause tissue damage. Increased levels of the pro-inflammatory cytokines TNF-α and IL-1 have also been detected in SF and serum from RA patients with active disease [32]. These cytokines have been blocked in RA with good therapeutic effect [33, 34]. In my thesis I have shown that RF-associated ICs from RA joints, but not from RA serum, induced TNF-α production that correlated with number of swollen and tender joints. These findings put RF in an IC-dependent pathological context, and couples RF to severe disease via IC-induced production of TNF-α in the inflamed joints. ICs formed with native human CII and anti-CII antibodies from RA serum induced production of the pro-inflammatory cytokines IL-1β and TNF-α. The RA ICs were further shown to induce cytokine production from monocytes via FcγRIIa. In this study the occurrence of antigen-specific ICs were coupled to inflammation in RA via IC-induced cytokine production.

Lymphoproliferative diseases such as WM and MM are characterized by increased plasma cell production with consequent antibody production. The antibodies produced are sometimes CGs that form ICs at low temperatures, but also with variations in pH and ionic strength. I have shown that CGs induce more cytokines at low temperatures compared to at body temperature. CG-induced cytokine production varied with changes in ionic strength in parallel to CG precipitation. IgG CG-induced cytokine production was also partly mediated via FcyIIa on monocytes. CGs are known to be strong activators of the complement system. Blockade of the complement system in our cell cultures, creating an in vitro system resembling the in vivo situation of constant complement consumption in CG-associated diseases, increased IgG CG induced production of IL-10 whereas TNF-α production decreased. If IgG CG-induced production of IL-10 will also be shown to be increased during periods of low complement function in vivo, this increased production of IL-10 might be a growth factor for malignant B cells in CG-associated multiple myeloma.
In conclusion, ICs from patients with SLE, RA and cryoglobulinemia induce cytokines of importance in the pathogenesis of the different diseases (figure 3). The main cytokine producers in our systems were monocytes/macrophages and blockade of FcγRIIa significantly reduced IC-induced cytokine production. IC-induced cytokine production might be important in the inflammatory process evident in these IC-associated diseases. Blockade of FcγRIIa or suppression of monocytes/macrophages might be a means of reducing pathogenic cytokine production in these diseases.

Immunology textbook examples of the effects of ICs mostly concern neutralization of antigen, classical complement activation or FcR-mediated phagocytosis, and not all of them mention the cytokine-inducing properties of ICs. In this thesis I have described how ICs can induce significant amounts of pathophysiologically important monocyte-derived cytokines in three major IC-dependent diseases. Further studies in this field are warranted.

Figure 3. Hypothesis for IC-induced cytokine production and role in RA, SLE and CG in lymphoproliferative diseases.
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