The role of anti-collagen type II antibodies in the pathogenesis and prognosis of rheumatoid arthritis

VIVEK ANAND MANIVEL
Abstract

Rheumatoid arthritis (RA) which affects 0.5-1% of the world population and is characterised by joint erosions and presence of the autoantibodies anti-citrullinated protein antibodies (ACPA) and rheumatoid factor. Collagen II (CII) is a joint-specific antigen and we have shown that antibodies against CII (anti-CII) are present in around 8% of RA patients. RA patients with anti-CII are characterized by acute RA onset with elevated CRP and early joint erosions at the time of RA onset. Polymorphonuclear granulocytes (PMN) and peripheral blood mononuclear cells (PBMC) are abundant in RA synovial fluids, where they can interact with anti-CII, thus forming immune complexes (IC) with CII. In my thesis I have shown that PMN upregulated the cell surface markers CD66b and CD11b and downregulated CD16 and CD32 after stimulation with anti-CII IC. These changes in CD66b and CD16 associated to joint erosions to a larger extent than did PBMC responses to anti-CII IC. PMN cocultured with PBMC and stimulated with anti-CII IC showed augmented chemokine production that was dependent on TLR4 and functionally active PMN enzymes. This mechanism can lead to accumulation of inflammatory cells in joints of RA patients who are anti-CII positive around the time of RA diagnosis, and may thus help explain the acute onset RA phenotype associated with anti-CII.

In a large Swedish RA cohort, anti-CII associated with elevations in clinical and laboratory measures of disease activity at diagnosis and until 6 months, whereas ACPA associated with late inflammation. Anti-CII seropositive RA was associated with improvements in clinical measurements and was negatively associated with smoking in contrast to ACPA that was associated with worseneing of clinical symptoms and associated positively with smoking. Anti-CII levels associated to HLADRB1*03 and HLADRB1*01 whereas ACPA showed negative association to HLA-DRB1*03. In a Malaysian RA cohort anti-CII also associated to elevated CRP at the time of diagnosis.

Anti-CII seropositive RA represents a distinct phenotype, in many respects representing the converse to the clinical, genetic and smoking associations described for ACPA. Early determinations of anti-CII in parallel to ACPA predict the inflammatory outcome in RA.

Keywords: Rheumatoid arthritis; Anti-collagen type II antibodies; HLADRB1*; Granulocytes; prognosis;

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dedicated
- to my family
List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.


*Equal Contributions- First Author.
# Equal Contributions- Last Author.

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### Abbreviations

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<th>Description</th>
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<tbody>
<tr>
<td>Ab</td>
<td>Antibody</td>
</tr>
<tr>
<td>ACPA</td>
<td>Anti-citrullinated protein/peptide antibodies</td>
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<tr>
<td>ACR</td>
<td>American college of rheumatology</td>
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<td>ADCC</td>
<td>Antibody-dependent cell-mediated cytotoxicity</td>
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<tr>
<td>Ag</td>
<td>Antigen</td>
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<tr>
<td>Anti-CII</td>
<td>Antibodies to collagen type II</td>
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<td>Anti-CCP</td>
<td>Antibodies to cyclic citrullinated peptide</td>
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<td>APC</td>
<td>Antigen presenting cell</td>
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<td>BSA</td>
<td>Bovine serum albumin</td>
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<tr>
<td>CIA</td>
<td>Collagen-induced arthritis</td>
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<tr>
<td>CAIA</td>
<td>Collagen-antibody induced arthritis</td>
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<tr>
<td>CII</td>
<td>Collagen type II</td>
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<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
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<td>ESR</td>
<td>Erythrocyte sedimentation rate</td>
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<tr>
<td>FcR</td>
<td>Fc receptor</td>
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<tr>
<td>FcγR</td>
<td>Fc gamma receptor</td>
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<tr>
<td>FcγRIIb</td>
<td>Fc gamma receptor IIb</td>
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<tr>
<td>FcγRIII</td>
<td>Fc gamma receptor III</td>
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<td>FCS</td>
<td>Fetal calf serum</td>
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<tr>
<td>HLA</td>
<td>Human leukocyte antigen</td>
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<td>HRP</td>
<td>Horseradish peroxidase</td>
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<td>HSA</td>
<td>Human serum albumin</td>
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<tr>
<td>IC</td>
<td>Immune complexes</td>
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<tr>
<td>ICA</td>
<td>Immune complex-mediated arthritis</td>
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<tr>
<td>IFN-γ</td>
<td>Interferon-gamma</td>
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<td>Ig</td>
<td>Immunoglobulin</td>
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<td>IL</td>
<td>Interleukin</td>
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<tr>
<td>ITAM</td>
<td>Immunoreceptor tyrosine-based activation motif</td>
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<tr>
<td>ITIM</td>
<td>Immunoreceptor tyrosine-based inhibitory motif</td>
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JIA  Juvenile idiopathic arthritis
LPS  Lipopolysaccharide
Mab  Monoclonal antibodies
MBL  Mannose-binding lectin
MCP-1  Monocyte chemoattractant protein-1
MHC  Major histocompatibility complex
MIP-1α  Monocyte inflammatory protein-1
NHS  Normal human serum
OD  Optical density
PBMC  Peripheral blood mononuclear cells
PBS  Phosphate buffer saline
P-IgG  Plate-bound IgG
PRR  Pattern recognition receptors
RA  Rheumatoid arthritis
RF  Rheumatoid factor
RPMI  Royal park memorial institute (culture medium).
SLE  Systemic lupus erythematosus
TLR  Toll-like receptor
TMB  Tetramethylbenzidine
TGF-β  Transforming growth factor beta
TNF-α  Tumor necrosis factor alpha
TT  Tetanus toxoid
TT-IC  Tetanus and tetanus toxoid antibody containing plate-bound immune complexes
Introduction

The Immune system

Our human body is exposed to various pathogens during its lifetime but is often defended by immune responses. Immune responses are enacted by the immune system against foreign substances that enters the body. The immune system consists of innate and adaptive components. The innate immune system is the first line of defense and is non-specific, containing anatomical barriers, physiological barriers, inflammatory barriers, and innate immune cells like natural killer cells, mast cells, eosinophils, basophils and phagocytic cells such as granulocytes and macrophages. Acute phase proteins like C reactive protein, serum amyloid apolipoprotein and complement fragment like complement fragment C3a assist in phagocytosis by opsonization. Unlike innate immunity, the adaptive immune system provides specific immune responses towards individual antigens. T cells and B cells are the major cells of the adaptive immune system, which recognize a wide range of antigens with specificity, diversity and immunological memory. Immunological memory helps to recognize antigens faster when the same antigen is exposed for the second time. The immune response is further distinguished based on the function as cell mediated immunity and humoral immunity. Cell mediated immunity involves direct contact between cells and antigens, while in humoral immunity, secreted molecules such as antibodies and complement proteins mediate the effect. T cells cannot recognize whole antigen and needs antigen presenting cells (APC) to process and present the antigens as small linear peptides. B cell recognizes epitopes present on antigenic surfaces based on their conformation, thus epitopes do not have to be linear. Although innate and adaptive immunity seems to function independently from each other, they often communicate through cytokines and other molecules and thus operate as an interacting network.

The immune system has the ability to distinguish between self and non-self antigens and this propensity of the immune response is provided by several mechanisms. In innate immunity the immune cells recognize certain structures in the pathogens, through pattern recognition receptors (PRR) present on their cell surfaces or in endosomal compartments (1, 2). In adaptive immunity the specificity is regulated through central and peripheral tolerance. Central tolerance takes place during the development of B and T cells in the bone marrow and thymus before cells reach the periphery. Self-
reactive B cells that react with self-surface antigens are deleted by clonal deletion before reaching the periphery. Mature self-reactive B cells that have escaped central tolerance are often not activated in the periphery as they only express immunoglobulin (Ig)D on their cell surfaces, and therefore become non-reactive (3). Similar to B cells, T cells undergo positive and negative selection based on what and how MHC molecules present to them. During positive selection the cells that bind self-antigens presented by major histocompatibility complex (MHC) with appropriate affinity are positively selected. In negative selection self-reactive T cells that bind MHC presenting self-antigens strongly are deleted (3). Regulatory B cells and regulatory T cells suppress inflammation by producing immune suppressive cytokines like IL-10 and TGF-β (4, 5). Failure in any of these mechanisms can lead to the development of autoimmune diseases.

**Antibodies**

Antibodies (also known as immunoglobulins) are glycoproteins produced by B cells and plasma cells. They are found both in free form and bound on the surface of B cells. Each antibody is formed with identical heavy and light chains. The N terminal of the antibody recognises various antigens with the help of high variability domains. Antibody are of five isotypes; IgG, IgA, IgM, IgE and IgD. IgG and IgA are further subclassed into IgG1-4 and IgA 1-2 respectively. The isotypes vary in their heavy chain constant regions. IgD and IgM are found on surface of B cells, and when the B cells are activated upon stimulation by antigens they start to form IgA, IgG or IgE by class switching. IgD have no known functions in general but are found in small amounts in the circulation. IgM antibodies are pentamers and are also activators of complement. IgA exists both in the circulation and as dimeric forms when secreated, providing immunity against mucosal infections. IgG is primarily involved to clear viruses and bacteria and to activate the complement cascade. IgE antibodies are involved in defense against parasites and, on the downside, also contribute to allergy. When free antibodies bind foreign antigens they can form immune complexes (IC) which activate effector mechanisms dependent on the classical complement pathway, Fc receptor-mediated phagocytosis and antibody-dependent cellular cytotoxicity (ADCC).

**Immune complexes**

Immune complexes (IC) are formed during immune responses in order to eliminate foreign antigens. They are formed between an antigen and any of the five classes of immunoglobulins. In some autoimmune diseases IC are
not cleared from the circulation and lead to inflammation after sequestration in tissues. As IgM is larger in size and has more valency, IgM-IC can even form very large lattices with monovalent antigens when antigen concentration is high and thus helps to clear monovalent antigens from the circulation (6). Phagocytic cells such as macrophages and polymorphonuclear neutrophil granulocytes (PMN) have Fc receptors and complement receptors that are important in clearing IC. IC can be soluble, insoluble or surface-bound and induce cytokine production from monocytes in various diseases (7-10).

Rheumatoid arthritis

Rheumatoid arthritis (RA) is a chronic systemic inflammatory disease affecting about 0.5 to 1% of the population worldwide characterized by morning stiffness, joint pain and destruction (11). The quality of life is very much affected and damages incurred can be irreversible, especially if not treated early. As for many other supposedly autoimmune diseases the cause of RA is unknown and a number of factors contribute to RA development. The definition of RA is based on classification criteria, and a system for classifying RA was established in 1958, which was thereafter revised by the American College of Rheumatology (ACR) in 1987. The ACR classification includes 7 criteria given in table 1(12). After the discovery of antibodies against citrullinated proteins (ACPA), serology became more important and included in the 2010 European League Against Rheumatism (EULAR)/ACR classification criteria as shown in table 2 (13). Smoking is an important preventable risk factor for RA development and disease severity (14-17). Diet modifications also have an impact in ameliorating the signs and symptoms of arthritis (18). Obesity increases the risk for developing RA (19). Alcohol consumption has been associated with a lower risk of developing RA (20). Treatment of RA involves disease modifying anti-rheumatic drugs (DMARDS) and administration of steroids into joints. Recently launched therapies include usage of antibodies against the pro-inflammatory cytokines TNF-α or IL-6, JAK inhibitors, depletion of CD20 positive B cells and blockade of T cell co-stimulation molecules (21).
Table 1. 1987 ACR classification criteria for RA (12).

| 1) | Morning stiffness in and around joints lasting at least 1 hour before maximal improvement |
| 2) | Soft tissue swelling (arthritis) of 3 or more joint areas observed by a physician |
| 3) | Swelling (arthritis) of the proximal interphalangeal, metacarpophalangeal or wrist joints |
| 4) | Symmetric swelling (arthritis) |
| 5) | Rheumatoid nodules |
| 6) | The presence of rheumatoid factor |
| 7) | Radiographic erosions and/or periarticular osteopenia in hand and/or wrist joints. |

Note: To fulfill the criteria, Criteria 1 through 4 should be present for at least 6 weeks. RA is characterized by the presence of 4 or more criteria, and no further qualifications are required (12).

Table 2. 2010 ACR/EULAR new classification criteria for RA (13).

| 1) | Patients with at least 1 joint with definite clinical synovitis (swelling)* |
| 2) | Patients with the synovitis not better explained by another disease † |

A. Joint involvement

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<tr>
<td>1 large joint</td>
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<td>2–10 large joints</td>
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<td>1–3 small joints (with or without involvement of large joints)</td>
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<tr>
<td>4–10 small joints (with or without involvement of large joints)</td>
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<td>&gt;10 joints (at least 1 small joint)</td>
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B. Serology (at least 1 test result is needed for classification)

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<tr>
<td>Negative RF and negative ACPA</td>
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<td>Low-positive RF or low-positive ACPA</td>
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<tr>
<td>High-positive RF or high-positive ACPA</td>
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C. Acute-phase reactants (at least 1 test result is needed for classification)

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<tr>
<td>Normal CRP and normal ESR</td>
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<td>Abnormal CRP or abnormal ESR</td>
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D. Duration of symptoms

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<tr>
<td>&lt;6 weeks</td>
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<tr>
<td>≥6 weeks</td>
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Note:*The criteria are aimed at classification of newly presenting patients. † An expert rheumatologist should be consulted to conduct the differential diagnosis. Joint involvement, serology, acute phase reactants and duration of symptoms are considered in the new 2010 criteria with scores given by an algorithm. 3/6 points are needed to classify RA criteria. Large joints refer to shoulders, elbows, hips, knees, and ankles. Small joints refer to the metacarpophalangeal joints, proximal interphalangeal joints, second through fifth metatarsophalangeal joints, thumb interphalangeal joints, and wrists (13).
Genetic susceptibility: HLADRB* alleles in RA

RA is not a Mendelian inherited disease. On the other hand, MHC class II is the most important genetic factor in determining the disease pathogenesis. Although there is not a single HLA gene reported to be the cause for RA, the major disease susceptibility lies within the HLADRB1* region (22, 23). RA development has a strong genetic association with certain HLADRB1* alleles, mainly HLADRB1*01, HLADRB1*04 and HLADRB1*10. These share a common epitope motif, collectively designated the “shared epitope”(24). Modern PCR techniques using sequence specific oligonucleotides permitted genotyping of HLA alleles and confers that the genetic susceptibility varies among ethnic groups (25), (26), while *04:05 is the commonest risk factor for the Asian RA populations (27-29). HLADRB1*04:05 is also evident in central Africa in Cameroon and Sudan (30) and Elshafie A., personal communication). Unknown antigens, hypothetical autoantigens, presented by these shared epitopes trigger auto reactive T cells and hypothetically contribute to the pathogenesis of RA, for which most published data concern HLA-DRB1*0401(31, 32).

Autoantibodies in RA

Although RA is linked with HLA-DRB1* alleles, disease pathogenesis is not restricted to cellular immunity. Autoantibodies have a crucial role in diagnosis and prognosis of RA. Rheumatoid factor (RF) is an autoantibody reacting against the Fc portion of IgG and is a diagnostic marker for RA.(33-35) Diagnostic specificity of RF in RA is limited since RF is also found in up to 5% of healthy individuals, and in higher percentages in patients infected with viral infections, and in other inflammatory diseases (12, 36). Early papers on anti-keratin antibodies and anti-perinuclear factor antibodies showed that these antibodies had very high diagnostic specificity for RA (37, 38). Later it was evident that anti-keratin antibodies recognized citrullinated forms of the stratum corneum protein filaggrin (39, 40).

Citrullination of proteins in RA occurs by conversion of arginine residues to citrulline residues by peptidyl arginine deiminase (PAD) enzymes. There are a number of PAD enzymes: PAD1, PAD2, PAD3, PAD4 and PAD6 found in epidermis, brain, macrophages, granulocytes, egg and ovary. PAD enzymes can be produced by activated PMN and macrophages in the inflamed synovium and contribute to RA pathogenesis (41). We have recently published findings that the PMN cathepsins might contribute to the pathogenesis of seropositive RA (42). ACPA are common in RA, and diagnostically more specific for RA than are RF (36, 43). Monoclonal antibodies (Mab) made from B cells isolated from joints of RA patients recognize citrullinated antigens (44). ACPA and RF appear years before RA onset, and
thus may help to diagnose RA very early (45, 46). The occurrence of ACPA or RF, especially if they occur together, are associated with a more severe prognosis and also with increased mortality in RA patients (47). Seropositivity of RA may determine the choice of treatment as RA patients with RF and/or ACPA respond better to anti-CD20 treatment (48). IgA-RF was on the other hand associated with poor response to TNF blockade (49). Hence after the discovery of ACPA, a new set classification criteria including ACPA was formed whereby serology (ACPA or RF) was given more importance. In the previous ACR criteria the autoantibodies were one out of 4 criteria (25%) in seropositive disease whereas in the new criteria serology can confer 3/6 of the points (50%) needed for RA classification (12, 13). Other naturally occurring RA-associated antibodies are anti-RA33 and anti-P68 have been reported to be associated with rheumatoid arthritis with low specificity, as they are found also in other autoimmune diseases (50).

**Collagen type II antibodies in humans**

Cartilage damage is a characteristic feature of RA. Cartilage contains chondrocytes, which produce the collagen matrix. There are many types of collagens such as fibrillar collagens, basement membrane-associated collagens, fibril-associated collagens with interrupted triple helices and short-chain collagens (51). Among the fibrillar collagens, collagen type II (CII) is a major component of the joint cartilage compassing >90%, and the rest of cartilage collagens consist of collagen IX and XI (52). Early studies done in pig state that CII has a stringent distribution with more than 50% in hyaline cartilage but is also found in ear, larynx, trachea and vitreous of the eye (53). CII humoral immunity has been reported in Meniere’s disease in Asia (54, 55). In 1980s cartilage specific antibodies against CII were observed in RA patients. The prevalence of anti-CII antibodies in RA in these early studies varied from 3-67% (56-62). The variation in anti-CII detection could be due to use of different approaches and techniques used in these papers. Recent human anti-CII measurements by others are done with denatured CII, synthetic CII peptides, native human CII and bovine CII. Both the Rikard Holmdahl and Cook groups have shown that CII peptides can be used detect anti-CII antibodies in about 77%-88% of investigated RA patients (63-65). Contrary to these high figures, our group has shown that anti-CII appear in 8.8% of RA patients using human native CII as antigen (66). Earlier studies from Möttönen showed that anti-CII do not precede the clinical appearance of RA, as do RF and ACPA (46, 67). However the study was done in a small number of patients and pre-ill individuals who later developed RA (67). In patients with very early synovitis, the occurrence of anti-CII was not more common among those developing RA than in patients developing other diagnoses (68). Cook et al have shown that anti-CII disappear soon after RA
diagnosis (69). Earlier studies by Kim et al have shown that anti-CII levels correlate to acute phase reactants and pro-inflammatory cytokines (70). Contrary to these findings, using a microarray, Huber et al showed that antibody reactivity against native CII predicted a less severe RA phenotype (71).

Our group has previously shown that immune complexes (IC) made with anti-CII and native human CII (anti-CII IC) stimulate peripheral blood mononuclear cells (PBMC) to produce pro-inflammatory cytokines such as TNF-\(\alpha\), IL-1\(\beta\) and IL-8/CXCL8 (7). Anti-CII levels are high in newly diagnosed RA patients when compared to healthy controls, and the levels of anti-CII are associated with increased laboratory signs of inflammation and more radiological destruction at the time of diagnosis, but not later (72). Previous studies from our group have defined the biological function of anti-CII, revealing that pro-inflammatory cytokines produced \textit{in vitro} by anti-CII IC stimulated PBMC were associated with acute phase protein CRP, and to ESR (66). When compared with conventional ACPA antibodies anti-CII serum levels vary in in parallel to inflammatory markers, whereas ACPA levels drop over time in contrast to the parallel clinical worsening in ACPA positive RA patients (66, 73). Thus available data show that anti-CII levels are high around the time of RA diagnosis, and drop later, and that anti-CII associates with an early RA phenotype with increased acute phase reacts and joint erosion early after diagnosis.

Figure 1. Difference between appearance of anti-CII and ACPA before RA onset, at diagnosis and after (adapted from paper II). The data on anti-CCP appearing before RA onset is based on data from Kokkonen et al This is in contrast to anti-CII levels which do not precede RA (data from Möttönen et al). The level of antibodies after diagnosis are based on data from Rönnelid et al and Mullazehi et al (66, 67, 73, 74).
Collagen immunity in mouse models

The pathogenicity of CII antibodies has been extensively studied in animal models. When bovine or rat collagen was injected in mice with Freund's complete or incomplete adjuvant the mice developed arthritis, a model known as collagen induced arthritis (CIA) (75). The propensity to develop CIA is linked to certain I-A beta chains of MHC II similar to HLA associations in RA (76). The pathogenesis of CIA was demonstrated to be dependent on MHC class II, T cells and antibodies reacting against CII (77-80). When the serum from injected mice was tested for specificity of anti-CII antibodies, most of the antibodies were against certain epitopes. After the discovery of immunodominant epitopes in mouse CII, the collagen antibody induced arthritis model (CAIA) was developed. When antibodies against CII with defined CII specificities are injected into mice, they develop the acute onset CAIA (81). The severity of CAIA was dependent on the specificity of anti-CII against certain CII epitopes, C1 J1 and U1, but was not dependent on MHC or autoreactive T cells as seen in CIA (63, 82). Mabs against these specific CII epitopes were potent in inducing arthritis and a combination of antibodies against three independent epitopes was required for the overt arthritis development (83). This could be due to antibodies binding to different epitopes on CII can induce complement activation (84). CAIA is characterized by symptoms of cartilage erosion, PMN infiltration and deposition of IgG antibodies and C3 fragments on cartilage surfaces. Notably, in CAIA the crucial cells involved in the pathogenesis are the PMN (85). These and other animal studies show the pathogenesis of anti-CII in arthritis and their possible relevance in RA (63, 86). It is our belief that the strictly antibody-dependent rodent arthritis model CAIA in which inflammation is already detected already after 5 days, and is aggravated by LPS, might be the animal model counterpart to acute onset pathology in the RA subgroup with elevated anti-CII levels at the time of diagnosis.
Figure 2. Figure made from information in Nandakumar et al. and Lindh et al. (63, 82). Schematic model showing the mechanism of CIA vs. CAIA in mouse. CIA model is dependent on autoreactive T cells and MHC whereas, (bottom) CAIA does not depend on T cells or MHC but on pathogenic antibodies specific to CII epitopes C1, U1 and J1. Antibody against F4 epitope of CII is shown to be protective for CAIA development.

Granulocytes in inflammation and RA

Inflammation is the body’s response against infection or injury and is normally an acute and self-limiting process. However in autoimmune diseases inflammation becomes chronic and sustained in autoimmune diseases. Inflammation involves 5 cardinal signs: redness (Greek: rubor), swelling (tumor), heat (calor), pain (dolor) and loss of function (functio laesa). The first signs of inflammation are increased blood flow through capillaries followed by swelling, eventually leading to heat and pain due to destruction of tissue. Initial cellular steps involve adhesion of white blood cells in the bloodstream to endothelial cells, followed by their transmigration from the vessels and chemotactic recruitment at the site of injury/infection. The granulocytes belong to the innate non-specific part of the immune system and consist of three major cell populations: the neutrophil, eosinophil and basophilic granulocytes. They are distinguished by the presence of granules and multi-lobed nuclei. Among the granulocytes the polymorphonuclear neutrophils (PMN) are of primary importance in the context of inflammation, and are very abundant at sites of acute inflammation (87, 88).

Human PMN are different from murine PMN in functional properties and hence PMN studies performed in mice cannot mirror PMN mediated patho-
PMN found in the human blood circulation are terminally differentiated with short half-life and cannot be genetically modified, demanding PMN isolation from fresh blood as a primary requirement in PMN research. PMN production from the bone marrow is strictly regulated by the cytokines G-CSF and IL-17. Under normal conditions only GM-CSF regulates PMN hemostasis, whereas during inflammation IL-17 recruits PMN and promotes hematopoiesis (90). Th17 cells that produce IL-17 at the site of inflammation recruit PMN by inducing the production of CXCL8 from endothelial cells (91). Although PMN have a very short half-life once they accumulate in inflammatory areas, they survive longer, for example in the synovium due to the local supply of growth factors and cytokines (92-94). It has been shown previously that synovial fluid can inhibit neutrophil apoptosis but do not interfere with PMN activation and hence activated PMN can survive longer in RA synovium (95). It is therefore plausible that PMN also survive longer in cultures provided with inflammatory stimuli.

PMN have 4 types of granules where MPO, neutrophil elastase, defensins and membrane-bound CD63 are found in primary granules, whereas LL-37 peptide, membrane-bound CD66b and lactoferrin are stored in secondary granules. The tertiary granules contain cathepsin and gelatinase. The fourth type, the secretory granules, store membrane receptors (96). PMN can also release chromatin threads with activating signals in their chromatin known as neutrophil extracellular traps (NETs) during the process of cell death called NETosis. If NETs are not cleared from the system they can contribute to autoimmunity (97). Based on their functional properties, distinct PMN subtypes have been proposed. As for the pathogenic macrophages and immunomodulatory macrophages, N1 (pro-inflammatory) and N2 (anti-inflammatory) PMN subtypes have been described (98, 99). Based on their ability to produce ROS, PMN can be categorized into subsets distinguished by CD makers: CD16\textsuperscript{dim}/CD62L\textsuperscript{bright} cells with high ROS production and CD16\textsuperscript{bright}/CD62L\textsuperscript{dim} cells with low ROS production. Some low-density granulocytes have also been reported with pro-inflammatory properties to produce enhanced NETs; this PMN fraction co-purifies with PBMC during Ficoll separation and thus represents a source of error (100). At the site of inflammation PMN may degranulate releasing ROS and neutrophil proteases that can alter pro-inflammatory cytokine regulation by cleaving cytokines such as TNF-α and interferon (INF)-γ (101, 102). The PMN enzyme neutrophil elastase can also augment CXCL8 production through TLR4 (103). NET formation is dependent on crucial steps that involve an oxidative burst initiated by formation of the NADPH complex followed by ROS production and MPO translocation to the nucleus (104). NETs contain IC and other complement-activating proteins that can activate PMN to produce more NETs and hence amplify NET production (104). PMN from serum and SF of RA patients exhibit enhanced NETosis when compared to healthy PMN. The rate of NETosis correlates with elevated CRP, ACPA and IL-17. PMN can
exhibit enhanced NETosis and may act as a source of citrullinated autoantigens after the release of NETs in RA (105). NETs not only promote inflammation, but also regulate immune function; when a crucial concentration of NETs is formed they aggregate and trap pro-inflammatory cytokines and thus resolve inflammation (106). Ncf1 is an important part of the NADPH oxidase complex that mediates the production of ROS (Figure 3). Polymorphisms in the Ncf1 gene are associated with T regulatory cell-mediated suppression of CD4+ cells that modify the T cell-dependent autoimmune response (107). Ncf1 regulates the severity of arthritis in rats and mice (107, 108) and RA patients have been reported with fewer copy numbers of the Ncf1 gene (109). PMN are very abundant in inflammatory synovial fluid, but are also found in the pannus tissue and thus are situated in close vicinity to cartilage (110). In this position close to cartilage, PMN might also promote and sustain cartilage-dependent inflammatory responses, this being the focus on the first two papers in this thesis. PMN in the synovial fluid of RA patients express elevated levels of activating FcγRI and FcγRIII and contribute to pro-inflammatory cytokine production (111, 112). PMN also contribute to the pathogenesis of juvenile idiopathic arthritis (113, 114). Thus PMN have an important role in both RA and juvenile arthritis and contribute to the pathogenesis by producing degrading serine proteases against connective tissues, modifying cytokine signals through Fc receptors, TLR, and the complement system.
Figure 3. Schematic picture of neutrophil containing PAD enzyme in the nucleus. It also shows phagolysosomes activated by IC, and the NCF1 subunit regulating the NADPH complex that produces ROS.

**Fc Receptors in autoimmunity**

Receptors for binding the Fc portion of antibodies IgA, IgM, IgD, IgE and the 4 subclasses of IgG are collectively called as Fc receptors (FcR). FcR are found on phagocytic cells such as monocyte-macrophages and PMN. FcR mediate antibody- and IC-dependent immune responses. Although FcR are involved in defense against infections they also play a role in allergy and in inflammatory diseases. FcγR are broadly classified into 4 groups based on
the ligands they bind, on their structure and function, whereby the main groups are FcγRI (CD64), FcγRII (CD32) FcγRIII (CD16), FcγRIV and neonatal receptor (FcRn). Some variants of Fc receptors are not found in mice but exist in humans (115, 116). FcR have either activating immunoreceptor tyrosine based activation motifs (ITAM) or immunoreceptor tyrosine based inhibitory motifs (ITIM) and these motifs determine FcR function. Intravenous immunoglobulin (IvIg) has been used to treat autoantibody- and IC-mediated inflammatory diseases (117, 118), and are thought to suppress inflammation by upregulating inhibitory FcγRIIb and downregulating activating FcγR (119, 120). FcγRI binds monomeric IgG and can be upregulated by pro-inflammatory stimuli such as INFγ or LPS, and downregulated by the inhibitory cytokines TGF-β and IL-4 (121-123). In contrast to FcγRI, FcγRIII and FcγRII are low affinity receptors for IgG, and preferably bind IC and thereby mediate IC-dependent effector functions. FcγRIV is a murine Fc receptor homologous to human FcγRIII. However, FcγRIV may also have functions similar to Fc IgE receptor and bind IgE containing IC and activate macrophages and neutrophils to produce inflammatory cytokines (124). FcRn is expressed in vascular endothelium, gut, APC, Central nervous system endothelium, kidneys and lungs. FcRn play a major role in determining the half-life of circulating IgG. Treatment with IvIg is also known to exhibit inhibitory function by saturating FcRn (125). Experiments in CIA show that Fc receptors play a crucial role in CIA development, as disease was abrogated after inhibition or blocking of Fc receptors (126-128). Studies of Fc receptor knockout animal models show the functional role of Fc receptors in the pathogenesis of autoimmune diseases (116, 129-131). Fcγ receptors are up regulated in blood monocytes of RA patients with high ESR (132), as well as in the synovium. In the immune-complex induced arthritis (ICA) model mice develops arthritis when IC are injected into joints. The mechanism was dependent on FcγRI and/or III as IC injected into joint of knockout mice lacking FcγRI and III did not develop arthritis (133). When macrophages isolated from RA patients show increased expression of FcγRI, II and III and the expression I correlated with MMP-1 production (134). The human FcγRIIa receptor contains an activating ITAM motif, and is not found in mice. When FcγRIIa is expressed in mice it promotes arthritis. The arthritis development was more rapid and was found only in FcγRIIa transgenic mice but not in non-transgenic mice. The pathogenesis was crucially dependent on FcγRIIa as the production of inflammatory cytokines was significantly reduced after blocking FcγRIIa (135). Previous research in our group has also shown that blocking of FcγRIIa receptors reduces the anti-CII IC-mediated production of pro-inflammatory cytokines (7).
Toll-like receptors (TLR) in autoimmunity

Toll-like receptors present in antigen presenting cells and other phagocytic cells play an important role both in innate immunity and adaptive immunity. In humans 10 different TLRs (TLR1-10) have been identified that can bind microbial peptides cell surface molecules and nuclear antigens including nucleic acids (136). TLR are a type of PRR that bind bacterial components and whose activation leads to induction of pro-inflammatory cytokines. TLR4, which is primarily involved in bacterial infections (137), is also involved in autoimmune diseases and contribute to immune pathogenesis (138). In SLE, DNA- and RNA-containing nuclear antigens stimulate plasmacytoid dendritic cells through TLR7 and TLR9 together with FcγRIIa (139). TLR-mediated recognition signaling in SLE can be either immune activating or suppressive, depending on the DNA or RNA amino acid sequences involved (140). TLR2 and TLR4 are upregulated in synovial tissue in RA patients (141). There are also various endogenous ligands such as neutrophil elastase (NE) that can activate NF-κB through TLR4 (103). Studies show that many endogenous TLR4 ligands are produced during inflammation (142, 143). High mobility group box 1 (HMGB1) is an endogenous ligand of TLR4 that can be secreted by activated monocytes (144, 145). Although there is evidence for endogenous TLR ligand expression in inflamed joints and that the involvement of TLRs in CXCL8 production via monocyte-endothelial interaction is known (146, 147), it remains unclear under what conditions these endogenous ligands are produced during inflammation. In IC-mediated autoimmune diseases in humans there is a crosstalk between TLR receptors and FcγRs that augments the response of inflammatory cells (148-150). Animal studies have shown that blocking TLR4 has a significant impact in diminishing the inflammatory response in IC-FcγR mediated pathogenesis of arthritis (151).

Chemokines and other cytokines

Cytokines are important for the integrated functionality of the immune system. They help in the communication between cells of the innate and adaptive immune systems. Activated immune cells produce cytokines that can regulate, activate, suppress and/or sustain immune responses. Chemokines are the low molecular weight proteins that recruit leucocytes. They are broadly classified as 2 groups based on the position of cysteine residues as CC and CXC chemokines, except fractalkine that has the unique group code CX3C. Chemokines such as CXCL8 (IL-8), CCL5 (RANTES), CCL2 (MCP-1), CCL3 (MIP-1α), CXCL2 (GRO-α) each attracts unique but diverse cell types like PMN, monocytes, memory T cells and dendritic cells (152). Among the monocyte-attracting chemokines are CCL2 (MCP-1), CCL7,
CCL8 and CCL13. CCL2 (MCP-1), which binds CCR2, is the most studied and shown to have a major role in monocyte recruitment and has been used as an intervention point in autoimmune diseases (153). Several chemokine blocking studies have shown the functional importance of different chemokines during RA disease pathogenesis (154).

Cytokines such as IL-1, IL-6 and TNF–α when released by macrophages in the synovium causes inflammation and cytokines also induce the production of acute phase reactants (155). The pro-inflammatory cytokines GM-CSF, IL-8, TNF–α and IL-6 are upregulated in RA patients regardless of therapy (156). Cytokines have also been implicated in causing disease-associated fatigue and pain. Treatment with anti-cytokine antibodies e.g. anti-IL-17 has been shown to decrease fatigue in RA patients (157). Several therapeutic targets like macrophage inhibitory factor (MIF) and RANK have been identified (158-162). Among the cytokine therapeutic targets in RA, TNF–α is a crucial cytokine as it drives the pathogenicity of RA. When human TNF–α was expressed in mice, they developed polyarthritis, which could be blocked by Mab against TNF–α (163, 164). Anti-TNF-α therapy has been a successful and well-established treatment in RA (165, 166). B cell depletion by rituximab results in diminishing cytokine production and T cell inactivation (167). IL-6 blockade is also an approved therapy for RA (168).

Cytokine regulation by collagens and cell interactions

Previous studies demonstrate that extracellular matrix surfaces (ECM) have a role in cytokine modulation and regulation (169, 170). and hence joint-specific collagen matrices may have a role in cytokine regulation. Interaction between PMN and collagen I inhibits CXCL8 production; this inhibition was collagen I specific and not found for other ECM proteins such as fibronectin or laminin (171). This shows that not all ECM proteins have the same regulatory properties. ECM proteins do not only down-regulate immune responses; it has been previously been reported that T cells reactive against CII when cocultured with synovial fibroblasts, enhance chemokine production (172). Pro-inflammatory TNF-α may adhere to ECM fibronectin and attract T cells activated by other chemokines (RANTES and SDF-1α) and thus TNF-a may act as an anchor to T cells during inflammation (173). Certain interactions between monocytes and endothelial cells can enhance CXCL8 production (147). Also chemokines interact with each other: RANTES and CCL2 create a chemokine network and contribute significantly to RA pathogenesis by enhancing the production of CXCL8 (154). RANTES and CCL2 can also activate osteoclast differentiation and can thereby have a role in the bone resorption that is found close to rheumatoid arthritis joints (174). Thus inflammatory cells and ECM might regulate cytokines and chemokines and contribute to pathogenesis of RA and other autoimmune diseases.
Aims of the thesis

**General aim**
To investigate the role of anti-CII in the pathogenesis and prognosis of RA.

**Specific aims**

a) To evaluate PMN activation after stimulation with anti-CII IC both in functional and clinical contexts.

b) To understand the mechanisms of PBMC-PMN coculture-associated enhancement of chemokine production after stimulation with anti-CII IC.

c) To evaluate the relation between anti-CII and laboratory and clinical measures of disease activity as well as HLA-DRB1* alleles in two large RA cohorts.
Materials and Methods

Patients and healthy controls

Sera from 72 RA patients were chosen from a well-established cohort containing 274 RA patients who had fulfilled the ACR criteria chosen was used in paper I. This group contained 24% men and 76% women. This cohort has been investigated in our previous studies (7, 66, 73). In paper II, 13 RA sera with high anti-CII antibodies were used to make anti-CII IC. All RA sera used were obtained from department of rheumatology at Karolinska University hospital, Solna. For cell culture studies described in papers I and II, heparinized blood was obtained from healthy donors and laboratory personnel at Uppsala University hospital after informed consent.

The Epidemiological Investigations of Rheumatoid Arthritis (EIRA) case-control study involves the majority of newly diagnosed RA patients in Sweden. In paper III, EIRA patients (n=2000) and controls (n=960) were included between 1996 and 2005. Smoking data was acquired by questionnaires and patients classified as either ever or never smokers. In studies on the relation to clinical follow-up data, exclusion was made of patients lacking AC-P A data (n=18), disease duration >365 days at diagnosis (n=170), patients lacking clinical data (n=650), more than 10 days between clinical diagnosis and inclusion in EIRA (n=226) and non-specific reactivity in the anti-CII ELISA (n=163). HLA association studies were performed in 1476 patients, after exclusion of patients lacking information on anti-CCP2 (n=18) or HLA-DRB1* (n=23), disease duration > 365 days at EIRA inclusion (n=163) or non-specific reactivity in the anti-CII ELISA (n=316). The EIRA study cohort consisted of 69.3% women and 30.7% men.

In the Malaysian Epidemiological Investigations of Rheumatoid Arthritis (MyEIRA) case-control study, 1260 patients with rheumatoid arthritis representing the three major ethnicities in Malaysia (Malay, n=530, 42.1%; Chinese, n=259, 20.6%; Indian, n=411, 32.6%; mixed-ethnicities, n=60, 4.8%) from various rheumatology centers in peninsular Malaysia were recruited between 2005 and 2009. Age, sex and ethnicity-matched controls (n=1569) were recruited to the study, and serves as an integral part of the MyEIRA cohort. RA cases aged between 18 and 70 years old were diagnosed according to the 1987 American College of Rheumatology (ACR) classification (12). MyEIRA patients consisted of 85.3% women and 14.7% men. After exclusion of 141 patients with > 1 year of disease duration at
inclusion, and 14 individuals with non-specific reactivity in the anti-CII ELISA, 1105 RA patients remained for further analysis. CRP data was available for 1045 RA patients (94.6%) of the RA patients and HLA genotyping data were available for all 1105 patients.

All patients had given informed consent to participate in the study. The investigations were described in papers I, II and III were performed after approval from the local ethical committee at University hospital Uppsala and at Karolinska University Hospital, Solna. All participants in paper IV were given information about the research, and written consent was obtained. The study was approved by the Medical Research and Ethics Committee, Ministry of Health, Malaysia.

### Preparation of IC and cell stimulation experiments

ELISA grade human type II collagen (Chondrex, Redmond, Washington, DC, USA; 10mg/ml) was coated on Maxisorp ELISA plate (Nunc, Roskilde, Denmark) overnight at 4°C. Plates were blocked with 1% human serum albumin (HSA; Alburex CSL Behring, Stockholm, Sweden) diluted with sterile PBS for 1 hour. After blocking, 50µl of 10% diluted (for PBMC and coculture studies) or undiluted (for PMN studies in paper I) patient and control sera were added to individual wells and incubated for 2 hours at room temperature. Before adding the responder cells the plates were washed with sterile PBS. In comparison studies plate-bound IgG was used as a control IC for most of the experiments. IC made with tetanus toxoid antigen (Statens Bakteriologiska Laboratorium, Solna, Sweden) and hyperimmune anti-tetanus serum (Tetagam, CSL Behring) (TT-IC) were also used as control IC to compare IC functional responses. Apart from IC stimulations, LPS and cytokine stimulation studies were also performed. PBMC were purified from heparinized blood from healthy blood donors using standard Ficoll-Paque (GE Healthcare, Uppsala, Sweden) density gradient. Purified cells were thereafter diluted to 0.5*10^6 cells/ml in RPMI-1640 medium (Sigma Aldrich, Saint Louis, MO, USA) supplemented with 10% fetal calf serum (FCS, Sigma Aldrich), 1% glutamine, 1% normal human serum (NHS), 1%HEPES and 1% penicillin-streptomycin (PEST). 12.5mg/ml of polymyxin B (Sigma Aldrich) was also added in some of the cell culture experiments in paper I and paper II.

PMN were initially purified from sodium-heparinized blood (Greiner bio-one GmbH, Kremsmünster, Austria) using Ficoll (GE Healthcare, Uppsala, Sweden) after osmotic lysis of the red blood cells. In subsequent experiments, PMN were isolated using Percoll (GE Healthcare) gradients with 72% Percoll at the bottom and 63% Percoll at the top. In this method the PMN were obtained at the interface between the two Percoll gradients. The purity and viability of PMN obtained from both the procedures were compa-
In paper II, coculture studies were performed with PBMC and PMN. Cell concentrations (0.5*10^6 cells/ml) of PBMC and PMN respectively were the same in papers I and paper II. The purity and viability of the cells were assessed before the experiments. Purity of PBMC and PMN was checked by Türk’s solution and was always >95%. Viability (trypan blue or flow cytometry using propidium iodide (PI), with comparable results using both methods) was >92% and >95% for PMNs and PBMCs, respectively.

**Figure 4.** Schematic model showing how anti-CII IC is made with anti-CII positive RA sera, and thereafter used to study anti-CII IC-induced PBMC and PMN responses. The lower part show the three in vitro models used. (Left) Anti-CII IC-induced PBMC production of TNF-α and CXCL8; the model was used in paper I and paper II, and has also previously been used by Mullazehi et al. (66) (Center) Anti-CII IC-induced PMN upregulation of CD11b and CD66b and production of MPO; the model was used in paper I and paper II. (Right) Anti-CII IC-induced coculture upregulation of chemokines and downregulation of TNF-α; that model was used in paper II.

**Anti-collagen type II ELISA**

The 96 well ELISA plates (NUNK, Roskilde Denmark) were coated with 50µl of ELISA grade human native CII at a concentration of 2.5µg/ml at 4°C overnight. Following incubation the coating solution was discarded and the plates were blocked with ELISA grade (essentially IgG-free) bovine serum albumin (BSA) for 1 hour. Sera were diluted at a concentration of 1:100 in PBS and added to CII-coated wells and incubated for 2 hours. The plates were washed with PBS-Tween and a goat detection antibody against the human IgG γ chain conjugated with alkaline phosphatase was added at a concentration of 1:10000 in 1% human serum albumin (HSA) and incubated for 1 hour. To minimize the risk of interference with any remaining bovine IgG in BSA or with rheumatoid factor, a F(ab’)2 detection antibody that had been pre-absorbed against bovine proteins was used. After washing, a sub-
strate solution containing 4-nitrophenyl phosphate was added and incubated for 45 minutes. The optical density (OD) was measured at 405 nm and anti-CII concentrations were calculated against a standard curve consisting of an RA serum with high level of anti-CII. After determining anti-CII positives using the 95th percentile among 100 healthy blood donors, the OD values of anti-CII positive sera were reevaluated by adding sera in parallel wells coated with CII and thereafter blocked with BSA, as well as to wells only blocked with BSA. After 2 hours the plates were washed and the OD values were obtained after substrate addition. Thereafter OD values from CII coated wells were subtracted from the BSA coated wells and sera with a positive difference were regarded as being anti-CII positive, whereas sera with a negative difference were regarded as having a non-specific binding. This latter group was large among the EIRA patients in paper III (163/2000, 8.2%) and the corresponding patients were statistically treated separately. The corresponding finding was much more uncommon in MyEIRA (14/1260, 1.1%) and these individuals were excluded from the analyses in paper IV. The serum dilutions used in the MyEIRA cohort (paper IV) was slightly modified from 1:100 to 1:50 and for the Malaysian patients, the 95th percentile among the MyEIRA controls served as cutoff. The assay produced stable results verified by internal controls that were evaluated on each ELISA plate. One control with high in anti-CII level and a another control with low anti CII level showed with low variations with intra-assay coefficient of variation <15%.

Anti-CCP2 and RF measurements:
Anti-CCP2 levels were measured by ELISA using a cut-off of 25 AU/ml as suggested by the manufacturer (Immunoscan-RA; Eurodiagnostica, Malmö, Sweden), the same method and cutoff was used in papers I, III and IV. RF in 72 RA patients described in paper I was measured using nephelometry (Image; Beckman Coulter, Stockholm, Sweden). In the MyEIRA patients described in paper IV, IgM and IgG rheumatoid factor (RF) were measured using ELISA kits (Immuno-Biological Laboratories, Hamburg, Germany) with a cutoff of 15 AU/ml for both isotypes, as suggested by the manufacturer.

Cytokine and chemokine profiling by ELISA and by addressable laser bead immunoassay (ALBIA)
Cytokine levels were measured in supernatants from cell cultures stimulated with IC. Antibodies against TNF-a and CXCL-8 were coated in ELISA plates (Maxisorb) and after blocking with 1% bovine serum albumin (BSA,
Sigma Aldrich) cell culture supernatants and dilutions of recombinant cytokines (R&D Biosciences, Abingdon, UK) as standard curve were added to the wells. Thereafter biotinylated secondary antibodies (R&D Biosciences) were added and incubated. After washing, streptavidin conjugated with horseradish peroxidase was added, followed by the substrate 3,3’-5,5’-tetramethylbenzidine. After incubation the OD values at 450 nm were obtained with an ELISA reader and results were analyzed using Deltasoft software (Princeton, NJ, USA). MPO was analyzed using an ELISA kit from Diagnostics AB, Uppsala, Sweden, according to the manufacturer’s instructions.

An array of chemokines CXCL8 (IL-8), CCL5 (RANTES), CCL2 (MCP-1), CCL3 (MIP-1α), CXCL2 (GRO-α), IP-10, fractalkine along with cytokines TNF-α, IL-1β, IL-10 and GM-CSF were measured using a custom-made ALBIA (Millipore, Stockholm, Sweden). The choice of cytokines was intended to study to what extent different chemokines might show upregulation in PBMC-PMN cocultures as was first shown for CXCL8 measured using ELISA. The magnetic beads were washed and sonicated for 30 seconds. Therafter the magnetic beads were added to the cell culture supernatants, standards and controls as recommended in the kit. After incubation the plate was washed using a magnetic rack and biotinylated secondary antibodies added and incubated. Later streptavidin-coupled phycoerythrin was added and after 30 minutes cytokine levels were measured using a Bio-Plex Magpix Reader (Bio-Rad, Berkeley, CA, USA). As levels of IL-10, IP-10 and fractalkine were undetectable in all cell cultures, they were excluded from further analysis.

**Blocking, neutralization and inhibition experiments**

For GM-CSF neutralization studies 0.5 µg/ml of polyclonal goat anti-GM-CSF antibody (R&D Biosciences) was added to cell cultures stimulated with anti-CII IC. A polyclonal goat IgG was used as a control in the GM-CSF neutralization studies whereas wells without any control reagent were used as controls for FcγR blocking studies. Granulocyte enzyme inhibition experiments were performed by incubating cells isolated from 8 healthy donors with inhibitory peptides against the granulocyte enzymes cathepsin S, cathepsin L (cathepsin1 inhibitor), MPO (MPO inhibitor) and elastase (elastase inhibitor) for 15 minutes before adding to the cell culture plate with the stimuli. All enzyme-inhibiting peptides were bought from Merck Chemicals and Life Sciences, Stockholm, Sweden and used in concentrations as recommended by the company. Polyclonal rabbit anti-human TLR4 antibodies and control polyclonal rabbit IgG were bought from Invivogen, Toulouse, France and used at the concentration 10 µg/ml as recommended by the pro-
vider and added to PMN, PBMC or cocultures for 15 minutes before adding to cell culture plate with anti-CII IC.

Flow cytometry analyses

Flow cytometry analysis of PMN was performed using fluorescein isothiocyanate-conjugated IgG1 antibodies against CD11b (Bcar clone), CD66b (80H3 clone) (Beckman Coulter, Marseille, France), CD16 (DJ130c clone; DAKO, Glostrup, Denmark) and phycoerythrin conjugated antibody against CD32 (2E1 clone; Beckman Coulter). The antibodies were incubated with the cells for 30 minutes at 4°C. Thereafter the cells were washed and fixed with 1% paraformaldehyde. Color compensation was performed for PE and FITC using compensation particle set from BD Biosciences. Isotype controls were used to assess for non-specific binding. Analysis was performed using a BD FACS Canto II flow cytometer, and the results were analyzed using FACS DIVA software (BD Biosciences, Stockholm, Sweden).

The Swedish rheumatology quality register (SRQ)

Swedish government-administered health registries are a great source of information for medical science, and together with the individual personal code numbers allow linking of sources of information. Sweden thereby has a unique advantage in amassing data for epidemiological studies. However, the government-administered registries do however often lack detailed data on disease state and treatment of individual patients, and they also lack information about patient reported outcomes and laboratory measures of disease activity including inflammation. For that purpose a number of healthcare quality registries have been instituted, with the aim of improving the delivery of healthcare. A recent survey listed 103 Swedish quality registries (175). SRQ was started in 1995 by the Swedish Rheumatology Society to improve healthcare for RA patients but has since been expanded to cover also other diagnoses (176). Data acquisition is an ongoing process in SRQ and is integrated with the health care system, and data are collected in conjunction with clinically motivated care visits. SRQ had been used in a number of research projects, including studies of time to discontinuation of different TNF inhibitors, usefulness of switching TNF inhibitors in cases of non-responsiveness, and the trend over time for clinical characteristics in patients receiving biologics (177-179). Registry data from SRQ have also been used to show that although the tuberculosis risk in increased in patients receiving TNF inhibitors, the new tuberculosis screening guidelines have conferred decreased risk (180, 181).
SRQ have however not previously been used to evaluate the prognostic impact of RA-associated autoantibodies. In collaboration with Saedis Saevarsdottir and Helga Westerlind at Karolinska University Hospital we have obtained linked SRQ data for 773 EIRA patients for whom we previously measured anti-CII levels in baseline sera. Data were obtained from 8 time points from time of diagnosis (baseline) up to five years concerning C-Reactive protein (CRP), erythrocyte sedimentation rate (ESR), tender joint count (TJC), swollen joint count (SJC), disease activity score28 (DAS28), DAS28 based on CRP instead of ESR (DAS28CRP), pain visual analog scale (VAS), global visual analog scale (VAS) and health assessment questionnaire (HAQ) (182, 183). Clinical data were available for 768 (99.4%) patients at baseline, 663 (85.8%) at 3 months, 627 (81.1%) at 6 months, 725 (93.8%) at 1 year, 669 (86.6%) at 2 years, 426 (55.1%) at 3 years, 265 (34.3%) at 4 years and 480 (62.1%) at 5 years.

**HLA genotyping in EIRA and MyEIRA.**

EIRA involves the majority of newly diagnosed RA patients in Sweden, and represent a unique possibility to evaluate the occurrence of anti-CII antibodies in a genetic (primarily HLA-DRB1*, but also GWAS data) and environmental (primarily smoking) context. In EIRA genotyping was performed using polymerase chain reaction (PCR) and sequence specific primers (Ole-rup SSP, Saltsjöbaden, Sweden) (184).

The HLADRB1* alleles 0101/0401/0404/0405/0408/10 were defined as shared epitope (SE) as described previously (25, 185, 186). In the study of EIRA in paper III, association to individual HLA-DRB1* alleles was performed on the two-digit level and on the four-digit level for HLA-DRB1*04 alleles as not all *04 alleles belong to SE.

In MyEIRA genotyping was done on the HLA-DRB1* four digit level by polymerase chain reaction (PCR) using sequence specific primers by LAB-Type® SSO Class II DRB1 and LABType® HD Class II DRB1 (One Lambda Inc., CA, USA), with Luminex Multi-Analyte Profiling System (xMAP, Luminex Corporation, Texas, USA), according to the manufacturer's instructions (187). Among the HLA-DRB1* alleles, the HLA-DRB1 shared epitope (SE) alleles group was defined by HLA-DRB1*01:01, *01:02, *01:07, *04:01, *04:04, *04:05, *04:08, *04:10, *10:01, and *10:03. More than 95% of the HLA-DRB1*10 subtypes were HLA-DRB1*10:01(188).
Statistical analyses:

Non-parametric statistics were used in most of the studies in papers I and II. Groups were compared using the Mann-Whitney U test, and paired investigations were performed using Wilcoxon signed-rank test. Clinical correlations studies were done using Spearman’s rank correlation test. In studies of changes in CD marker mean fluorescence intensities (MFI) in paper I, the response from the corresponding control wells were subtracted from the anti-CII wells. In paper I the functional responses from the cell stimulation experiments were associated with the clinical symptoms using the 85th percentile of changes in CD markers, which was found to be the most appropriate discriminatory cut off in preliminary calculations.

In papers III and paper IV, two-way ANOVA was used to compare the clinical association to the occurrence of anti-CII and anti-CCP2, and to study the relation between anti-CII levels and the two alleles HLA-DRB1*01 and HLA-DRB1*03. Chi-square was used to find the co-occurrence of autoantibodies. Stepwise regression analysis (backward and forward) was used to find the HLA association to autoantibodies. All calculations were performed using Graphpad prism 6 and JMP softwares.
Results and Discussion

Results papers I and II

PMN stimulated with surface bound anti-CII IC upregulated CD11b, CD66b and downregulated CD16 and CD32 compared to PMN controls. When comparisons were made between PMN reactivity and PBMC reactivity it was found that PMN required higher anti-CII levels for activation. Undiluted sera were used to stimulate PMN whereas sera were diluted 1:10 for PBMC stimulations in accordance with previous studies in the group. When functional potential of each IC (anti-CII IC and plate-bound IgG) was compared at different concentrations of IgG in the surface-bound IC, the responses from anti-CII IC were higher than from plate-bound IgG at similar IgG concentrations, both concerning PBMC TNF-α production, as well as PMN upregulation of CD66b and downregulation of CD16. Isolated PMN cultures never produced measurable levels of any cytokines/chemokines, irrespective of stimuli.

In a separate study baseline sera from 72 RA patients who were clinically well characterized were used to stimulate PMN. In parallel we also investigated PBMC production of TNF-α after stimulation with identical anti-CII IC. Among the 72 RA patients 24 were anti-CII positive and 48 were anti-CII negative (24 RF positive and 24 RF negative). PMN CD markers CD16 and CD66b were chosen as they were found to react in a more sensitive way to IC stimulation than did CD11b and CD32. The anti-CII IC-induced CD marker changes correlated with anti-CII levels among the 72 RA patients. Both PMN and PBMC functional responses were associated with laboratory signs of inflammation (CRP and ESR) at the time of diagnosis but not later, as shown for anti-CII antibodies in previous studies from the group (66, 69, 72). Especially intriguing was that anti-CII IC-induced changes in PMN surface expression of CD16 and CD66b were related to joint erosions, which was not the case for PMBC TNF-α production stimulated by the same anti-CII IC. MPO levels produced by PMN correlated to anti-CII levels, but supernatant MPO levels were not associated with any clinical variables. Occurrence of RF and anti-CCP associated with joint erosions late during the follow-up period in agreement with previous studies (73).
Table 3. Adapted from paper I. Association between *in vitro* granulocyte responses, PBMC responses stimulated with anti-CII IC and baseline autoantibody levels with baseline inflammatory markers, and Larsen score and changes in Larsen score during the first 2 years after RA diagnosis. Larsen score was performed as described in previous studies (72).

<table>
<thead>
<tr>
<th></th>
<th>Baseline CRP</th>
<th>Baseline ESR</th>
<th>Baseline Larsen score</th>
<th>1 year Larsen score</th>
<th>2 years Larsen score</th>
<th>Δ Larsen score 1-0 years</th>
<th>Δ Larsen score 2-0 years</th>
<th>Δ Larsen score 2-1 years</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD16 (PMN)</td>
<td>35/16.5 (0.08)</td>
<td>35.5/19.5 (0.26)</td>
<td>14.4/5.5 (0.024)</td>
<td>22.5/11.0 (0.034)</td>
<td>24.0/31.1 (0.046)</td>
<td>7.3/3.0 (0.08)</td>
<td>9.0/6.3 (0.88)</td>
<td>3.0/2.3 (0.27)</td>
</tr>
<tr>
<td>CD66b (PMN)</td>
<td>49/14 (0.004)</td>
<td>33/19 (0.063)</td>
<td>13.5/6.0 (0.15)</td>
<td>22.0/11.0 (0.071)</td>
<td>24.0/13.2 (0.059)</td>
<td>7.5/2.6 (0.017)</td>
<td>10.4/5.2 (0.016)</td>
<td>5.1/2.3 (0.012)</td>
</tr>
<tr>
<td>MPO (PMN)</td>
<td>14/18 (0.6)</td>
<td>19/21 (0.61)</td>
<td>5.4/7.0 (0.69)</td>
<td>17.0/11.0 (0.99)</td>
<td>17.7/13.2 (0.97)</td>
<td>6.9/2.8 (0.27)</td>
<td>9.6/5.3 (0.41)</td>
<td>2.5/2.3 (0.37)</td>
</tr>
<tr>
<td>TNF-a (PBMC)</td>
<td>28/17 (0.18)</td>
<td>43/19 (0.049)</td>
<td>9.3/6.3 (0.57)</td>
<td>11.6/11.8 (0.75)</td>
<td>15.3/13.2 (0.52)</td>
<td>5.6/3.0 (0.50)</td>
<td>9.4/5.3 (0.43)</td>
<td>2.9/2.3 (0.48)</td>
</tr>
<tr>
<td>Anti-CII</td>
<td>36.5/14 (0.012)</td>
<td>29/19 (0.10)</td>
<td>11.5/6.3 (0.75)</td>
<td>16.0/11.0 (0.44)</td>
<td>15.0/13.5 (0.44)</td>
<td>4.3/3.6 (0.21)</td>
<td>8.1/5.3 (0.22)</td>
<td>2.6/2.3 (0.29)</td>
</tr>
<tr>
<td>Anti-CCP</td>
<td>15/18.5 (0.20)</td>
<td>23/20 (0.57)</td>
<td>6.8/7.0 (0.76)</td>
<td>13.0/10.5 (0.76)</td>
<td>15.8/12.0 (0.42)</td>
<td>4.6/2.5 (0.41)</td>
<td>8.3/5.3 (0.12)</td>
<td>3.0/1.5 (0.024)</td>
</tr>
<tr>
<td>RF</td>
<td>15/18 (0.65)</td>
<td>23/20 (0.66)</td>
<td>6.0/7.9 (0.17)</td>
<td>11.0/12.0 (0.52)</td>
<td>14.0/13.5 (0.64)</td>
<td>4.4/3.6 (0.71)</td>
<td>6.3/7.0 (0.44)</td>
<td>3.0/1.8 (0.049)</td>
</tr>
</tbody>
</table>
Chemokines and cytokines are crucial players in mediating inflammation. As PMN alone did not produce detectable levels of cytokines or chemokines, PMN were cocultured with PBMC and stimulated with anti-CII IC to see if PMN could alter anti-CII IC-induced PBMC cytokine and chemokine production. When PMN were added to PBMC in anti-CII IC-stimulated cultures they synergistically augmented supernatant levels of the chemokine CXCL8 but downregulated supernatant levels of TNF-α. When other chemokines and cytokines were measured after stimulation with identical anti-CII IC, the production of RANTES and MCP-1 was also increased in cocultures in parallel to CXCL8. This upregulation was unique to chemokines, as the cytokines TNF-α, GM-CSF and IL-1β were downregulated in cocultures as compared to in isolated PBMC cultures. For subsequent mechanistic studies CXCL8 and TNF-α were chosen among other chemokines and cytokines listed above. Blocking studies showed the coculture-associated enhancement of CXCL8 in anti-CII IC-stimulated cocultures to be totally dependent on TLR4 and partly dependent on functional PMN enzymes. The anti-TLR4-mediated cytokine suppression was unique to anti-CII IC-stimulated cell cultures, as anti-TLR4 did not suppress cytokine production in plate-bound IgG-stimulated cell cultures. As opposed to CXCL8, TNF-α levels were downregulated compared to PBMC in all coculture system, irrespective of stimuli. Endotoxin measurement revealed very low levels in the CII preparation, close to what is the approved level in dialysis fluids and hence endotoxin contamination is an unlikely reason for chemokine upregulation in anti-CII IC-stimulated cocultures. Addition of LPS either on a CII surface or on a human serum albumin-coated surface, could also not repeat the CXCL8-enhancing effect of anti-CII IC on PMN+PBMC cocultures. We also tried to mimic the anti-CII-associated effect by adding gradually increased levels of CII to surfaces pre-coated with irrelevant IgG, but the only effect was that increasing CII concentrations gradually blocked the Fc-mediated cytokine responses, without any CXCL8 enhancements in cocultures.

The enhancement of CXCL8 levels in anti-CII IC-stimulated cocultures was also at least partly mediated via GM-CSF and FcγR. Anti-GM-CSF reduced the fold increase in anti-CII IC cocultures when compared to control antibody cocultures but did not totally abolish the increase in CXCL8 production.

Discussions papers I and II

In paper I we found that anti-CII IC-induced higher TNF-α levels from PBMC than did plate-bound IgG. There are a number of conceivable causes for this discrepancy. A close proximity binding of antibodies on major epitopes on the CII surface could affect the functional response of the cells.
Fc glycosylation of the antibodies could also affect the binding affinity to Fc receptors and affect the functional response (189-192).

Only the PMN reactivity towards anti-CII IC was related to joint erosions; measurement of PBMC-derived TNF-α against identical anti-CII IC did not show such an association to joint erosions. These findings argue that PMN might be more important than PBMC in anti-CII IC mediated pathogenesis in RA, in agreement with animal studies (193, 194).

We found downregulation of TNF-α and other cytokines in all cocultures in paper II irrespective of cell culture system. This could be due to degradation of monocyte-derived cytokines by PMN enzymes, as has been previously shown for the action of elastase and cathepsin G on TNF-α and IFN-γ (101, 195, 196). But our own data do not favor this hypothesis as the down-regulation of TNF-α levels in anti-CII IC-stimulated cocultures were unchanged after addition of an elastase inhibitor. To our knowledge, there are no reports on degradation of chemokines by PMN enzymes. It is therefore very intriguing that the enhancement of CXCL8 in anti-CII IC-stimulated coculture supernatants is abrogated by addition of PMN enzyme inhibitors. Neutrophil elastase has however been show to upregulate CXCL8 via TLR4, and the elastase-induced CXCL8-production could be blocked with an anti-TLR4 antibody (103). We hypothesize that similar functions might be applicable for cathepsin S/L and MPO for which enzyme inhibition almost reversed the anti-CII IC-induced CXCL8 enhancement in cocultures.

When increasing concentrations of CII were used to coat wells previously coated with human IgG, cytokine responses to IgG gradually declined, but we could not reproduce the anti-CII IC-induced augmentation of CXCL8 in PBMC+PMN cocultures. We therefore think that CII and/or antibodies against CII from RA patients contain a hitherto unknown endogenous ligand for TLR4.

GM-CSF-stimulated cocultures incubated on HSA or CII surfaces yielded increased CXCL8 levels compared to PBMC cultures, whereas TNF-α levels were generally increased but with maintained downregulation in cocultures. Thus the effect of GM-CSF mimicked the effect of anti-CII IC on PBMC+PMN cocultures, and we hypothesized that the CXCL8 augmentation in anti-CII IC-stimulated co-cultures might be mediated via GM-CSF. Subsequent GM-CSF neutralization experiments lowered the CXCL8 enhancement in anti-CII IC-stimulated cocultures, but did not totally abolish the increase in CXCL8 production in cocultures as compared to in PBMC cultures. However, when data were expressed as fold change between cocultures and PBMC cultures stimulated with anti-CII IC, GM-CSF neutralization significantly diminished CXCL8 production but not TNF production. Collectively these findings argue that the enhancement of CXCL8 levels in anti-CII IC-stimulated cocultures might be at least partly mediated via GM-CSF.
Anti-CII antibodies are produced by B cells in synovial tissue and joint fluid of RA patients, but not detected to comparative levels in the blood (197, 198). It is therefore probable that locally produced anti-CII antibodies are almost directly bound to exposed CII epitopes in joint cartilage, and that anti-CII levels in serum represent the excess of anti-CII not directly binding to CII but accessing the circulation. The previous study that showed local anti-CII production by B cells from almost all (12/13) synovial tissue specimens could not be reflected by elevated anti-CII levels in serum in any of the investigated RA patients (197). We therefore believe that our in vitro model represent a mechanism of action that can be of significance in far more patients than in the very small group of patients with very high levels of serum anti-CII (72). As anti-CII antibodies are at high levels at time of diagnosis and disappear or get considerably lower after 6-12 months, the mechanism of anti-CII IC-induced chemokine upregulation via TLR4 in cocultures might represent a mechanism sustaining the acute onset inflammatory phenotype in early anti-CII positive RA patients (figure 5).

![Figure 5](image_url)

*Figure 5. Adopted from paper II. Schematic figure showing a proposed model of how anti-CII IC induced PBMC and PMN interact to augment chemokines through TLR4.*

**Results paper III and paper IV**

Among 1476 EIRA patients, 97 (6.6%) were anti-CII positive, and 855 (57.9%) were anti-CCP2 positive. Thirty-nine patients (2.6%) had only anti-CII, 797 (54%) had only anti-CCP2, 58 (3.9%) were double positive and 582 (39.4%) lacked both antibodies. Among the EIRA controls 15/926 (1.6%) were anti-CII positive (34 showed non-specific binding) and 16/958 (1.7%) were anti-CCP2 positive. The occurrence of anti-CII was higher among RA
patients than among controls (p<0.0001). Anti-CII levels were significantly higher among RA patients than among EIRA controls (median [mean] 13.3[38.4] vs. 9.3[21.6] AU/ml, p<0.0001). There was no association between the occurrence of anti-CII and anti-CCP2 among the RA patients (p=0.7). When clinical parameters were compared with occurrence of anti-CII and anti-CCP2, anti-CII at the time of diagnosis associated with elevated CRP, ESR, SJC, DAS28 and DAS28CRP at diagnosis and up to six month but not later, whereas anti-CCP2 associated with SJC and DAS28 from 6 months and up to 5 years, but not earlier. The 773 patients with available clinical follow-up data where then divided into patients expressing only anti-CII (n=20), only anti-CCP2 (n=432) or both (n=36), and each group was compared to double negative patients (n=285). Patients expressing only anti-CII or only anti-CCP mirrored the phenotypes described above. The anti-CII-associated phenotype was stronger than the ACPA phenotype, and predominated in anti-CII/anti-CCP2 double positive patients.

The changes in clinical and laboratory measures were calculated after subtraction from baseline values. When these changes were compared with the occurrence of anti-CII and anti-CCP2 in baseline sera, anti-CII was associated with improvements in CRP, ESR, SJC, TJC and DAS, whereas anti-CCP2 was associated with deteriorations in SJC and DAS28 over time.

HLA analysis in EIRA showed that anti-CII occurrence was positively associated with HLADRB1*03 and negatively associated with HLADRB1*04, but the latter negative association disappeared when CCP2 positives were excluded. When the anti-CII positivity cutoff was raised to 200AU/ml, anti-CII associations with HLADRB1*03 became more clear and we also found that HLADRB1*01 was associated with anti-CII, although the negative HLADRB1*04 association was lost. Using a backward stepwise regression model and starting with all 13 HLA-DRB1* two digit alleles we confirmed that anti-CII were associated with both HLADRB1*03 and HLADRB1*01 but not with any other HLA-DRB1* alleles. We then performed a two-way ANOVA to see whether there was any interaction between the two HLA-DRB1* alleles. This analysis confirmed a strong individual association between anti-CII levels and each of the HLA-DRB1*01 and HLA-DRB1*03 alleles (p<0.0001 for both), but also a highly significant interaction (p<0.0001). Mean anti-CII levels in HLA-DRB1*01/03 double negative patients (n=818) were 21.1AU/ml, 77.7AU/ml for individuals only positive for HLA-DRB1*01 (n=338) and 38.6 AU/ml for individuals only positive for HLA-DRB1*03 (n=268). The statistically significant interaction was manifest as strikingly increased anti-CII levels in patients with both HLA-DRB1*01 and HLA-DRB1*03 (n=52); mean 330.1 AU/ml.

In MyEIRA the anti-CII levels were higher in RA patients when compared to all Malaysian healthy controls and also when investigated individually for each of the three different ethnicities. Among the 1,105 RA patients, 106 (9.6%) were anti-CII positive as compared to 705 (63.8%) who were
anti-CCP2 positive. Among the controls 75/1565 (4.8%) were anti-CII positive, after exclusion of 4 controls showing non-specific binding. Anti-CII alone, without concomitant anti-CCP2, was found in 44 RA patients (4.0%), and anti-CCP2 alone in 643 (58.2%). Double antibody positivity (anti-CII/anti-CCP2+) was found in 62 patients (5.6%), while 356 (32.2%) had none of the antibodies. IgG RF was found in 567 (51.3%) and IgM RF in 562 (50.9%) of the RA patients. Anti-CII positive RA patients had higher baseline CRP than did anti-CII negative patients. Anti-CCP, IgG RF and IgM RF were also associated with elevated CRP at baseline. The occurrence of anti-CII did not associate with the occurrence of anti-CCP, IgG RF and IgM RF, whereas these other three autoantibodies showed a larger degree of co-occurrence, and anti-CII was independently associated to elevated CRP as compared to other antibodies. When patients with only one of the antibodies were compared to antibody negative patients, only anti-CII associated to elevated CRP. Hence anti-CII is an independent marker associated with an early inflammatory RA phenotype not only in Caucasian, but also in an Asian RA population.

Genetic association studies in MyEIRA showed that RA patients with the HLA-DRB1*12:01 allele (n=218) had higher anti-CII levels when compared to RA patients without HLA-DRB1*12:01 allele (n=887; 14.7 vs. 12.1 AU/ml; p=0.0005, or 0.01 after correction for multiple comparisons). Conversely, HLA-DRB1*12:02 was negatively associated with anti-CII antibodies in the 73 RA patients with the genotype (9.9 vs. 12.6 AU/ml; p=0.0030, or 0.06 after correction).

Discussion papers III and IV

We found anti-CII in 6.6% in the Swedish RA patients and 9.6% in the Malaysian RA patients; figures rather close to the 8.8% reported previously from our group in Swedish RA patients.(66) This low diagnostic sensitivity argues against using anti-CII as a tool for RA diagnosis. We are aware that these rather low figures are in disagreement both with a number of early studies on reactivity against the full CII molecule (56, 60), as well as more recent studies showing up to 77%-88% using CII fragments and synthetic CII peptides (63-65).

We have excluded a high number of patients (n=316 in EIRA cohort) from our analysis considering that these sera reacted more strongly to a BSA than to a CII surface that had been blocked with BSA. When these sera were investigated in a clinical context they showed reactivity between the negative and true anti-CII positive patients, with clinical associations closest to the anti-CII negative group. Patients showing non-specific binding did also not show any HLA associations. Collectively the results from both these investigations argue that patients with non-specific binding should not be
regarded as anti-CII positive. Non-specific binding can thus obscure the group with true anti-CII reactivity, and if the 316 patients showing non-specific binding had not been excluded, a much higher percentage of anti-CII positives would be reported in EIRA: (97+316)/(1476+316) or 23%. Given the strength of the clinical and genetic associations reported in paper III, probably many of these associations would still be there on the group level if patients showing non-specific binding were included as anti-CII positive. But on the individual patient level, such handling of data would misdiagnose most of the allegedly anti-CII positive RA patients.

Using the same anti-CII ELISA, our group has also recently described that almost all (93%) patients with active visceral leishmaniasis from Sudan showed non-specific binding, whereas the same kind of non-specific reactivity was uncommon among healthy Sudanese controls (2%) (199). Thus this kind of background reactivity might interfere with ELISA measurement in different inflammatory diseases, and might constitute a sizeable and not previously acknowledged source of error in inflammatory diseases including RA.

As anti-CCP2 occurrence was positively associated with SE/HLADRBI*04 and negatively associated with HLADRBI*03 in contrast to anti-CII occurrence, and as anti-CII associated with early and anti-CCP2 with late appearing measures of inflammations, our data argue that these two antibodies associate not only with different and partly opposite phenotypes as shown previously in a smaller cohort, (66, 73) but also have different genotypes that show opposite associations to HLA-DRB1*03. Opposite associations were also seen for smoking. Ever smoking was associated with elevated anti-CCP2 levels (median ever smokers 159 AU/ml vs. never smokers 18.71 AU/ml; p=<0.0001) whereas smokers had lower anti-CII levels than non-smokers. However there was only a modest difference in anti-CII levels (median ever smokers 15.2 AU/ml compared to median never smokers 16.5 AU/ml; p=0.0264). Thus anti-CII seropositive RA patients have a distinct phenotype and HLA-DRB1* genotype, and modest negative association to smoking. Collectively these findings in many respects represent the converse to the clinical, genetic and smoking associations described for ACPA. Also in the Malaysian study we showed that anti-CII associated with elevated CRP at the time of RA diagnosis, and that this association was stronger than for the conventionally RA associated antibodies anti-CCP2, IgM RF and IgG RF.

The EIRA study in paper III shows that although anti-CII positive RA is a minor group as compared to the ACPA/anti-CCP2 positive group, the anti-CII associated phenotype is so profound that also the diminutive group of patients single positive for anti-CII (2.2%; 20/773) show a strong active onset anti-CII phenotype as compared to antibody negative patients, and in patients with both autoantibodies, the early onset anti-CII-associated phenotype predominates over the anti-CCP2-associated phenotype characterized
by elevated clinical and laboratory measures of activity from 6 months and onwards.

As compared to antibody negative patients, anti-CII was associated with improvements in CRP, ESR, SJC, TJC and DAS, whereas anti-CCP2 was associated with deteriorations in SJC and DAS28 over time. As we previously have shown anti-CII to be a functionally active antibody in RA, we think that the anti-CII phenotype involves a self-limiting part that is associated with declining anti-CII levels during the months after diagnosis.(66) This indicates that anti-CII detection together with ACPA detection might serve as prognostic tool where the detection of ACPA could argue for more aggressive treatment, and the detection of anti-CII could be an argument for less aggressive treatment, as compared to antibody negative patients. Hence anti-CII positive RA might represent a phenotype with good prognosis, in relation to the rather high degree of inflammation that these patients show around the time of RA diagnosis.

In EIRA, we reported anti-CII associate both with HLA-DRB1*01 and HLA-DRB1*03. Whereas the association with HLA-DRB1*03 has been reported also by others, (200-202) the association with HLA-DRB1*01 has not been described previously. This is remarkable as the DRB1*01 single positive RA patients had mean anti-CII levels twice as high as the HLA-DRB1*03 single positive RA patients, and as both HLA types were roughly equally common in EIRA. Both HLA-DRB1*01 and HLA-DRB1*03 are however rare in Malaysia, and only 4 MyEIRA patients co-expressed HLA-DRB1*01 and HLA-DRB1*03, although two of them had clearly elevated anti-CII levels (215 and 1254 AU/ml, respectively).

The only positive HLA association we found in MyEIRA was to HLADRB1*1201. Such an association between anti-CII and HLADRB1*1201 has previously been described in a Korean cohort with Meniere’s disease, another disease that has been associated to anti-CII antibodies (54). Hence HLADRB1*1201 may be associated with humoral anti-CII immunity in Asia.

Although both the anti-CII associated clinical phenotype and the positive HLA-DRB1*03 association represent the counterpart to ACPA, the occurrence of the two antibodies was not statistically inversely related in EIRA as previously described in a smaller cohort by our group and concerning anti-CII and RF by Ferriss et al (66, 203).
Conclusions

In my thesis I have investigated the role of PMN in acute onset anti-CII positive RA patients. PMN responses to anti-CII IC associate with the clinical outcome of RA patients, especially with the degree and rate of change of joint destruction during two years after diagnosis. PBMC and PMN cocultures stimulated with anti-CII IC show enhanced production of chemokines. These findings are limited to stimulation with anti-CII IC, thus depending on IC consisting of an autoantigen and autoantibodies both mainly found in RA joints, and where anti-CII levels peak around the time of RA onset. The mechanism therefore seems to be strictly joint-specific and associated to events happening in RA patients around the time of diagnosis. This argues that the PMN/chemokine pathway is probably most important in early RA when anti-CII levels peak. In two studies on clinically well-characterized RA cohorts from Sweden and Malaysia I have replicated and extended findings showing that anti-CII positive RA is associated with an acute onset rheumatoid arthritis phenotype. Swedish data show that elevated levels of anti-CII at the time of diagnosis prognosticates lowered degree of inflammation during five years follow-up. Malaysian RA patients which are anti-CII positive around the time of diagnosis also have elevated CRP levels, thus arguing that the anti-CII-associated RA phenotype is found in different ethnic RA populations.

My studies may implicate that more focus should be placed on the role of the joint-specific protein CII, both as an autoantigen for IC formation and as an endogenous TLR ligand. It also emphasizes the role of PMN-associated chemokine responses during the early phases of RA, and suggests ways to block the accumulation of inflammatory cells to joints early in the disease process, especially in patients with elevated levels of anti-CII.

In summary, anti-CII antibodies are probably pathogenic in humans, and anti-CII positive RA represents a distinct RA phenotype characterized by elevated clinical and laboratory measures of disease activity and inflammatory activity at early stages, but with good prognosis. This is in contrast to ACPA-positive RA, associated with worse prognosis as compared to autoantibody-negative patients. Anti-CII is detected in a rather low percentage among RA patients, and levels soon fall after diagnosis. Detection of anti-CII is therefore probably not diagnostically useful in RA. Early detection of anti-CII in patients recently diagnosed with RA may have a vital role as a prognostic marker, however, especially when combined with a conventional ACPA test.
Future Perspectives

I have thus far defined a possible mechanism explaining part of the anti-CII associated acute inflammatory phenotype in RA. The major findings were that PMN were crucial effector cells as they enhance PBMC-stimulated cytokine production through a mechanism dependent on TLR4. Anti-CII positive RA also represents an acute onset RA phenotype with elevated levels of the acute phase reactant CRP in Caucasian and Asian RA populations. As compared to the time of diagnosis, anti-CII detected around the time of RA diagnosis prognosticates lower degree of inflammation during five years of follow-up.

I would like to extend these studies in the following ways:

1. TLRs play a crucial role in autoimmune diseases. Anti-TLR4 has diminished the anti-CII IC-induced production of pro-inflammatory cytokines (Paper II). More evidence has been reported for the presence of TLR ligands in inflamed RA synovium and TLR2, TLR7 and TLR8 have been shown to have a role in RA (204, 205). We have shown TLR4 is important in anti-CII positive RA in diminishing chemokine response. We aim to investigate more TLR mediated mechanisms involved in the anti-CII-dependent pathogenesis of early RA.

2. Previous studies have shown that CII has a modulatory role in cytokine production as T cells incubated with CII when cocultured with synovial fibroblasts enhanced cytokine production (172). In paper I we observed that PMN cultured on a CII surface had a higher expression of FcγRIII compared to PMN cultured on a human serum albumin-coated surface. These findings implicate that CII per se might regulate cell surface receptors and activation markers. FcγRIIa is an important activation receptor found on human macrophages and PMN for pro-inflammatory cytokine production (206). Our group has previously shown that blocking FcγRIIa diminished pro-inflammatory cytokine production from monocytes (7, 207). We therefore aim to study the role of collagen II in the context of FcR and cytokine regulation with individual PMN cell cultures and PMN cocultures with T cells or macrophages. We intend to study TLRs and Fc receptors suggested to be involved in RA pathogene-
sis after incubation with human CII and thereafter stimulating with IC. To examine if the results obtained from the above mentioned study are restricted to CII, similar studies will be conducted with antigens with triple helical structure similar to CII, like native collagen I, and also with denatured CII and denatured collagen I.

3. In paper III we have shown that the anti-CII-associated RA phenotype represents the opposite to the anti-CCP2-associated phenotype in many respects including associations to HLA-DRB1*, clinical associations and smoking. We now would like to focus on treatment response associated with anti-CII. In paper III, patients were included from the early EIRA 1 cohort between 1996 and 2001, thus before the launch of many modern biologic anti-rheumatic therapies. I will now also analyze anti-CII in the more recent EIRA 2 cohort in which many patients have been treated with biologics targeting TNF, IL-6, B lymphocytes and T-cell activation. The study will be done in collaboration with Saedis Saevarsdottir at Karolinska Institutet (KI).

4. Until now, my genetic association studies have been confined to HLA-DRB1*. Now we aim to investigate what SNPs might be associated with anti-CII positive RA. Previous studies, including those our group, have reported that the occurrence of anti-CII and anti-CCP or RF are inversely related (66, 203). My own findings presented in this thesis also show that anti-CII and ACPA have contrasting associations to HLA-DRB1*, clinical phenotype and associations to smoking. Hence we primarily aim to look for SNPs associated with RA in the group that are seronegative for anti-CCP and RF. The study will be done in collaboration with Leonid Paduykov at KI.

5. As anti-CII is associated with acute onset clinical symptoms in RA, we asked ourselves whether anti-CII might not be an RA specific marker, but instead a marker for acute onset arthritis in general. To investigate this, we have collaborated with René Toes in Leiden and Karim Raza in Birmingham to investigate sera from patients with other diagnoses with an acute onset arthritis phenotype. We have studied patients with acute onset gouty arthritis, pseudogout, reactive arthritis, remitting seronegative symmetrical synovitis with pitting edema and compared them to the previously investigated RA cohort. A manuscript is in preparation based on the results showing that anti-CII is not generally increased in these other diagnoses. Instead the outlier group with very high anti-CII levels is only found only in RA, as shown in figure 6.
Figure 6. Level of anti-CII antibodies from other arthritides in comparison to RA. The dotted line represent the cutoff based on 95\textsuperscript{th} percentile over controls (n=100).
Acknowledgements

My journey as a PhD student in Uppsala would have not been possible with timely help and support from many people in my life. I would like to take this opportunity to thank the people involved during my PhD studies.

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