Anti-Collagen Type II Autoantibodies in an Acute Phenotype of Early Rheumatoid Arthritis

MOHAMMED MULLAZEHI
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Abstract

Rheumatoid arthritis (RA) is an autoimmune disease with systemic inflammatory features that primarily affects small peripheral joints. Type II collagen (CII), is the most abundant collagen type in joint cartilage. Antibodies against CII (anti-CII) are found in a subpopulation of RA patients. Anti-CII can form surface-bound immune complexes (IC) in inflamed joints, which might intensify joint inflammation and destruction. In this thesis I have studied the functional effects of surface-bound anti-CII–containing IC in vitro and correlated the results to clinical parameters.

Anti-CII IC induced TNF-α, IL-1β and IL-8 production from monocytes via FcyRIIa. Anti-CII levels were dichotomously distributed in RA patients where a small outlier group (3.3%) with very high anti-CII levels showed in vitro induction of pro-inflammatory cytokines by anti-CII-containing IC. These patients also had a distinct phenotype with elevated laboratory signs of inflammation and increased radiological erosions at the time of diagnosis.

In another in vitro model, co-cultured macrophages and RA synovial fibroblasts stimulated with anti-CII IC induced the production of matrix metalloproteinases (MMP)-1 and MMP-8, enzymes responsible for the initial cleavage of CII during cartilage degradation. This was mediated via production of TNF-α and IL-1β, and especially anti-CII IC-induced IL-1β supported the production of MMP-1. The presence of anti-CII antibodies in patients with early synovitis was not predictive for future RA development.

In summary, I have shown how anti-CII-containing IC may explain part of the early pathogenesis and can define a distinct clinical phenotype in RA patients with high levels of anti-CII.

Keywords: Rheumatoid arthritis, antibodies, autoantibodies, collagen type II, anti-collagen type II antibodies, immune complexes, cytokines, inflammation, erosion

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To my parents
List of Papers

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


III Mullazehi M, Wick MC, Klareskog L, van Vollenhoven R, Rönnelid J. Anti-type II collagen antibodies are associated with early erosions in rheumatoid arthritis. Manuscript


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<tr>
<td>Ab</td>
<td>Antibody</td>
</tr>
<tr>
<td>ACPA</td>
<td>Anti-Citrullinated Protein/peptide Antibodies</td>
</tr>
<tr>
<td>ACR</td>
<td>American College of Rheumatology</td>
</tr>
<tr>
<td>ADAM</td>
<td>A Disintegrin And Metalloproteinase</td>
</tr>
<tr>
<td>ADAMTS</td>
<td>A Disintegrin And Metalloproteinase with ThromboSpondin motifs</td>
</tr>
<tr>
<td>ADCC</td>
<td>Antibody-dependent cell-mediated cytotoxicity</td>
</tr>
<tr>
<td>Ag</td>
<td>Antigen</td>
</tr>
<tr>
<td>Anti-CII</td>
<td>Antibodies to Collagen type II</td>
</tr>
<tr>
<td>Anti-CCP</td>
<td>Antibodies to Cyclic Cirullinated Peptide</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen Presenting Cell</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>CIA</td>
<td>Collagen-Induced Arthritis</td>
</tr>
<tr>
<td>CAIA</td>
<td>Collagen-Antibody Induced Arthritis</td>
</tr>
<tr>
<td>CI</td>
<td>Collagen type I</td>
</tr>
<tr>
<td>CII</td>
<td>Collagen type II</td>
</tr>
<tr>
<td>CIII</td>
<td>Collagen type III</td>
</tr>
<tr>
<td>CIX</td>
<td>Collagen type IX</td>
</tr>
<tr>
<td>CXI</td>
<td>Collagen type XI</td>
</tr>
<tr>
<td>COMP</td>
<td>Cartilage Oligomeric Matrix Protein</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic Cells</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular Matrix</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-Linked ImmunoSorbent Assay</td>
</tr>
<tr>
<td>ESR</td>
<td>Erythrocyte Sedimentation Rate</td>
</tr>
<tr>
<td>FACIT</td>
<td>Fibril-Associated Collagens with Interrupted Triple Helices</td>
</tr>
<tr>
<td>FcR</td>
<td>Fc Receptor</td>
</tr>
<tr>
<td>FcγR</td>
<td>Fc gamma Receptor</td>
</tr>
<tr>
<td>FcγRIIa</td>
<td>Fc gamma Receptor IIa</td>
</tr>
<tr>
<td>FcγRIIb</td>
<td>Fc gamma Receptor IIb</td>
</tr>
<tr>
<td>FcγRIIIa</td>
<td>Fc gamma Receptor IIIa</td>
</tr>
<tr>
<td>FcγRIIib</td>
<td>Fc gamma Receptor IIIb</td>
</tr>
<tr>
<td>FcγRIV</td>
<td>Fc gamma Receptor IV</td>
</tr>
<tr>
<td>FCS</td>
<td>Fetal Calf Serum</td>
</tr>
<tr>
<td>FGF</td>
<td>Fibroblast Growth Factor</td>
</tr>
<tr>
<td>HLA</td>
<td>Human Leukocyte Antigen</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>HSA</td>
<td>Human Serum Albumin</td>
</tr>
<tr>
<td>IC</td>
<td>Immune Complexes</td>
</tr>
<tr>
<td>ICA</td>
<td>Immune Complex-mediated Arthritis</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Interferon-gamma</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>ITAM</td>
<td>Immunoreceptor Tyrosine-based Activation Motif</td>
</tr>
<tr>
<td>ITIM</td>
<td>Immunoreceptor Tyrosine-based Inhibitory Motif</td>
</tr>
<tr>
<td>JRA</td>
<td>Juvenile Rheumatoid Arthritis</td>
</tr>
<tr>
<td>kDa</td>
<td>kiloDalton (measurement unit)</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>mAb</td>
<td>monoclonal Antibodies</td>
</tr>
<tr>
<td>MAC</td>
<td>Membrane-Attack Complex</td>
</tr>
<tr>
<td>MBL</td>
<td>Mannose-Binding Lectin</td>
</tr>
<tr>
<td>MCP</td>
<td>Metacarpophalangeal (joints)</td>
</tr>
<tr>
<td>MHC</td>
<td>Major Histocompatibility Complex</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloproteinases</td>
</tr>
<tr>
<td>MT-MMP</td>
<td>Membrane-type matrix metalloproteinases</td>
</tr>
<tr>
<td>NHS</td>
<td>Normal Human Serum</td>
</tr>
<tr>
<td>OD</td>
<td>Optical Density</td>
</tr>
<tr>
<td>PBMC</td>
<td>Peripheral Blood Mononuclear Cells</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffer Saline</td>
</tr>
<tr>
<td>PBS-Tween</td>
<td>Phosphate Buffer Saline with 0.02%Tween</td>
</tr>
<tr>
<td>PDGF</td>
<td>platelet-drive growth factor</td>
</tr>
<tr>
<td>PEST</td>
<td>Penicillin Streptomycin</td>
</tr>
<tr>
<td>PIP</td>
<td>Proximal Interphalangeal (joints)</td>
</tr>
<tr>
<td>PG</td>
<td>Prostaglandins</td>
</tr>
<tr>
<td>RA</td>
<td>Rheumatoid Arthritis</td>
</tr>
<tr>
<td>RASF</td>
<td>Rheumatoid Arthritis Synovial Fibroblast</td>
</tr>
<tr>
<td>RBC</td>
<td>Red Blood Cell</td>
</tr>
<tr>
<td>RF</td>
<td>Rheumatoid Factor</td>
</tr>
<tr>
<td>RPMI</td>
<td>Royal Park Memorial Institute (culture medium)</td>
</tr>
<tr>
<td>SLE</td>
<td>Systemic Lupus Erythematosus</td>
</tr>
<tr>
<td>TCR</td>
<td>T Cell Receptor</td>
</tr>
<tr>
<td>TMB</td>
<td>Tetramethylbenzidine</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming Growth Factor beta</td>
</tr>
<tr>
<td>TIMP</td>
<td>Tissue Inhibitors of Metalloproteinases</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumour Necrosis Factor alpha</td>
</tr>
</tbody>
</table>
Introduction

The immune system

From birth to death humans are exposed to a variety of microorganisms and other agents such as bacteria, toxins, viruses and other foreign substances that are potentially harmful to us and cause diseases. Without effective protective mechanisms each of us would soon succumb to infectious diseases. Humans and other living organisms have therefore developed protective mechanisms collectively called the immune system to defend themselves against pathogens.

Pathogenic microorganisms and substances that are recognized being foreign by the immune system and that induce immune responses are called antigens (Ag). Antibodies (Ab) or immunoglobulins (Ig) are glycoproteins produced in membrane-bound or secreted form from specific cells of the immune system (B cells and plasma cells) in response to antigens. Ig consists of two identical heavy chains and two identical light chains that recognize a particular site (epitope) of an antigen.

The immune system can be divided into two main components: the innate and the adaptive immune systems.

The innate immune system

Innate immunity refers to the mechanisms that an individual is born with, which exists before the actual immune activation. These mechanisms include anatomical (skin and mucous membrane) and physiological (temperature, low pH and chemical mediators). Phagocytic cells such as macrophages and neutrophil granulocytes also internalize, kill, and digest microorganisms. Innate defense mechanisms comprise the first line of host defense against invading pathogens. When such invaders escape the nonspecific host defense mechanisms or when nonspecific host defenses are not sufficient to protect against these invaders the acquired immune system takes over.

The acquired immune system

Specific or acquired immunity develops in response to e.g. microbial infection and is capable to recognize and selectively eliminate foreign microorganisms and antigens. This specific immune system does not function inde-
pendently of innate immunity but rather the two systems interplay and complement each other in such a way that a more effective total immune response is produced. Unlike innate immune responses, acquired immune responses are adaptive and exhibit four characteristic features: antigenic specificity, diversity, the capacity to remember an encountered antigen and respond much more strongly and quickly to a second exposure and the ability to distinguish self from non-self. Acquired immunity is divided into cell-mediated and antibody-mediated (humoral) immune responses.

**Cells of the immune system**

The specific immune system comprises two major groups of cells: lymphocytes and antigen presenting cells (APC). On the basis of function and cell membrane components lymphocytes are divided into the three major populations namely, B-lymphocytes, T-lymphocytes and natural killer (NK) cells. B- and T-lymphocytes are the key cells of the adaptive immune system with their own specific antigen receptors while NK cells are large and granular lymphocytes that are part of the innate immune system and normally do not possess cell surface markers characteristic for B- and T-cells. B-cells are produced in the red bone marrow during the process of hematopoiesis, and also mature in the bone marrow. They have the capacity to synthesize and express membrane-bound and soluble Ig.

When a naive B-cells interact with their specific antigen, the cells begins to divide rapidly and differentiate into memory B-cells and effector B-cells called plasma cells that have the ability to produce and secrete large amounts of soluble antibodies in response to encountered antigen during the immune response. Plasma cells are responsible for antibody-mediated immune responses. Antibody-mediated immunity is mostly effective against extracellular antigens such as bacteria present in the body fluids.

T-lymphocytes also originate from hematopoietic stem cells. Unlike B-cells, which complete their development into mature cells in the red bone marrow, T-cells mature in the thymus gland. During maturation of T-cells within the thymus, they come to express unique antigen-binding receptors on their membrane called the T-cell receptor (TCR) which can only recognize antigens associated with cell membrane proteins known as either class I or class II major histocompatibility complex (MHC) molecules.

There are at least three major distinct populations of T cells: CD4+ T cells often called T helper (T\(_h\)) cells, CD8+ T cells often called cytotoxic T (T\(_c\)) cells, and T regulatory (T\(_{reg}\)) cells. CD8+ T cells recognize antigen through MHC class I (i.e. they are MHC class I-restricted) and develop into mature cytotoxic cells that can directly attack infected cells or cancer cells. Conversely T\(_h\) cells are class II MHC-restricted. When a CD4+ cell encounters and interacts with an antigen-MHC II molecule complex, the cell is activated
and becomes either an effector cell that secretes various growth factor known collectively as cytokines, or differentiates into memory cells. T<sub>reg</sub> cells comprise a diverse group of lymphocytes with regulatory properties that control pathological immune responses and actively suppress activation of the immune system. T<sub>reg</sub> cells are identified by the presence of cell surface molecules among them CD4, CD25, and transcription factor Foxp3 (1). T<sub>reg</sub> cells directly suppress B-cell Ig response (2). There are three subtypes of T<sub>H</sub> cells namely, T<sub>H</sub>1, T<sub>H</sub>2 and T<sub>H</sub>17 subtypes. Through secreting different ranges of cytokines, T<sub>H</sub>1 cells broadly promote cellular immunity and T<sub>H</sub>2 cells activate B cells and promote antibody-mediated immunity. T<sub>H</sub>17 cells promote host defense against certain extracellular bacteria, fungi, protozoa and viruses. It is also believed that these cell types are important in development of autoimmunity (3). Antigen-presenting cells are specialized cells that include macrophages, B cells and dendritic cells that can process whole antigens and present peptide fragments of antigens associated with class II MHC molecules to T-cells.

**Monocytes and macrophages**

The total amount of monocytes in the bloodstream is 3-8% of the total white blood cells or about 300-600 cells/mm³. Monocytes in the blood and macrophages in the tissues build up a system known as the mononuclear phagocyte system, which is an important part of the non-specific immune system against invading pathogens. Peripheral blood monocytes are produced in the red bone marrow during hematopoiesis. Monocytes are a group of large round cells with a diameter of about 10 to 18 μm. They enter the bloodstream and circulate in the peripheral blood for about 8-72 hours. Monocytes leave the circulation and migrate into various tissues in response to stimuli and chemotactic factors such as complement factor 5a (C5a) or interleukin-8 (IL-8). These molecules are produced at inflammatory sites in response to inflammation. Under the influence of a variety of cytokines such as interferon gamma (IFN-γ), interleukin-1β (IL-1β) and tumor necrosis factor alpha (TNF-α) monocytes at the inflammatory site differentiate into tissue-specific activated macrophages. Macrophage stimulation and activation results in a number of changes and activities such as increased cell-size, increased phagocytic ability, increased production and secretion of proteolytic enzymes and increased secretion of a variety of inflammatory cytokines such as IL-1β and TNF-α. These cytokines have the ability to induce each other and they can also induce production of interleukin-8 (IL-8), a potent chemoattractant for neutrophil granulocytes. Monocytes and macrophages express various cell surface receptors e.g. complement receptors, cytokine receptors and Fc gamma receptor (FcγR). The latter can specifically recognize the constant region (Fc part) of IgG antibodies.
Monocytes and macrophages initiate phagocytosis, antibody-dependent cell mediated cytotoxicity (ADCC), and the release of proinflammatory mediators such as cytokines, reactive oxidants and proteases upon stimuli via FcγR. FcγR-mediated activation of these cells is an important link between Ab-mediated humoral immunity and cellular immunity (4).

The anti-microbial and cytotoxic activities of macrophages are due to the production of reactive oxygen and nitrogen radicals such as superoxide anion, hydroxyl radicals and nitric oxide.

**Antibodies**

Antibodies (Ab) also called immunoglobulins (Ig) are large glycoprotein molecules that are produced in vertebrates in response to foreign or autologous structures called antigens. Antibodies are produced by B-lymphocytes and plasma cells. Antibodies are produced as membrane-bound antibodies on the cell surface of B-lymphocytes and in this form function as receptors for antigen. They can also be secreted into the bloodstream or other body fluids and various tissues. Most characteristic features of antibodies are their diversity, specificity and their ability to recognize and bind with high affinity to their antigens.

Antibodies are symmetrical structures made up of two identical heavy chains and two identical light chains that are linked to each other by disulfide bonds (`Figure 1`). Each antibody chain is composed of an amino-terminal region that is highly variable (V) in the first 110 amino acid sequence and that participates specifically in antigen recognition, and a constant carboxy-terminal region (C) that is highly conserved and mediates various effector functions when the Ab interact with other serum proteins or cell membrane receptors.
Five classical major effector functions are mediated by domains of the constant region when antibodies bind to their antigens:

1. Neutralization of foreign substances through opsonization.
2. Activation of the classical complement pathway.
3. Fc receptor (FcR)-mediated phagocytosis.
4. Mediate ADCC, which can kill antibody-coated target cells.
5. Activation of mast cells, eosinophils, and basophils exclusively mediated via IgE.

To this can be added a sixth function which is one of the foci of the present thesis:

6. Induction of cytokine production from Ab cross-linked on surface-bound antigens or in the form of soluble immune complexes.

Antibodies are classified on the basis of differences in the heavy chain C regions. There are five distinct classes of antibodies: IgA, IgM, IgD, IgG, and IgE which correspond to five different heavy chain constant regions namely, α, μ, δ, γ and ε. In humans, further differences in the amino acid sequences of α and γ chains results in the subclasses IgA1, IgA2 and IgG1-4 respectively. The various antibody classes and subclasses have specific bio-
logical activities, and different average serum concentrations and half-lives. They are furthermore differently distributed in body tissues.

IgA accounts for 10-15% of the total Ig in the serum. IgA exist primarily as monomer in serum but in external secretion such as breast milk, tears, saliva, and mucus in the gut, it mostly occurs as a dimer. Two IgA molecules are covalently linked to each other via their Fc regions through a Joining (J) chain. IgA is the main antibody class present in external secretion. It plays an important role in mucosal immunity as well as activation of complement through the lectin or alternative pathways.

IgM accounts for 5-10% of the total serum Ig. This antibody is the first Ig produced in primary response to an antigen both in adults and neonates. As monomeric molecules IgM are expressed on the surface of B-cells, while serum IgM is always a pentamer consisting of five monomeric IgM molecules linked by disulfide bonds via their heavy chain domains. Because of its pentameric structure with 10 antigen-binding sites, serum IgM can more efficiently activate the complement system and facilitate clearance of foreign invaders.

IgD antibodies are present at low levels in the serum but together with IgM it is the most abundant membrane-bound Ig on naïve B cells. The biological effects of these serum antibodies remain unclear.

IgG antibodies are the most abundant antibodies in the serum. About 80% of the total serum Ig is of the IgG class. In humans, four different IgG subclasses can be distinguished. They differ in their γ-chain constant region-sequences. IgG subclasses are numbered according to their decreasing serum concentration: IgG1, IgG2, IgG3, and IgG4. IgG subclasses have different effector functions depending on slight amino acid differences.

IgG1, IgG3, and IgG4 are important in neonatal immunity, as they are actively transported from the mother to the fetus via the placenta.

IgG3 and IgG1 and less efficiently IgG2 activate complement system. IgG4 antibodies are not able to activate the complement system.

IgG1, IgG3, IgG4 and IgG2 bind with decreasing affinity to Fc receptors on phagocytic cells and mediate phagocytosis.

IgE antibodies are present at low levels in the serum and play an important role in defense against parasites as well as mast cell degranulation, which give rise to allergic reactions.

**Immune complexes**

Immune complexes (IC) consist of antibodies that associate with their respective antigens and form larger complexes that have a broad effect on cells and tissues in the body. IC are mostly known to cause and contribute to the emergence of acute and chronic inflammation, with resulting tissue damage (e.g. in rheumatic diseases and cancer). The formation of IC is part of the
antibody-mediated immune response that facilitates recognition of non-self molecules. Circulating IC and IC deposited in the tissues may be involved in the pathogenesis of a number of diseases and conditions such as autoimmune diseases (Rheumatoid arthritis (RA) and Systemic lupus erythematosus (SLE)), drug reactions (e.g. allergy to penicillin), and infectious diseases (e.g. meningitis, hepatitis and malaria).

Besides their ability to cause acute and chronic inflammation, IC can mediate immunosuppressive effects and influence the balance of T H1/ T H2 responses by inducing production of anti-inflammatory cytokines such as IL-6 and IL-10 (5, 6). The intensity and emergence of IC-caused tissue damage is dependent on the antigen/antibody ratio at which IC are formed, mainly because this ratio determines the solubility, clearance and site of IC deposition (5).

IC activates the complement system mainly via the classical pathway. IC bound to complement proteins can activate type III immune reactions leading to chronic inflammation when they deposit in tissues such as joints of RA patients. The complement proteins C1q, C4 and C3 can modify IC structure and thereby inhibit precipitation of IC in tissues or resolve already deposited IC. Such modifications also results in a variety of inflammatory effects, including the opsonization of IC for phagocytosis (7). Antibodies within IC may also via their Fc part bind to FcR present on monocytes and macrophages resulting in activation of these cells. It has been demonstrated that high molecular weight IC isolated from juvenile rheumatoid arthritis (JRA) synovial fluid can induce secretion of pro-inflammatory cytokines such as IL-1β and IL-6 from human PBMC (8).

**Human receptors for immunoglobulin G (FcγR)**

Hematopoietic cells such as monocytes and macrophages express a variety of cell surface receptors, among these being the human Fc receptors for IgG (FcγR) that specifically recognize the constant region (Fc part) of IgG heavy chains. Differences in specificities or affinities of each FcR for the various IgG isotypes are based on differences in the structure of this Fc-binding polypeptide chain called the γ chain. FcγR genes are a collection of 8 genes encoded by members of the immunoglobulin super-family located on the long arm of chromosome 1. Depending on the cell type, FcγR initiate different effector functions such as phagocytosis, ADCC by NK cells, antigen presentation, reactive oxidant production and cytokine release by monocytes and macrophages (4). FcγRs expressed on the cell surface of monocytes and macrophages may be either stimulatory or inhibitory.

Human FcγRs can be divided into four different classes on the basis of ligand affinity, monoclonal antibody reactivities and genetic structure:
FcγRI (CD64) is a high affinity receptor for monomeric IgG1 and IgG3 in humans occurring on monocytes, dendritic cells, myeloid progenitor cells and macrophages. The Fc-binding γ chain of FcγRI is associated with a disulphide-linked homodimer of a signaling protein called the FcR γ chain. This γ chain is also found in the signaling complexes associated with FcγRIII, FcεR and FcεRI. The FcR γ chain contains an immunoreceptor tyrosine-based activation motif (ITAM) that couples receptor clustering to activation of protein tyrosine kinase.

FcγRII (CD32) is a low affinity receptor for IgG1 and IgG3. Monomeric IgG are not able to bind to FcγRII. FcγRII are only able to bind aggregated IgG or when antibody is part of an IC. In human FcγRII is divided into three subclasses FcγRIIa FcγRIIb and FcγRIIc that arise through alternative RNA splicing. These receptors have similar intracellular domains and ligand specificities but differ in cytoplasmic tail structure, cell distribution and functions. FcγRIIa and FcγRIIc are single chain stimulatory receptors containing (ITAM) in their cytoplasmic tail, triggering cell activation.

FcγRIIa is expressed on the surface of monocytes/ macrophages, neutrophils, eosinophils, platelets, and endothelial cells and mediate phagocytosis of opsonized particles.

FcγRIIb is expressed on all immune cells and it is the only Fc receptor present on B cells. It is a single chain inhibitory receptor with an immunoreceptor tyrosine-based inhibitory motif (ITIM) that via co-crosslinking inhibits activation signals through receptors containing ITAM, resulting in inhibition of release of inflammatory mediators and inhibition of phagocytosis.

FcγRIII (CD16) is also a low affinity IgG receptor for IgG1 and IgG3 and occurs in two isoforms, FcγRIIIa, and FcγRIIIb. The FcγRIIIa is a stimulatory multichain-receptor composed of a ligand-binding α subunit with a cytoplasmic tail associated with a transmembrane ITAM subunit that triggers cell activation. FcγRIIIa is present on macrophages, eosinophils, dendritic cells (DC) and NK cells (9). The FcγRIIIb isoform, which is expressed on neutrophils, is a lipid-linked receptor that lacks an intracellular domain but when it is cross-linked with FcγRIIa or FcγRIIIa it can induce cell activation.

FcγRIV is a receptor with intermediate affinity and restricted subclass specificity and it binds with intermediate affinity to IgG2 subclasses of mouse IgG (10). Based on its sequence similarity it could be considered the mouse homologue to human FcγRIIIa (10, 11). This receptor is expressed on the surface of various cells such as monocytes, neutrophils, and DC but not on T cells, B cells, NK cells or granulocytes.
FcγR receptor expression is significantly upregulated on monocytes by IFNγ and lipopolysaccharide (LPS) and is downregulated by transforming growth factor beta (TGF-β) and IL-4 (12-14).

Macrophages present in the synovium of RA patients express higher levels of FcγRII and FcγRIII compared with macrophages from healthy controls (15). Furthermore FcγRIIb and FcγRIII are upregulated in synovia of RA patients as compared to in healthy individuals (16). Induced expression of FcγRII on RA macrophages results in higher production of pro-inflammatory cytokines such as TNF-α following IC stimulation (15). The involvement of FcγRs on monocytes and macrophages in IC-mediated events has been investigated in several experimental arthritis models and in RA (17). Blom et al have determined that the FcγR expression on monocytes and macrophages is correlated to severity and chronicity of inflammation and cartilage destruction during experimental IC-mediated arthritis (ICA). When they induced ICA in knee joints of DBA/1 mice, which expressed significantly higher levels of FcγRs than C57BL/6 mice, they observed very severe, chronic inflammation and cartilage destruction. In contrast, when they induced ICA in knee joints of C57BL/6 gene deleted mice, which lack functional FcγRs (FcγRI and FcγRIII) they could not observe any synovial inflammation and cartilage destruction. This finding may indicate a crucial role for IC, macrophages and FcγRII and FcγRIII in the pathology of RA (15).

The major histocompatibility complex

The major histocompatibility complex (MHC) is a set of highly polymorphic and polygenic genes, encoding cell-surface molecules that are necessary for antigen presentation to T-cells. MHC molecules are called the human leukocyte antigen (HLA) complex in humans. The MHC complex plays a central role in the development of both humoral and cell-mediated immune responses. It is a collection of genes located on the short arm of chromosome 6 in humans, organized into regions, in which class I, class II and class III HLA genes are located in distinct gene clusters. Class I MHC molecules consist of a large polymorphic 45-kDa α chain with three α1, α2, α3 segments that are associated non-covalently with a much smaller 12-kDa β2-microglobulin molecule which is encoded by a gene outside the MHC. These glycoproteins are presented on almost all nucleated cells and present self-peptide antigens to CD8 positive cells. Unlike class I MHC, class II MHC molecules contain two different membrane-bound chains, a 33-kDa α chain and a 28-kDa-β chain, which associate non-covalently to each other. These glycoproteins are exclusively expressed on antigen-presenting cells, macrophages, dendritic cells and B-cells, where they present processed exogenous peptide antigens to CD4 positive cells. Class III MHC genes encode mole-
cules that are essential for immune functions. These molecules include complement proteins and several inflammatory cytokines such as TNF-\(\alpha\) and TNF-\(\beta\).

The complement system

The complement system is a major effector arms of the antibody-mediated defense mechanisms of the immune system. It consists of more than 30 soluble and 10 membrane-bound proteins that play an important role in antigen clearance. The complement components react with one another in a highly regulated enzymatic cascade, by opsonizing pathogens and generating a series of inflammatory responses (18-20). The main source of complement proteins and glycoproteins are hepatocytes, but also monocytes, macrophages, endothelial cells, epithelial cells and fibroblasts can produce complement proteins. There are three distinct pathways of complement activation: the classical pathway, which is initiated by binding of C1q directly to pathogen surfaces or indirectly via IC, thus providing a key link between the innate and adaptive immune systems. The second is the alternative pathway, which is initiated by binding of C3b to activating surfaces such as microbial cell walls. Finally there is the mannan-binding lectin pathway, which is initiated by binding of the mannan-binding lectin, a serum protein, to mannose-containing carbohydrates on bacteria or viruses (18-20). The terminal components of the complement system generate the membrane-attack complex (MAC) responsible for cell lysis by creating holes in the cell membranes of the pathogens. The main consequences of complement activation are:

- Opsonization of pathogens. When antibodies bind antigens and thereafter activate complement or when complement fragments have been directly activated on antigen surfaces, the complement-coated antigen can bind to cells expressing complement receptors (CR1, CR3 and CR4). Thereafter this binding results in enhanced clearance of antigen.
- Recruitment of inflammatory cells via fragments of complement activation (e.g. C3a and C5a).
- Direct killing of pathogens, mediated by MAC formation.
- Enhanced clearance of soluble IC. C3b-coating on IC facilitate their binding to CR1 on erythrocytes, which transport the IC to the liver and spleen. IC are then removed from erythrocytes via FcR-mediated mechanisms and are phagocytosed (21).
Connective tissue and joint cartilage

Connective tissues and extracellular matrix (ECM) are components that serve to connect and bind the cells and organ together and ultimately provide structural support and form of the body. Two major connective tissue macromolecules are collagens and elastin, which constitute bone, skin, tendons, ligaments and cartilage.

Cartilage is a specialized form of connective tissue. There are three different forms of cartilage: hyaline cartilage, fibrocartilage and elastic cartilage. Hyaline cartilage is the most common form of cartilage that is composed predominantly of water, collagen fibers and proteoglycans. In adult mammals, hyaline cartilage is found in the articular surfaces of the movable joints, the walls of large respiratory tissue, the ventral ends of ribs and in the vitreous of the eye. In the joint, hyaline cartilage is responsible for protecting the underlying bone by absorbing transmitted forces and facilitating bone movement with minimal friction and protection against permanent distortion.

The collagen super-family

Collagens are a group of structural macromolecules that are present in connective tissue including bone, skin, hyaline cartilage, blood vessels, synovial membrane and liver among others. Forty-two different genes are responsible for production of at least 28 different types of collagen, which have been identified in different tissues of vertebrates (22, 23). Collagens are numbered by roman numerals in the order of discovery.

The collagen super-family can be classified into the following subclasses, depending on their structure, supramolecular structures, tissue distribution and function (23):

- Fibrillar collagens.
- Basement membrane-associated collagens.
- Fibril-associated collagens with interrupted triple helices (FACIT).
- Short-chain collagens.
- Collagen-like proteins such as acetyl cholinesterase, C1q, fibrinogen, macrophage receptor and surfactant protein.

Here I have focused on the fibrillar collagens, especially on CII, CXI and fibril-associated collagen type IX that are the main collagen components of articular cartilage.
Fibrillar collagens

Fibrillar collagens as their name intimate form fibrils consisting of monomers of polypeptide chains.

Each fibril α chain comprises about 1000 amino acids which when they assemble makes up triple helical domains. The α chain of the fibrillar collagens contains highly repetitive sequences of GLY-X-Y. The glycine is the smallest amino acid that is packed into a confined area in the core of the triple helix. The X position is almost always proline and the Y-position is often occupied by hydroxyproline. The presence of proline and hydroxyproline, both of which are saturated ring amino acids, are essential for the formation of hydrogen bonds that keep the α chain in an extensive configuration and contribute to the stability of the triple helix.

Collagen molecules are secreted into the extracellular space as procollagens, with preserved C- and N-terminal propeptides. Cleavage of the C- and N-terminal propeptides reduces the solubility of the protein, resulting in self-assembling into highly ordered, quarter-staggered, triple helical fibrils (23, 24).

The major fibrillar collagens, type I (C1), II (CII) and III collagen (CIII) are the most abundant collagen types in the body. The fibrillar collagens also include the minor types V, XI and the new members collagens XXIV and XXVII.

CII is quantitatively the most abundant fibrillar collagen in the body and is the major structural protein of bone and teeth, tendons, the endomysium of myofibrils and scar tissue.

CIII is found in the bone, cartilage, tendon, inner ear, blood vessels and other connective tissue.

Collagen XI is a small fibril molecule that in association with CII is uniformly distributed in articular cartilage, where it accounts for 5-20% of the total collagen. Like CII, it is also expressed in the vitreous body of the eye and the intervertebral disc (25, 26).

Type II collagen

Type II collagen (CII) is the predominant type in joint cartilage. More than 50% of all protein in cartilage and 90% of hyaline cartilage consists of CII (27). It is also present in the ear, larynx and trachea, vitreous of the eye, intervertebral disc and many non-chondrogenic tissues during development (28).

CII is encoded as propeptide by the COL2A1 gene located on the long (q) arm of chromosome 12. Due to alternative splicing of the COL2A1 gene it exists in two isoforms, namely type IIA and IIB. Type IIA mRNA contains an additional 207 bp exon (exon 2) encoding a 69-amino- acid cysteine-rich domain present in the N-terminal propeptide of the procollagen molecule (29, 30). Type IIB procollagen is the shorter isoform due to lack of this exon.
2-encoded domain. Type IIA procollagen is synthesized by pre-chondrogenic and non-cartilaginous epithelial and mesenchymal cells (29, 30) and it has also been localized in the somites, notochord, neuroepithelia and pre-chondrogenic mesenchyme of mouse (29) and human (31) embryos as well as in pre-cartilaginous condensations and perichondrium during the development of long bones. The propeptide of type IIA procollagen have been localized in the ECM of human pre-chondrogenic and epithelial tissues. Type IIB collagen is expressed in chondrocytes. The expression of type IIB collagen is spatially correlated with the high level expression of the cartilage proteoglycan, aggrecan, establishing type IIB procollagen and aggrecan as markers for the chondrocyte phenotype (29).

Native CII is a triple-helix composed of three identical α chains. When CII is denatured (e.g. by heat) the chains are separated. Antibodies against native CII recognize epitopes dependent on the native triple helix and these epitopes are destroyed by denaturation. CII can act as an autoantigen in RA (32-36). It has been demonstrated that the proteinaceous layer covering the intact articular surface inhibits anti-CII binding in healthy cartilage. During joint inflammation cartilage CII epitopes are exposed to anti-CII antibodies due to disruption of this proteinaceous layer that protect the intact cartilage surface. Therefore prerequisites exist for the formation of cartilage surface-bound anti CII containing IC in joint inflammation in a rabbit arthritis model (37) and in the RA (38) but not in healthy joints. In contrast, data from in vitro experiments argue that anti-CII can also bind to the surface of intact cartilage (39, 40).

Basement membrane-associated collagens
Type IV and VII collagens are the two major components of all basement membranes. Monomers of the type IV protein bind to each other through globular extension resembling a mesh network, serving as filtration barriers. Type VII collagen is found in the upper layers of the dermis serving to anchor fibrils between the basement membrane of the skin and dermis.

FACIT collagens
The members of the FACIT collagen subclass are type VII, IX, XII, XIV, XIX, XX, XXII and XXVI collagens, which contain interrupted triple helical domains and large NH2-terminal domains. Collagen types VII, IX, XIV and XVI are associated with CI and CII. Type IX, together with type XI collagen are particularly associated with type II fibrils in articular cartilage (41, 42).
Cartilage oligomeric matrix protein (COMP)

The extracellular matrix of articular cartilage contains a variety of non-collagenous proteins and proteoglycans. Cartilage oligomeric matrix protein (COMP) is a structural protein occurring in the extracellular matrix. It is present in all articular cartilage and is produced by chondrocytes (43). It has also been detected in tendon, meniscus, ligament and synovium but at much lower levels. The COMP gene is located on mouse chromosome 8 and in human on chromosome 9. This glycoprotein belongs to the thrombospondin protein family that comprises five members. COMP is a homopentameric molecule with five identical subunits and with a total molecular mass of 524 kDa (43, 44). The subunits are composed of an amino-terminal domain, four epidermal growth factor-like domains, eight thrombospondin type III repeats, and a COOH-terminal globular domain (45). Several studies demonstrate that serum COMP levels are correlated with cartilage destruction in inflamed joints (46-51). Increased levels of COMP have been detected in human synovial fluid, in osteoarthritis, progressive arthritis and following cartilage injury (52-58). COMP has variety of functions including:

- Through its COOH-terminal domain COMP binds to CII fibrils and stabilise the collagen-fibril network in the articular cartilage.
- COMP is a storage and delivery protein for vitamin D$_3$ and retinoic acid.
- EGF-like domains and type III repeats in COMP have calcium-binding properties.

The abundant expression of COMP in normal cartilage, its relatively low expression in other tissue along with the fact that levels of COMP in sera and blood are measurable using ELISA technique make COMP a candidate to determine cartilage destruction in human arthritis.

Cytokines

Cytokines are a group of soluble low-molecular-weight glycoproteins (approximately 150 amino acids) that are secreted by white blood cells and a variety of other cells in response to e.g. microbes and other foreign antigens and to self-antigens. Up to now more than 150 cytokines have been identified and cloned (59). Cytokines do not belong to a single gene family. They can be divided into interleukins (IL-)1-34, interferons (IFN-α, IFN-β and IFN-γ), hematopoietic colony-stimulating factors and tumor necrosis factors (TNF-α, TNF-β). Cytokines mediate and regulate innate and adaptive immune responses and inflammatory reactions. They are small proteins that function at picogram concentrations and that usually act on nearby target cells (paracrine action) as well as on the cells that secret them (autocrine...
action). When cytokines are secreted in large amounts they can also enter the blood circulation and act on distant cells (endocrine action). They have the ability to act on many different cell types and therefore have many different physiological and biological effects on the cells and on the body as a whole, such as inflammation, cell growth, proliferation and differentiation, apoptosis, angiogenesis, and tissue injury and repair. Cytokines exert their biological effects by binding to specific receptors expressed on the membrane of target cells. Cytokine receptors are classified into several families of receptor proteins based on their structures. Most of them have very high binding affinity for their cytokines and only very small amounts of cytokines are needed to occupy the specific receptors and mediate transduction of an activating or inhibiting signal across the membrane (60).

The cytokines studied in this thesis are:

**Tumour necrosis factor-α**

Tumour necrosis factor alpha (TNF-α) is a soluble 51-kDa protein composed of three identical 17 kDa subunits and is mainly produced by monocytes and macrophages but also by B cells, T cells (61-63) and fibroblasts in response to many stimuli e.g. other cytokines or IC. This potent cytokine has diverse effects on a variety of cells, including synovial cells in the joints of RA patients. TNF-α is an autocrine stimulator and it is also a potent paracrine inducer of other inflammatory cytokines such as interleukin-1 (IL-1), interleukin-6 (IL-6), interleukin-8 (IL-8), and colony-stimulating factors (monocyte-CSF, granulocyte-CSF and granulocyte-macrophage-CSF). Induced secretion of these CSF stimulates hematopoiesis, resulting in temporary increase production of white blood cells required to fight infection. The concentration of TNF-α is elevated both in synovial fluid and sera of RA patients. TNF-α and other cytokines such as IL-1β play an important role in the pathogenesis of RA by stimulating mesenchymal cells such as synovial fibroblasts, osteoclasts, and chondrocytes that release tissue-destroying matrix metalloproteinases (MMP) (64). It also promotes inflammation by stimulating fibroblasts to express adhesion molecules such as intercellular adhesion molecule 1, which interact with their respective ligands on the surface of leukocytes, resulting in increased transport of leukocytes into inflammatory sites, e.g. in the joints in RA patients.

**In vivo** studies have demonstrated that transgenic mice constitutionally expressing the human TNF-α gene spontaneously developed an inflammatory and destructive polyarthritis similar to RA (65). TNF-α is now considered a major cytokine in RA pathogenesis, and treatment of RA patients with TNF-α neutralizing agents has become a breakthrough in RA therapy (66-70). TNF-α also indirectly inhibits inflammation by stimulating the release of corticotrophin hormone from the pituitary, which in turn stimulates the adrenal cortex to release cortisol, which inhibits inflammation (64).
Interleukin-1

Interleukin-1 (IL-1) is a 17 kDa protein that similar to TNF-α mediates inflammation in response to infection and other inflammatory stimuli. IL-1 is primarily produced by monocytes, macrophages, B cells, activated T cells and endothelial cells. There are two subtypes of IL-1, IL-1α and IL-1β, which have less than 30 % homology, but that bind to the same receptors on cell surfaces and mediate the same biological effects (71). The IL-1β concentration is higher than IL-1α in the circulation.

The major biological effects of IL-1 are very similar to those of TNF-α, but the signaling system involved is more complex than for TNF-α. There are two types of cell-surface IL-1 receptors, type I and type II. Only type I receptors can mediate intracellular signaling (72). Type II receptors lack cytoplasmic tails and therefore cannot mediate intracellular signals. They are decoy receptors that bind circulating IL-1 without producing a signal cascade (73).

There are several mechanisms for the inhibition of IL-1 activity, such as naturally occurring IL-1 receptor antagonist that binds to the type I receptor with high affinity without triggering a signal, thus providing inhibition of IL-1 activity. Another inhibitory mechanism of IL-1 is when soluble forms of both types of IL-1-receptors compete with cell surface receptors to bind circulating IL-1 and thereby decrease IL-1 mediated activation of cells. In vivo studies strongly implicates that IL-1 is a key mediator in RA. Injection of IL-1 into the knee joint of rabbits results in the degradation of cartilage (74). Expression of human IL-β using an ex vivo gene transfer method of delivery into the knee joints of rabbits resulted in a severe, highly aggressive form of arthritis reproducing some of the features of RA in humans (75).

Like TNF-α, IL-1 may cause damage by stimulation of mesenchymal cells such as synovial fibroblasts, osteoclasts, and chondrocytes to release tissue-destroying MMP (64, 76).

Interleukin-8

Interleukin-8 (IL-8) belongs to the chemokine family, a group of low-molecular weight cytokines that play an important role in the inflammatory response by recruiting white blood cells to the sites of infection or inflammation. IL-8 is secreted by macrophages and endothelial cells and neutrophils are its primary target cells. Along with IC and chemotactic peptides such as C5a, IL-8 contributes to the recruitment of polymorphonuclear leukocytes into the inflamed joints of RA patients. The neutrophil chemoattractant activity of IL-8 in synovial fluid can be reduced about 40% by addition of anti-IL-8 neutralizing antibodies. IL-8 activates neutrophils through protein G-linked receptors, triggering an activating signal. This signal induces a conformational change in the integrin molecules (receptors on the neutrophils)
enabling them to adhere firmly to Ig-superfamily molecules (receptors on the endothelial cells), resulting in the trans-endothelial migration of neutrophils to the site of infection or inflammation. IL-8 is also a potent angiogenesis factor.

**C-reactive protein**

C-reactive protein (CRP) is an acute phase protein that is produced in the liver in response to tissue injury, inflammation and viral or bacterial infection. CRP consists of five identical non-covalently linked polypeptides and can be detected in the plasma of all individuals. IL-1β and TNF-α induce production of IL-6 that then subsequently can induce CRP production (77, 78). Blood levels of CRP are known to rise rapidly from normal baseline levels of < 3 mg/L to as high as 500 mg/L as part of the body’s non-specific innate inflammatory response to infection or injury. It binds to a broad spectrum of microorganisms and activates the complement cascade, resulting in opsonization and elimination of microorganisms by phagocytic cells from the site of infection and blood circulation. Serum CRP levels are routinely followed as a laboratory measure of inflammatory activity in RA patients and other inflammatory diseases.
Autoimmunity and autoimmune disease

Autoimmunity is a response of the immune system against self-components and self-antigens. The mechanisms of self-tolerance protect us from potentially self-reactive B and T lymphocytes. Most of the self-reactive lymphocytes are eliminated during B and T-cell maturation in the red bone marrow and thymus gland respectively. Activities of those self-reactive lymphocytes that escape clonal deletion are strictly regulated in normal individuals.

But when a breakdown of this regulation occurs, activation of self-reactive clones of B or T cells, generating humoral or cell-mediated immune response against self-antigens may cause severe damage to cells and tissues, sometimes with serious outcome and progression to disease. Such diseases are collectively called autoimmune diseases and can be divided into organ-specific and systemic diseases. In organ-specific diseases such as insulin-dependent diabetes mellitus and Graves’ disease, the immune response is primarily directed against a target antigen unique for a single tissue or organ. In systemic diseases such as RA and SLE, the immune responses are directed against several tissues and organs and encompass cell-mediated responses and cellular damage caused by T-cells, autoantibodies or IC.

Rheumatoid arthritis

Rheumatoid arthritis (RA) is a chronic, systemic inflammatory disease primarily affecting small peripheral joints in a symmetrical way.

RA is one of the most common human autoimmune diseases, affecting about 0.5-1% of the population worldwide. The word rheumatism comes from the Latin term *rheuma*, which means flow and flux. In ancient times it was believed that the composition of body fluids were of importance for the initiation of disease.

RA affects both genders and all ages, but women between 30-50 are three times more often affected than are men. The health economical consequences of RA are significant for health care system and society. Despite intensive research efforts the etiology, origin and cause of RA are still not known. Several factors such as bacterial or viral infection, hereditary factors and autoimmune mechanisms have been vigorously discussed, but the primary cause is still uncertain. The classification of RA for research purposes is based on a set of classification criteria developed for this purpose. The
first criteria for classification of RA were published in 1958 and revised in 1987 by the American College of Rheumatology (ACR) and known as the ACR classification criteria for RA (79). This classification system is based on clinical symptoms and physical examination and consists of seven criteria (table 1).

Table 1. The 1987 revised ACR criteria for RA (79). A Patient is classified as having RA if at least four of the seven criteria are fulfilled. Criteria 1-4 must have been present for at least 6 weeks.

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

MCP= metacarpophalangeal joints, PIP= proximal interphalangeal joints

A vast majority of RA patients experience functional decline, rigidity and work disability due to deterioration, atrophy and destruction of small peripheral joints (7). The best-studied genes associated with RA reside within the MHC. In the 1970s Stastny reported that RA was associated with an antigen of the HLA-DR4 gene (80). He demonstrated that RA lymphocytes proliferate when stimulated by lymphocytes from healthy controls, but did not re-
spond when stimulated by lymphocytes from other RA patients. This finding indicated genetic similarities between RA patients. Many reports have thereafter confirmed this finding of MHC association, but not only to HLA-DR4 but also to other HLA-DR alleles. These RA-associated HLA-DR alleles have been shown to have a shared epitope on the β chain, i.e. an epitope common to DR molecules that predispose for development of RA (81). This shared epitope is located on the third hypervariable region of the DRβ chain and found in multiple RA-associated DR genes including DR4, DR14 and DR1 (82, 83). Recent findings confirm the well-documented association of the HLA–DR1 (HLA-DRB1*0101, *0102) and HLA-DR4 (HLA-DRB1*0401, *0404, *0405 *0408), loci with RA (84) and implicate two additional non-shared epitope HLA-DRB1 susceptibility loci, DRB1*0701 and *0301 (85).

Synovitis is the most significant pathological process of RA, and is defined as severe proliferation of the synovial membrane with increased vascularization and simultaneous invasion of activated inflammatory cells. These produce a variety of cytokines, enzymes, proteases and vasoactive substances that contribute to atrophy and destruction of cartilage and bone, a characteristic of RA. Considerable knowledge gained of the pathogenesis, modulating factors and molecular mechanisms mediating inflammation and tissue destruction in RA during the past 10 years have resulted in many new approaches to therapy and treatment of RA patients.

There have been several theories presented regarding the mechanisms underlying RA. In the 1960s the IC theory was introduced according to which IC containing rheumatoid factor (RF) and other autoantibodies (86) are formed in the joints (87). The IC activate and recruit inflammatory cells to the inflamed joint. But auto-Ab and RF are however not specific for RA so the IC theory can not be unique for RA.

The T cell theory was then introduced, by the end of 1970s, when CD4+ T cells were detected in the inflamed joints of RA patients (88). Later it was determined that levels of macrophage and fibroblast cytokines such as TNF-α, IL-1β and IL-6 were elevated, instead of the T cell cytokines as had been expected. Recent therapeutic interventions using anti-TNF-α (67, 70), soluble TNF receptors (89) and IL-1 inhibitors (90, 91) have demonstrated the importance of macrophage cytokines in RA.

**Synovial fibroblasts**

Synovial fibroblasts originate from mesenchymal stem cells and together with macrophage-like synoviocytes line the superficial layer of the synovial tissue of joint capsules. In the normal human synovium there are two phenotypes of fibroblast cells, intimal and subintimal fibroblasts. Intimal fibroblasts express a wide range of cell surface molecules such as vascular cell
adhesion molecule-1 that binds to α4β1 integrin on the surface of mononuclear leucocytes. The deeper subintima contains relatively unspecialised counterparts (92).

Synovial fibroblasts are involved in many processes in the synovial tissue. They have barrier function and provide the joint cavity and the adjacent cartilage with lubricating molecules such as hyaluronic acid. They also maintain the integrity of structural connective tissue as they are constantly involved in synthesis and resorption of ECM and collagen fibrils. Synovial fibroblasts mediate neoangiogenesis via release of pro-angiogenic cytokines. Vascular endothelial growth factor is one of the most potent angiogenic factors, and is expressed constitutively in synovium by synovial fibroblasts, its secretion being induced by IL-1β and hypoxia (93). Synovial fibroblasts also mediate subsequent recruitment of inflammatory cells into the synovium by secreting chemokines such as macrophage chemotactic protein and macrophage inflammatory protein (MIP)-1α. Synovial fibroblasts can also secrete IL-8, RANTES and MIP-1β and can attract CD4 positive cells into the synovium by antigen-independent mechanisms through the release of IL-16 (94).

In RA, synovial fibroblast populations differ from normal human synovial fibroblasts and are called RA synovial fibroblast (RASF). Apart from secretion of chemotactic proteins, RASF produce a wide range of proinflammatory cytokines and effector molecules such as cyclo-oxygenase and thereby contribute to the inflammation (95). RASF contribute to hyperplasia, chronic inflammation and joint destruction either through direct mechanisms or indirectly by producing factors activating neighboring cells (96).

One of the distinctive features of RA is joint destruction. RASF plays a critical role in this process by producing a wide variety of matrix-degrading enzymes in particular the classic fibroblast collagenase MMP-1 the mesenchymal form of MMP-8 and MMP-13 (97). Pannus formation is a chronic condition of inflamed, edematous synovial membrane when synovial membrane grows into the cartilage and covers the cartilage surface. Although various inflammatory cell populations and their interactions contribute to pannus growth and thereby to the pathogenesis in rheumatoid synovitis, this vascularised granulation tissue is rich in activated macrophages, monocyte-derived osteoclasts and fibroblasts derived from synovial tissue.

Pannus tissue formation is also the most significant inflammatory process that occurs in the inflamed joints of RA patients. This pannus formation finally leads to the destruction of articular cartilage and subchondral bone. (98).

Matrix metalloproteinases

Matrix metalloproteinases (MMP) also collectively called matrixins are enzymes that can degrade components of the ECM. In humans there are 24 matrixin genes including the MMP-23 gene that occur in duplicate (99). The
MMP family consists of more than 25 structurally related enzymes that share a catalytic zinc-binding domain with a conserved sequence motif. MMP exist in either a secreted form or attached to cell membranes.

On the basis of substrate specificity, sequence similarity, and domain organization, vertebrate MMP can be divided into six major groups, namely; collagenases, gelatinases, stromelysins, matrilysins, membrane-type MMP (MT-MMP) and a heterogeneous subgroup including macrophage metalloproteinases (100).

There are two more enzyme families structurally related to MMP: the ADAM (A Disintegrin And Metalloproteinase) and the ADAMTS (A Disintegrin And Metalloproteinase with ThromboSpondin motifs). Over 25 ADAM genes and 19 ADAMTS genes have been described. ADAM are usually membrane-linked proteinases with diverse functions conferred by the addition of different protein domains. The disintegrin domain can bind to integrins and prevent cell-cell interactions (101, 102). Two members of the ADAMTS family (ADAMTS-4 and -5) are known to be involved in proteoglycan cleavage (103, 104).

Inflammatory cytokines, growth factors and other MMP molecules regulate the expressions of MMP (105) but they are also regulated by endogenous inhibitors, tissue inhibitors of metalloproteinases (TIMP) (99). IL-1 β is the central inducer of MMP-1, -8, -13 and -14 (106). The fibroblast growth factor (FGF) and platelet-derived growth factor (PDGF) induce MMP and thereby they potentiate the effect of IL-1β on the expression of MMP (107). TGFβ produced by RASF have proven to induce MMP-13 (108).

Important collagen-degrading MMP in RA are MMP-1, MMP-8 and MMP-13, also called collagenases. The collagenases make the initial cleavage in the collagen triple helix and unwind the collagen chains and make these denatured molecules susceptible for further degradation by other MMP (99, 109-111).

MMP-1 and MMP-13 are synthesized by macrophages, fibroblasts and chondrocytes when these cells are stimulated by inflammatory mediators. MMP-8 is predominantly released from neutrophils upon stimulation of the cell but is also produced by chondrocytes. All three collagenases are present in diseased cartilage (112).
Autoantibodies in RA

There are several autoantibodies of pathogenic importance in RA such as RF, anti-CCP antibodies and anti CII-antibodies.

Rheumatoid factor

About 75% of the RA patients produce a group of autoantibodies called rheumatoid factors (RF) that react with determinants in the Fc region of IgG (113, 114). The classical RF is an IgM antibody but RF associated with RA includes not only IgM RF but also IgG, IgA, and IgE RF variants with higher affinity. RF is not however, a specific marker for RA. It is also found in the sera from patients with other diseases such as viral infections, acute and chronic inflammatory diseases and in other rheumatic diseases (115) as well as up to 5% of normal individuals. (79). In many human and experimental conditions RF production has been proven to be the result of IC stimulation (116-119).

Anti-citrullinated protein/peptide antibodies (ACPA)

A specific serological diagnostic marker in RA is antibodies to cyclic citrullinated peptides (CCP), the most common of the antibodies against citrullinated proteins or peptides (ACPA). CCP is a synthetic peptide containing the modified amino acid citrulline that is formed by a post-transcriptional modification of arginine residues through action of the enzyme peptidyl arginine deiminase (115, 120). Antibodies against CCP (anti-CCP) has been shown to be highly specific and with similar sensitivity as RF in RA (115). Anti-CCP has become frequently used as an aid in diagnosis of RA patients being particularly useful as a diagnostic and prognostic marker in very early RA. RA patients with anti-CCP antibodies develop more severe joint damage than patients without anti-CCP (121, 122). Moreover, anti-CCP antibodies enhance and contribute to the pathogenesis of inflammatory arthritis in mice with experimental arthritis (123). All these findings and data from other studies strongly demonstrate the important role of anti-CCP antibodies in RA. Patients with/without anti-CCP have often same disease activity at disease onset, while anti-CCP positive patients later develop more inflammation and
radiological destruction compared to anti-CCP negative patients (124, 125). Furthermore HLA SE and smoking are exclusively associated with anti-CCP positive patients (126).

Collagen type II autoantibodies (Anti-CII)

It was in the early phase of the 1970s that collagen antibodies were noted in the sera and synovial fluids of patients with RA and the idea of collagen autoimmunity in RA was introduced by Andriopoulos et al. (127, 128).

CII antibodies are a group of autoantibodies detected in the serum, synovial fluid and cartilage of RA patients (37, 129-142). In addition to these, anti-CII-producing B cells in high numbers were also found in the rheumatoid synovium and synovial fluids (32, 143-145), indicating the presence of local antigen-driven immune reaction and importance of the adaptive immune system. These antibodies consist primarily of complement-fixing IgG subclasses with capacity of binding to homologous cartilage and of converting C5 to C5a (142, 146-148). Anti-CII occur in a subpopulation of RA patients. In different investigations, the frequency of elevated anti-CII among RA patients differ between 3 and 27% (130, 135, 142, 149, 150). Early after diagnosis the level of serum CII-antibodies is high in anti-CII positive early RA patients, but rapidly decline among patient with erosive RA (136, 151, 152). One possible explanation for this rapid decline of CII-antibodies might be due to formation of surface-bound IC within the joint when cartilage is gradually eroded, thus exposing collagen (37, 153-155). RA patients with elevated levels of anti-native human CII have increased levels of TNF-α and IL-6, together with higher erythrocyte sedimentation rate (ESR) and CRP levels, as compared to anti-CII negative patients (154).

It has also been reported that CII antibodies are correlated with disease severity and radiological changes (135). CII antibodies have been reported to emerge in the serum prior to RF by one group (151), which is in contrast to data that have been presented by Möttönen et al. (156). This is in contrast to RF levels, which have been shown to be increased years before RA diagnosis (157, 158) as are ACPA levels (157, 159). Antibodies against the denatured CII molecule also occur in other diseases (160), whereas antibodies against the intact native CII conformation is more closely associated with RA and a few other disease states like relapsing polychondritis (161). Elevated level of antibodies against intact human collagen IX, which interact with CII have also been reported in the sera of patients with RA (162).

It has also been demonstrated that arthritogenic mouse anti-CII antibodies that recognize specific epitopes in mice are shared with those identified by human RA sera. These antibodies contribute significantly to the development of arthritis independent of inflammation both in vitro and in vivo (40, 163).
Lessons on anti-CII to be learned from animal studies

Numerous different animal models, including genetically modified models, have been used to help understand some of the underlying mechanisms in CII pathogenesis in RA. Collagen-induced arthritis (CIA), collagen-antibody induced arthritis (CAIA) and transgenic models such as K/BxN are among the widely used experimental models resembling human RA. While these models are easily reproducible and well defined and proven to be useful in the development of new therapies, they still cannot completely reproduce the condition of human RA. Both innate and adaptive immune systems are involved in the CIA, CAIA and K/BxN models.

CIA is created when susceptible mice are actively immunized with CII and develop an autoimmune polyarthritis with autoantibodies to CII (164, 165). Passive vaccination by injection of anti-CII into experimental animals might either create a mild self-limiting synovitis (166) or severe joint-destructing arthritis (167) probably depending on the CII epitope specificity of the injected antibodies. To induce severe arthritis in the CIA model a panel of different mAb is required (141, 168, 169). It has also been demonstrated that single anti-CII mAb injected into naive DBA/1 mice can induce persistent arthritis (170). Moreover as for RA, CIA is also associated with specific MHC class II genes. In addition it is also associated with different gene loci outside the MHC both in mice and rats (171, 172). More than 25 different quantitative trait loci have been reported which regulate the severity of CIA in different strains of rats (171).

A functional B-cell response is essential for the development of CIA since it has been proven difficult to transfer CIA with CD4+ T cell alone (173, 174). B-cell depletion inhibits the development of clinical and histological arthritis in CIA (175, 176). The same is also true in human RA. The successful use of B-cell depletion for the treatment of RA has showed the importance of B-cells in RA pathogenesis but not all our questions about the roles of B-cells in this disease are as yet answered (177).

CAIA has some features of CIA and resembles human RA as the animals manifest the cardinal symptom of bone erosions, neutrophil infiltration and deposition of IgG and C3 on the articular cartilage surface (170). However unlike the CIA model the CAIA is not persistent and it is also less severe, although injection of panels of mAb induces a severe arthritis (141, 163, 168, 169). These findings have demonstrated the pathogenesis of CII-antibodies in initiating joint disease and mediating joint inflammation (141, 167, 170, 176, 178).

Monoclonal anti-CII antibodies alone can cause cartilage damage in vitro manifested by degradation of proteoglycan, loss of collagen and even complete matrix destruction (39, 40).
The FcγR system plays an essential role in the CAIA pathogenesis since FcγRIIb deficient mice develop a more rapid and severe arthritis when immunized with a single injection of monoclonal CII-antibodies (170, 179). Transgenic mice expressing human FcγRIIa develop a spontaneous arthritis with erosions and growth of pannus tissue as evident in RA patients (180, 181). Interestingly, it has also been proven that plasma or sera from RA patients containing CII-antibodies can induce arthritis in FcγRIIb deficient mice and the IgG rich fraction was recognized as being the pathogenic factor (182). These data indicate that the balance between inhibitory FcγRIIb and stimulatory FcγRIIa is important in RA pathogenesis.

The complement system also plays an essential role in RA pathogenesis since C5-deficient mice did not develop arthritis when injected with CII-antibodies, in spite of abundant IgG and C3 deposition on the cartilage surface (183). Using other Ab to elicit CAIA, C3 deficient knockout mice developed less severe disease as compared to C3 sufficient mice (184). In summary, valuable lessons can be learned from the CAIA model. Findings from animal studies might be relevant, applicable and adaptable into human RA and might partly shed light over the mechanisms underlying human pathogenesis of anti-CII in RA, investigated in the current thesis.
Aims of the present study

General aims

The aim of this thesis was to investigate the functional effects of anti-CII-containing IC and the possible mechanisms underlying anti-CII IC-induced cytokine production in vitro. Furthermore I wanted to investigate the association between these functional effects in vitro and the corresponding clinical signs and symptoms in RA.

Specific aims

**Paper I**
To investigate whether it was possible to create an in vitro model to study the functional effects of anti-CII-containing IC.
Our findings in this paper led to the follow-up aims for paper II.

**Paper II**
To determine if anti-CII IC-induced cytokine production was correlated with clinical indices in a well-characterized cohort of patients with early RA.

**Paper III**
To study if anti-CII antibodies and anti-CII IC-induced cytokine production was not only associated with early inflammation as shown in paper II, but also with early radiological destruction in RA.
Our findings in paper III gave rise to the follow-up aims in paper IV.

**Paper IV**
To develop an in vitro model that could explain the early anti-CII-associated radiological changes in RA patients.

**Paper V**
The aim of paper V was to investigate if high levels of anti-CII antibodies in patients with very early synovitis could predict future development of RA.
Materials and methods

Patients and healthy controls

In paper I, initially two RA sera with high serum levels of anti-CII and two healthy control sera without elevated anti-CII were selected to develop a method whereby IC-induced production of TNF-α from PBMC could be studied. Thereafter, sera from sixty-five patients with arthritis (47 women and 18 men, mean age 59 years, range 28-82), and from ten healthy controls were selected for investigation.

In paper II and III 274 RA patients from a prospective cohort of early (<12 months of disease duration) RA at Karolinska University Hospital were included between January 1995 and October 2000. Mean age was 56 years, and 70% were females. All patients fulfilled the 1987 ACR classification criteria for RA (79). One hundred healthy blood donor sera from the Blood Center at Uppsala University Hospital were investigated to define a reference range for anti-CII. Control subjects consisted of 62 men and 38 women with an age range between 21-69 and 20-66 years respectively and with no history of arthritic or other inflammatory disease. Sera obtained from patients and healthy controls were separated and stored at –70°C within four hours of sampling.

As responder cells in cell culture experiments, mononuclear cells were purified from buffy coats obtained from healthy blood donors or from heparinized blood from laboratory personnel.

All patients and controls had given informed consent to participate in the studies. The investigations were approved by the respective ethical committees of the Uppsala University Hospital and the Karolinska University Hospital in Stockholm. Clinical patient characteristics had earlier been collected at the Unit of Rheumatology, Karolinska University Hospital in Stockholm. For Larsen score calculations, identical X-rays of hands and feet were collected in posterior-anterior and tangential views at baseline, 1 year and 2 years. The radiographs were quantified blinded to treatment in pairs (hands and feet), and in chronological sequence applying Larsen erosion score (185). The quantifying procedure was performed by an experienced investigator (Marius C, Wick) as described in (124), and documented using the ”X-Ray RheumaCoach”-software (186). In each case, 32 joints were scored.

In paper IV one serum sample from an RA patient with very high levels of anti-CII was used. In paper V 177 patients with early synovitis (less than
3 months of duration) were recruited. Ethical permission was obtained and all patients gave written informed consent. Patients were followed for 18 months and assigned to their final diagnostic groups.

Preparation of peripheral blood mononuclear cells

Buffy coat samples were diluted 1:4 and heparinized blood diluted 1:2 in sterile phosphate buffer saline (PBS) at room temperature and then gently added on top of a Ficoll- Paque density gradient (GE Healthcare, Uppsala, Sweden) in a centrifuge tube. The samples were centrifuged for 20 minutes, 400 g and peripheral blood mononuclear cells (PBMC) separated from granulocytes and red blood cells (RBC). After centrifugation, the cloudy layer was gently collected using a sterile Pasteur pipette. After two subsequent washes conducted in sterile PBS (centrifuged for 5 minutes at 200 g), the cells were counted using a Bürker chamber and diluted to 1×10⁶ PBMC/ml in RPMI-1640 medium with L-glutamine, (GIBCO™, Invitrogen-Corporation, UK) supplemented with 1% penicillin streptomycin (PEST), 1% HEPES buffer, 12,5 μg/ml of polymyxin B sulfate, (SIGMA-Aldrich Chemical CO GMBH) and 1% Ultroser® G (Flow Laboratories, Irvine, Scotland, UK). Ultroser® G is a serum supplement for in vitro cell cultures. In previous studies in our laboratory, Ultroser® G has been shown to have positive effect on the IC-induced cytokine production in otherwise serum-free systems (187).

During the studies we ran out of our pre-screened Ultroser® G-batch and none of the new tested batches was as good as the earlier one. In paper IV we instead used a selected batch of fetal calf serum (FCS) with comparable property.

Previous experiences in the laboratory revealed that different PBMC donors might differ considerably concerning their PBMC responses to artificial or patient-derived IC. Two different PBMC donors were therefore often investigated in parallel in each experiment and data from the best responder PBMC donor were chosen.

Surface-bound immune complexes and cell culture experiments

In initial experiments we created anti-CII-IC with biotinylated antigen and human anti-CII in fluid phase. Thereafter we stimulated responder PBMC with IC either in soluble form or after binding to streptavidin-coated plates. But none of these two systems worked.
In the finally used system with surface bound CII anti-CII IC which could induce the production of pro-inflammatory cytokines, I simply bound the CII direct to 96 well ELISA plates by direct coating of antigen without biotinylation. Then the plates were blocked with human serum albumin (HSA; Albumin Behring, Aventis Behring, Stockholm, Sweden), followed by addition of RA sera containing anti-CII antibodies or healthy control sera. Finally healthy PBMC and/or RASF were added and cultured for 20 hours on top of the surface-bound anti-CII-containing IC (Figure 2). Cell culture experiments were performed with human CII in native form (Chondrex®, Redmond, WA, USA). As the native collagen was not proven to be endotoxin-free, these experiments were performed with the addition of polymyxin B. Control experiment without addition of polymyxin B did however yield comparable results.

![Diagram](image)

*Figure 2*) Simplified outline of the assay with surface-bound IC and direct coating of antigen, followed by the addition of responder PBMC/RASF cells.

**Monocyte depletion and enrichment (purification)**

To investigate whether monocytes/macrophages were the responder cells of CII-IC-induced cytokine production, cell depletion and enrichment studies were performed using specific tetrameric antibodies from Stem Cell technologies.

There are several techniques for separation and isolation of enriched and purified monocyte populations. We used the RosetteSep™, a rapid and easy cell separation method, which is based on negative selection without the need of magnetic beads or other particles attached to the select monocyte populations, as such particles might be phagocytosed by macrophages with ensuing non-specific cell activation.

In the negative selection method specific tetrameric antibody complexes cross-link unwanted cells to multiple RBC during a short incubation period,
forming rosettes. In the subsequent Ficoll separation, these unwanted cells pellet along with the free RBC leaving the desired cells untouched and purified. In the monocyte depletion study we eliminated monocytes by addition of 50 μl of Rosette Sep monocyte depletion cocktail/ml blood and incubated the blood for 20 minutes at room temperature (RT) and then Ficoll separated the blood as usual (see above). In the monocyte enrichment study we eliminated all other leukocytes except monocytes using a similar procedure. The results of monocyte depletion and enrichment were then assessed by FACS analysis of the percentage of CD14+ monocytes.

Cell surface receptor (FcγRIIa/III) blocking experiments

We have earlier demonstrated that IC from patients with cryoglobulinemia (188), SLE (189) and RA (190) induce cytokine production via FcγRIIa. One hypothesis in paper I was that cytokine production induced by CII-containing IC is also mediated via Fcγ receptors. To investigate this, FcγRIIa and FcγRIII were blocked using mAb Fab/F(ab’)2 fragments. We chose mAb Fab/F(ab’)2 fragments in order to avoid un-intentional cross linking of Fcγ receptors on responder cells by the Fc part of the blocking antibodies.

The cell surface receptors on purified PBMC were blocked by addition of 1.5 μg/ml anti-FcγRIIa-mAb (clone IV.3, F(ab)2) or anti-FcγRIII-mAb (clone 3G8, F(ab’)2) respectively which were obtained from Medarex (Nutley, NY) and incubated at 4°C for 30 minutes. These PBMC were added and cultured for 20 hours on top of the surface-bound anti-CII-containing IC in 96 well plates in parallel with control cultures without blocking antibodies. Supernatants were harvested after 20 hours incubation and cytokine ELISAs for IL-1β, IL-8 and TNF-α were performed to measure cytokine release from stimulated PBMC.

Cytokine Enzyme Linked Immunosorbent Assay (ELISA)

Supernatants from PBMC cultures were harvested after 20 hours incubation and cytokine ELISAs for IL-1β, IL-8 and TNF-α were performed to measure cytokine release from stimulated PBMC. The antibodies and cytokines used in the assay were purchased from R&D System (Abingdon, UK).

96-well plates (Nunc Maxisorb, Denmark) were coated with 50 μl of monoclonal anti-human cytokine antibodies (primary antibody) diluted in PBS and then incubated for two hours at room temperature or at 4°C overnight. After incubation, the wells were blocked with 100μl of PBS 1% BSA (PBS-BSA) for one hour at room temperature. Fifty μl of supernatants from stimu
lated PBMC cell cultures or diluted standards (diluted in RPMI) were added in duplicates and incubated for two hours at room temperature. The plates were subsequently washed three times in PBS with 0.05% Tween 20 (PBS-Tween; Life Technologies) to remove the excess of antigens. After washing, 50 μl of biotinylated anti-human cytokine antibodies (secondary antibody) diluted in PBS-1% BSA were added and incubated for two hours. After further washing 50 μl of streptavidin-horseradish peroxidase (R&D system, USA) was added and incubated for at least 20 minutes on a shaker. Following additional washing, 50 μl of substrate solution, 3,3’-5,5’-tetramethylbenzidine (DAKO® Glostrup, Denmark) was then added and the plates were placed in dark at room temperature until a bright blue colour developed. The reaction was stopped with 50 μl of 1M H2SO4 and the plates were finally read at 450 nm on a microplate reader (Labsystems OY, Helsinki, Finland).

**IgG anti-CII ELISA**

Anti-CII ELISA investigations in papers III and V were performed using a standardized and quantitative ELISA. Maxisorb plates were incubated with 100 μl of human native CII (2.5 μg/ml; ELISA grade native human CII; Chondrex, Redmond, WA; concentration determined in preliminary titration experiments), blocked with 150 μl of PBS-BSA and subsequently incubated with 100 μl of sera diluted 1:100 in PBS-BSA. The standard curve was constructed from a high titer anti-CII serum from an RA patient, and defined as containing 10,000 arbitrary unit (AU) anti-CII/ml, prediluted in PBS-BSA, aliquoted and thereafter stored at –70°C for single use. The detection antibody was an alkaline phosphatase-coupled goat F(\(\text{ab}'\))2 antibody against human \(\gamma\) chain adsorbed against bovine immune globulins (Jackson ImmunoResearch Europe Ltd, Cambridgeshire, UK) diluted 1:10000 in PBS-BSA. After washing, 100 μl/well of substrate (\(p\)-nitrophenyl-phosphate tablets (Sigma) 1 mg/ml in diethanolamine buffer, pH 9.8) was added and the reaction was read after 45 minutes at 405 nm in a spectrophotometer. Two internal control sera were diluted once in PBS-BSA, frozen aliquoted at –70°C before commencement of the study, and used as controls on each occasion. While running the anti-CII investigations in this study and thereafter the low control serum (mean 19.5 AU/ml, within the normal range), showed an intra-assay CV of 13%, whereas the high control serum (332 AU/ml) showed an intra-assay CV of 6%.

Wells only blocked with PBS-BSA but without CII coating were investigated in parallel, and only anti-CII reactive samples showing higher OD values in CII coated wells as compared to control wells were regarded as positive.
Rate nephelometry
Serum levels of RF and CRP in paper II were determined using rate nephelometry technique on the Beckman Immage® nephelometer (Beckman Coulter). The rate nephelometer measures the increase in the dispersion of light as it passes through a solution of light scattering particles suspended in a cuvette. The light source for the rate nephelometer is a 670 nm laser. The detector is placed at a 90° angle from the laser beam to measure scattered light. The light scattering properties depend on the sample through which the light is passed. When antibodies are mixed with their specific antigens (in this case, RF or CRP), IC are formed entailing increased dispersion of the laser beam. The formation of light scattering complexes is dependent on the presence of antigen and antibodies in optimal proportions. The amount of IC formed is dependent on the amount of antigen in the sample, because the antibody concentration is kept constant. The light scattered at 90° increases with time due to the kinetic appearance of IC.

Maximal light scattering will be highest at optimal antigen-antibody concentration, and followed by decreased maximal dispersion of light at antigen excess. Antigen excess will yield smaller IC and lower light scattering properties. The apparatus cannot determine the amount of antigen in the sample if the IC are formed at antigen excess. To assess if the IC are formed at antigen excess the apparatus performs an antigen excess test in which more antibodies are added to the sample. If there is antigen excess the light scattering will increase due to formation of new IC. In the case of antigen excess the sample has to be further diluted and measured again.
Statistical analyses

In paper I, Pearson’s product-moment correlation with Fisher’s r-to-z transformation was used for correlation analyses. The Wilcoxon test was used to analyze the effect of FcγR-blocking antibodies in a paired design. In paper II differences between anti-CII-positive and anti-CII-negative patients were analyzed using unpaired t test, whereas differences between proportions were analyzed using the χ² test or Fisher’s exact test. For evaluation of changes with time in the group of nine patients with high anti-CII levels, the Wilcoxon test was performed.

In paper III, the Mann-Whitney’s U test was used for the comparison of Larsen score between different groups. Two alternative cut-off values based on the 95th percentile among the controls (29 AU/ml) or between anti-CII levels supporting or not supporting IC-induced cytokine production in vitro (200 AU/ml) have been used.

In paper IV, Non-parametric methods have been used throughout the study to avoid inappropriate impact of outliers. The Wilcoxon signed rank test was applied to analyze the effect of cell stimulation, monocyte depletion and cytokine neutralization in a paired design.

In paper V, the Mann-Whitney’s U test was used for the comparison of differences between the groups whereas differences in proportion between the groups were analyzed using the χ² test. The Spearman rank test was used for correlation analysis between the groups.

In all studies P values less than 0.05 were considered to be significant.
Results

Paper I

In this paper we demonstrated that IC containing anti-CII from arthritis patients induced the production of TNF-α, IL-1β, and IL-8. We found a close correlation between anti-CII OD ELISA values and induction of TNF-α, IL-1β, and IL-8 respectively. The anti-CII-containing IC density threshold needed for cytokine induction differed among peripheral blood mononuclear cell donors, but was around 200 AU/ml. In monocyte depletion studies we succeeded in eliminating the amount of monocytes by 99% and the amount of cytokines induced by CII-IC also decreased by almost 99% compared to controls. In the monocyte enrichment experiments we succeeded in enriching monocytes up to 8 times and the amount of cytokines increased up to 6 times compared to controls. As monocyte depletion substantially eliminated anti-CII IC-induced cytokine production and monocyte enrichment instead enhance anti-CII IC-induced cytokine production, we concluded that monocytes/macrophages are probably the most important producers of anti-CII IC-induced cytokines in our model.

In FcγRIIa/III blocking experiments, FcγRIIa blockade significantly diminished anti-CII IC-induced cytokine production whereas FcγRIII blockade had no effect. The TNF-α and IL-1β production induced by anti-CII containing IC was reduced by up to 92% when blocking FcγRIIa but we could not record the blocking effect for FcγRIII. In summary, CII-IC induced cytokine production by PBMC is dependent on monocytes/macrophages, and is predominantly mediated via FcγRIIa. In this methodological investigation we had used samples chosen on the basis of anti-CII levels. We thereafter went on to study a clinically well-defined RA cohort in paper II.

Paper II

In paper II we investigated whether surface-bound IC containing CII and anti-CII might have a clinical impact in early RA. For this purpose we developed a quantitative anti-CII ELISA.

Analysis of baseline levels of anti-CII antibodies among 274 patients with RA and 100 controls revealed that 5/100 healthy controls and 24/274 (8.8%) patients with RA exhibited increased levels >29 arbitrary units (AU)/ml of
anti-native CII antibodies, a non-significant difference. 9/274 (3.3%) patients with RA and no controls comprised a discrete group with high anti-CII levels >450 AU/ml. These high anti-CII level sera but no other sera were associated with induction of proinflammatory cytokines by anti-CII-containing IC formed in vitro. 8/9 patients with high baseline anti-CII levels exhibited a parallel decline in antibody levels, IC-induced cytokines, CRP and ESR. Serum from the 9th patient did not induce cytokines, and this patient never had elevated levels of CRP or ESR. In the total RA cohort anti-CII-positive patients had significantly increased levels of CRP and ESR at baseline, but not later during the follow-up.

In this study, we demonstrated that elevated levels of anti-native CII clearly correlated with anti-CII IC-induced cytokine production, CRP and ESR. This indicates that levels of IgG anti-CII are associated with laboratory signs of inflammation, mediated by cytokine production via anti-CII-containing IC in joint cartilage

Paper III
In paper III we investigated if anti-CII antibodies and anti-CII IC-induced cytokine production were associated with erosions in RA. The same early RA cohort as in the paper II was selected for investigation, and X-ray plates from baseline, 1 and 2 years were available for 256 of them.

Comparison of median Larsen scores during the first 2 years after diagnosis revealed that anti-CII antibodies were significantly associated with increased Larsen score at disease onset using a high cut-off level (anti-CII > 200 AU/ml) corresponding to anti-CII IC-induced cytokine production in vitro. Sizeable but non-significant differences between the groups sustained at all investigated time points. When we instead used a conventional 95th percentile cut-off (anti-CII > 29 AU/ml) there was a sizeable but non-significant difference at baseline between anti-CII positive and negative patients. This difference vanished rapidly during follow-up.

We concluded that anti-CII antibodies and anti-CII IC-induced cytokine production in vitro were associated with early erosions in RA.

Paper IV
In this paper we investigated whether the association between early joint destructions and high levels of anti-CII could be explained by in vitro functions of anti-CII. For this purpose we needed a model containing relevant cells and stimuli in the RA pannus tissue. We therefore established an in-
vitro model in which the pannus-relevant cells monocytes (from PBMC, instead of tissue macrophages) and synovial membrane fibroblasts were stimulated with anti-CII containing IC.

We could observe that anti-CII IC–induced levels of TNF-α and IL-1β stimulated the production of MMP-1 and MMP-8, enzymes that can degrade CII in joint cartilage. Moreover we demonstrated that IC-induced production of MMP-1 was more strongly associated with IL-1β than with TNF-α. Baseline production of IL-1β and MMP-1 increased significantly in PBMC/fibroblast co-cultures as compared to cultures containing the individual cell preparations, whereas this was not the effect for TNF-α and MMP-8. Monocyte depletion decreased TNF-α, IL-1β and MMP-1 production in co-cultures, while the effect on MMP-8 production was variable. Cytokine neutralization in co-cultures revealed that IL-1β was a stronger inducer of MMP-1 than was TNF-α.

In this paper we presented an experimental model that might explain the findings of anti-CII-associated early radiological destructions in paper III.

**Paper V**

We had the aim of investigating if high levels of anti-CII antibodies in patients with very early synovitis could predict future development of RA. Among 177 patients with early synovitis 64 patients developed RA and 113 did not during 18 months of follow-up. The prevalence of anti-CII antibodies in patients who subsequently developed RA was 5%, whereas it was 8% among very early synovitis patients who did not subsequently develop RA. In this heterogeneous group here was no association between the ESR, CRP or swollen joint count and anti-CII levels. None of the patients exhibited the very high anti-CII levels distinguishing the outlier group defined in paper II. Our data demonstrated that the presence of anti-CII antibodies in patients with synovitis of less than 3 months duration was not predictive for future development of RA.
Discussion

In this thesis I have demonstrated that surface-bound IC containing native human CII and anti-CII antibodies from arthritis patients induced TNF-α, IL-1β and IL-8 production from monocytes via FcγRIIa. I then designed a quantitative ELISA, and determined that anti-CII levels were dichotomously distributed in RA patients where a small outlier patient group (3.3%) with very high anti-CII levels was associated with in vitro induction of pro-inflammatory cytokines by anti-CII-containing IC. RA patients with elevated levels of anti-CII also had a distinct RA phenotype with elevated laboratory signs of inflammation at the time of diagnosis. Very high levels of anti-CII were also associated with early radiological erosions at the time of RA diagnosis. Another in vitro model for the rheumatoid pannus tissue was thereafter developed to explain this latter finding. Co-cultured macrophages and RASF stimulated with anti-CII-containing IC induced the production of MMP-1 and MMP-8, enzymes responsible for the initial cleavage of CII during cartilage degradation. This IC-driven increase in MMP production was mediated via production of TNF-α and IL-1β, and especially anti-CII IC-induced IL-1β supported the production of MMP-1.

Commonly studied RA-associated autoantibodies such as RF and ACPA (e.g. anti-CCP and anti-MCV) are associated with late increase in clinical inflammation and late increased rates of radiographic destruction, as compared to autoantibody-negative patients. In the presently investigated RA cohort we have conversely recorded anti-CII to be associated with increased laboratory signs of inflammation and of radiographic destructions at the time of RA diagnosis, but not at later time points. We can thus distinguish between two different phenotypes of RA patients, namely a group with traditional RA-associated antibodies characterized by late inflammation and late increased radiographic destructures and another group of RA patients positive for anti-CII antibodies that are characterized by early inflammation and early erosions. The fact that all antibodies have been investigated in the same group of RA patients (124, 191, 192; paper II in this thesis) make these findings even more intriguing. Even though these two different phenotypes (ACPA/RF and anti-CII respectively) are statistically inversely correlated to each other in the presently studied RA cohort they are not mutually exclusive. The patient cohort that we have investigated is rather limited (n=274). This, together with the fact that the findings of early rise in CRP/ESR as well
as the finding of early radiological erosions attributed to elevated anti-CII levels both were observed in the same RA cohort, argues for future follow-up studies to investigate whether an anti-CII-dependent acute RA phenotype can also be defined in other RA populations.

One may speculate whether these two different RA phenotypes are also genetically inversely correlated to each other, and such investigations are currently underway in a larger genetically well-characterized RA cohort.

The Larsen scoring system measures bone destruction, while the Sharp-van der Heide scoring system measures both cartilage and bone destruction and these two measures (cartilage and bone destruction) have been shown to be highly correlated with each other (193, 194).

Previous studies in our research group have shown that serum IC can induce the production of various cytokines in other diseases such as SLE and cryoglobulinemia (188, 189), and also that synovial fluid IC from RA patients induced TNF-α especially in RF positive patients (190). Having these results in mind, I initially tried to develop a similar soluble IC model with artificial anti-CII-containing IC. I then combined pure or biotinylated CII with human anti-CII in fluid phase and thereafter stimulated responder PBMC with IC either in soluble form or after binding to streptavidin-coated plates. Neither of these approaches worked out, and I never obtained cytokine responses that exhibited a dose response to anti-CII levels in IC. It was not until I turned to the present surface-bound model that has similarities to an ELISA that I obtained reliable functional cellular responses. One might therefore question the terminology “surface-bound IC”. The presently used model more resemble an antibody mediated type II reaction where antibodies specific for antigens on cells or tissues attach to these cells or tissues. In contrast the traditionally IC-mediated type III reaction occur when antibodies bind to their antigens and form IC in circulation after which these IC subsequently might deposit in tissues, e.g. in blood vessels. One close similarity between type II and type III reactions are involvement of FcγRs. We have also identified an FcγRIIa dependency of cytokine responses in the surface-bound anti-CII IC assay that parallels the FcγRIIa dependency that has previously been reported concerning cytokine responses to soluble RA IC in fluid phase (189, 190).

It is conceivable that the presently used in vitro model can depict antibody sequestration at the cartilage surface or in the cartilage tissue in vivo. If so, the model is in concordance with data from Jasin et al, showing that in joint inflammation CII epitopes are exposed to anti-CII due to disruption of the proteinaceous covering that protects the intact cartilage surface from antibodies in the synovial fluid. According to the authors, this reaction
should then take place during a short period of acute joint inflammation but not in healthy joints (37, 38). However there are contradictory data from \textit{in vitro} experiments arguing that anti-CII can also bind to the surface of intact cartilage (39, 40). We and other groups have demonstrated that early after RA diagnosis the levels of serum anti-CII-antibodies is high, but thereafter rapidly decline among patient with erosive RA (136, 151, 152). Irrespective of whether anti-CII binding to joint cartilage is restricted to acutely inflamed joints or not, the appearance of high levels of anti-CII at the time of RA onset or diagnosis and later decreasing anti-CII levels might therefore together with data generated in our \textit{in vitro} models explain the appearance of an anti-CII-associated acute RA phenotype.

Cytokine production is not a general phenomenon occurring with any antigen–antibody combination. Wells treated with only HSA yielded considerably different OD values in ELISA using different sera, without parallel differences in cytokine induction. When we used the citrulline-containing peptide cfc9 (120) together with sera containing high levels of anti-CCP we could not record cytokine production. Recently another group has however reported a similar mechanism using citrullinated-fibrinogen as antigen (195). We have shown that surface-bound CII-containing IC can induce the production of cytokines of pathogenetic importance in the rheumatoid joint, and this may explain the early laboratory signs of inflammation evident in RA patients with elevated serum levels of anti-CII. This hypothesis is corroborated by Kim \textit{et al.} who reported that RA patients with elevated levels of anti–native human CII have increased levels of TNF-\(\alpha\) and IL-6, together with higher ESR and CRP levels, compared with anti-CII–negative patients (154).

The fraction of very high anti-CII antibody-positive patients with RA corresponds to the most conservative prevalence (3\%) of anti-CII published in a study using haemagglutination for detection of anti-CII antibodies (150). Both haemagglutination and our functional cell stimulation assays use techniques requiring cellular attachment via anti-CII, indicating that few patients have sera with such properties. Interestingly this earlier study (150) also elucidated an association between anti-CII and erosions. For the first time we have shown that anti-CII in RA patients are dichotomously distributed. A minority of patients with early RA (3-4\%) have very much increased levels of anti-CII (anti-CII levels 470-3520 AU/ml, \textit{Figure 3}), clearly distinguished from the otherwise normal distribution (anti-CII levels \(\leq 29\) AU/ml, \textit{Figure 4}) and thereby comprising a separate outlier subgroup.
Figure 3) Figure showing dichotomously distribution of anti-CII antibodies among 274 RA patients with two alternative cut-off values. The 95\textsuperscript{th} percentile among healthy controls (left arrow) and the cut-off based on anti-CII IC induced cytokine production in our \textit{in vitro} models (right arrow).

Figure 4) Anti-CII antibodies distribution among 100 healthy blood donors.

These patients who comprised the separate outlier group exhibited a drop in antibody levels with time, in parallel with reductions in CII-IC-induced production of pro-inflammatory cytokines \textit{in vitro} and reductions in laboratory signs of inflammation \textit{in vivo}. Our findings thereby argue that anti-native
CII-containing IC can be a link between humoral anti-CII responses and acute RA onset via the IC-induced and FcγRIIa-dependent production of key proinflammatory cytokines by monocytes and macrophages.

Even if anti-CII levels are dichotomously distributed in our RA cohort, we cannot at present determine whether such high autoantibody levels are needed for the in vivo expression of an anti-CII-associated acute RA phenotype or not. Our data in paper II might argue that this is not the case. Baseline CRP and ESR levels were increased among anti-CII positive patients using the statistical 95th percentile cut-off, but not using the higher functional cut-off separating sera with and without cytokine inducing properties in vitro. In paper III it was conversely the high functional cut-off that separated the newly diagnosed RA patients with increased amounts of radiological destructions. One possibility might be that our in vitro models are insensitive as compared to the hypothesized in vivo situation, and that similar mechanisms might work in RA patients even with lower anti-CII levels than those needed to obtain functional responses in vitro.

The FcγRIIa receptor mediating anti-CII IC-induced cytokine production in our model is not expressed in rodents, and transgenic mice expressing human FcγRIIa have been developed to study the importance of this receptor (180, 196). Transgenic mice expressing human FcγRIIa develop a spontaneous syndrome with erosions and growth of pannus tissue as evident in RA patients (181). These data, together with our data point to an important role for FcγRIIa in development of arthritis. Even if our FcγR blocking experiments in the PBMC/RASF co-culture system were inconclusive, we indicate that the anti-CII IC-induced MMP-1 production is dependent on cytokines, in particular IL-1β. As we demonstrated in our first paper that the anti-CII IC-induced production of IL-1β is FcγRIIa dependent, and as IC-induced IL-1β induced MMP-1, we speculate that FcγIIa also is important in anti-CII IC-driven cartilage destruction in vivo.

It is evident that both RA and CIA are not purely antibody-mediated diseases. Nonetheless, in rodents anti-CII serum from CIA animals, different individual monoclonal anti-CII antibodies or combinations thereof can transfer self-limiting joint symptoms (149, 166, 197, 198) or cartilage destruction (141) depending on choice of antibodies. Such antibody transfer experiments can be experimental models for the acute RA phenotype associated with anti-CII antibodies in man discussed in this thesis. Since our hypothesis for the action of anti-CII according to our experimental model is one of a indirect action by cellular activation, it is interesting that different investigators of anti-CII–transferred arthritis have noted an accumulation of transferred anti-CII at the cartilage surface as well as a rapidly evolving pathology directly involving cartilage and without pannus devel-
development (141). Both our *in vitro* models rely on responding cells, and our hypothesized cartilage-degrading effect is indirect via the IC-induced MMP production from PBMC/fibroblast co-cultures. It would be interesting to investigate whether human anti-CII also has a direct and cell-independent effect on cartilage.

In contrast with ACPA levels which increase during years before RA diagnosis (157-159) and thereafter remain rather stable (124, 125), anti-CII antibodies are absent before the onset of RA (156) and decrease during the first few years of disease (135). Anti-CII antibody levels thus appear to peak around the time of diagnosis of RA when they are associated with active inflammation. In paper V we have investigated whether the occurrence of anti-CII in patients with very early synovitis but without definite diagnosis could predict later development of RA, and determined that this was not the case. Instead, moderately increased anti-CII levels were rather common also in patients who later developed other diagnoses than RA, or who remained unclassified. One may speculate that an acute anti-CII phenotype characterized by early inflammation might not only be restricted to RA but may also occur in other arthritides with sudden onset. Concerning RA we consider that the anti-CII phenotype can be superimposed on other RA phenotypes and can give rise to acute disease onset in both ACPA positive and ACPA negative patients.

Whether the presence of anti-CII in arthritis patients is a primary event caused by B-cell activation by unknown causes or secondary to exposure of collagen epitopes (e.g. due to acute joint inflammation or increased joint load) is presently unknown. Association between anti-CII and specific genes including strong MHC association might point toward a primary cause, but without proving that this is the case. An association to environmental factors might instead point toward a secondary cause. We plan such studies in a large genetically and environmentally well-characterized RA cohort.

In this thesis I have studied the potential of surface-bound anti-CII–containing IC to induce proinflammatory cytokines and collagen-degrading enzymes of importance in RA pathogenesis using two *in vitro* models. These studies have implicated a functional relationship between anti-CII antibodies and acute onset of RA, and possibly also other arthritides. Further studies are needed in order to extend our understanding of anti-CII IC driven inflammation and cartilage destruction *in vivo* in arthritis patients.
General conclusions

The studies in this thesis have shown the potential of surface-bound anti-CII–containing IC to induce proinflammatory cytokines of importance in RA pathogenesis. In conclusion we have determined that:

I Anti-CII-IC induces production of proinflammatory cytokines such as TNF-α and IL-1β from monocytes/macrophages via FcγRIIa. Therefore blockade or suppression of FcγRIIa or suppression of synovial macrophages might be conceivable treatment options in such patients.

II High levels of anti-CII-antibodies are associated with laboratory signs of acute inflammation at the time of disease onset and this can be explained by the findings above.

III High level of anti-CII and anti-CII IC-induced cytokine production are associated with early erosions in RA.

IV Anti-CII IC–induced levels of TNF-α and especially IL-1β can stimulate production of MMP-1 and MMP-8. This might explain the early joint destructions evident in RA patients with high levels of anti-CII.

V The presence of anti-CII antibodies in patients with early synovitis is not predictive for future development of RA.
Future perspectives

I The anti-CII phenotype is unique as it comprises a subgroup of RA patients (3-4%) with very high levels of anti-CII antibodies together with early laboratory signs of inflammation and early erosions. This RA subgroup has a completely different phenotype compared to the phenotype found in ACPA positive patients. In collaboration with the Unit of Rheumatology at Karolinska Institutet, we will investigate the association of anti-CII with genes implicated in autoimmune diseases (e.g. HLA and PTPN22) as well as data from wide genome scan in the Epidemiological Investigation of Rheumatoid Arthritis (EIRA) cohort.

II In our collaboration with Karim Raza, we have shown that the presence of anti-CII antibodies in patients with synovitis of less than 3 months duration was not predictive for the development of RA. Subsequent diagnoses among the anti-CII positive non-RA patients suggested that anti-CII might rather be a phenomenon of acute arthritis irrespective of diagnosis (Figure 5) and therefore we want to investigate anti-CII in patients with other defined acute arthritides.

III In collaboration with other researchers we are going to study whether anti-CII IC stimulate production of inflammatory prostaglandins (PGE) in our in vitro PBMC/fibroblast co-culture model resembling rheumatoid pannus tissue. Preliminary data support this hypothesis.

IV We have analyzed COMP levels in the same patient group as investigated in the paper II and V. Initially high COMP level were associated with ACPA negative RA and showed a trend for association with anti-CII positive RA, thus with similarities to the anti-CII phenotype. Now we want to extend the study and investigate association of anti-CII with additional destructive cartilage and bone markers including collagen fragments.

V Neutrophil granulocytes in the inflammatory synovial fluid are the most abundant cells that might come in contact with cartilage-associated IC. We plan to develop another in vitro model to study the effect of granulocytes stimulation with CII-containing IC.
VI  We are going to study association between anti-CII and environmental factors like work-related load on joints.

VII  As we have shown in paper II that not all sera from RA patients with high anti-CII levels induced cytokine production from PBMC as part of IC. This might indicate that cytokine induction depends on collagen epitopes and/or usage of different IgG subclasses and not only on the levels of anti-CII. We want to investigate collagen epitopes and IgG subclass distribution and correlate these to biological function in our *in vitro* models.

VIII  In collaboration with other researchers we will extend our *in vitro* models to study anti-CII IC effects on rodent and human chondrocyte cell lines.

IX  We will also study longitudinal anti-CII IC responses in a patient earlier treated with oral collagen.

*Figure 5*) Antibody levels to native human type II collagen in 177 patients with very early synovitis split according to later diagnosis. Horizontal bars show median levels. Patients were the ones studied in paper V.
Summary of the thesis in Swedish

Autoimmuna sjukdomar är vanligt förekommande i samhället och orsakar stort lidande för patienterna. De medför också stora samhällsekonomiska kostnader. Reumatoid Artrit (RA) är en kronisk inflammatorisk ledssjukdom som drabbar 0.5% -1 % av vuxna. RA är en sjukdom där både autoantikroppar, immunkomplex (IC) och cytokiner har central betydelse i sjukdomsprocessen. Kollagen typ II (CII) som utgör en stor del av ledbrosket, har länge studerats som en källa för autoimmuna reaktioner i RA. Antikroppar mot nativet CII (anti-CII) kan påvisas i cirkulationen, i ledvätska och i ledbrosket hos en del RA-patienter.


Vi har visat att anti-CII-innehållande IC kan inducera produktion av TNF-α, IL-1β och IL-8, tre inflammationsdrivande cytokiner av betydelse vid RA. Cytokinproduktionen kommer från monocyter och är beroende av cellyte receptor FcγRIIa. Serumivåerna av anti-CII var dikotomt fördelade hos RA-patienterna. Medan de flesta patienterna hade samma anti-CII-nivåer som hos en frisk kontrollgrupp, fann vi hos RA-patienterna en liten avvikande grupp (3.3%) med mycket höga anti-CII-nivåer. När dessa sera med mycket höga anti-CII-nivåer användes för att blida IC in vitro inducerade dessa IC proinflammatoriska cytokiner, vilket inte skedde med övriga sera. Dessa patienter med mycket höga anti-CII-nivåer hade också en distinkt RA-fenotyp med höga serumnivåer av C-reaktivt protein och hög sänkereaktion, laboratoriemetoder som mäter inflammatorisk aktivitet. De förhöjda nivåerna fanns i samband med diagnos men inte senare under uppföljningstiden.
Patienter med mycket höga anti-CII-nivåer vid diagnos hade också ökad grad av ledförstöring mätt med röntgen i samband med diagnosstillfället, men inte senare under uppföljningstiden. För att kunna förklara dessa fenomen, utvecklade jag en annan in vitro-modell för den reumatiska pannusvävnaden, den inflamerade vävnad som bildas brosk- och bennära in den reumatiska ledhinnan. I denna modell där mononukleära celler och synoviala fibroblaster odlades tillsammans och stimulerades med anti-CII-innehållande IC producerades enzymerna MMP-1 och MMP-8, vilka är ansvariga för den initiala klyvningen av CII under brosknedbrytning. Denna enzymproduktion var beroende av TNF-α och IL-1β producerat i cellkulturen, och speciellt IC-inducerad produktion av IL-1β understödde produktion av MMP-1.

Förekomst av antikroppar mot citrullinerade proteiner eller peptider (ACPAs) hos patienter med tidiga ledsymtom har tidigare visat sig tala för att patienterna senare har stor risk att utveckla RA. Vi undersökte om förekomst av anti-CII hos patienter med mycket tidig ledhinneinflammation skulle vara associerat med att patienterna utvecklade RA efter 18 månaders uppföljning. Förhöjda serumnivåer av anti-CII hos patienter med mycket tidig ledhinneinflammation kunde inte förutsäga senare utveckling av RA.

Den anti-CII-associerade RA-fenotypen innebär alltså att patienterna med förhöjda nivåer av denna antikropp har kraftigare laboratoriresultat på inflammation och mer leddestruktion mätt med röntgen tidigt, d.v.s. i samband med diagnos, än patienter utan förhöjda anti-CII-nivåer. Detta till skillnad från RA-patienter med ACPA, vilka jämfört med ACPA-negativa RA-patienter får mer inflammationstecken och mer leddestruktioner senare under sjukdomsförloppet. Även om förekomsten av de båda antikropparna i den nuvarande studien visar en viss statistiskt signifikant invers korrelation så överlappar de med varandra. Det är möjligt att anti-CII-fenotypen kan finnas tillsammans med både ACPA-positiv och ACPA-negativ RA, och eventuellt också tillsammans med andra artriter.

Sammanfattningsvis tyder resultaten på att antikroppar mot CII har betydelse för utveckling av tidig inflammation och tidiga leddestruktioner hos den subgrupp av RA patienter som har förhöjda serumnivåer av anti-CII. Anti-CII-innehållande IC kan hos dessa patienter förstärka de tidiga sjukdomssymtomen genom att stimulera produktionen av proinflammatoriska cytokiner som TNF-α, IL-1β och IL-8 från monocyter och makrofager via FcγRIIA, och dessa cytokiner kan därefter stimulera inflammation. Sekundärt till denna IC-stimulerade cytokinproduktion kan även produktionen av brosknedbrytande enzymer i pannusvävnaden stimuleras. Det behövs ytterligare studier för att öka vår förståelse avseende den anti-CII IC-associerade tidiga akuta RA-fenotypen.
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