To detect the potential role of collagen type II immune complexes in granulocyte activation and acute inflammatory arthritis.

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ABBREVIATIONS:

ACPA  Anti-Citrullinated Protein/Peptide Antibody
ADCC  Antibody Dependent Cellular Cytotoxicity
Anti-CII Antibodies to Collagen type II
APC   Antigen Presenting Cell
ACR   American College of Rheumatology
BSA   Bovine Serum Albumin
CAIA  Collagen Antibody Induced Arthritis
CD    Cluster of Differentiation
cELISA Cytokine ELISA
CII   Collagen Type II
DHR   Dihydrorhodamine
DMARDS Disease Modifying Antirheumatic Drugs
ELISA Enzyme Linked Immunosorbent Assay
FACS  Fluorescence Activated Cell Sorting
FC    Flowcytometry
FcyR  Gamma receptor for the Fc part of IgG
FITC  Fluorescein IsothioCyanate
GM-CSF Granulocyte Macrophage Colony Stimulating Factor.
h    Hour
HLA   Human Leukocyte Antigen
HSA   Human Serum Albumin
IC    Immune Complexes
Ig    Immunoglobulin
IL    Interleukin
mab   Monoclonal antibody
MFI   Mean Fluorescence Intensity
MHC   Major Histocompatibility Complex
NHS   Normal Human Serum
PAD   PeptidylArginine Deiminases
PBMC  Peripheral Blood Mononuclear Cells
PBS   Phosphate Buffered Saline
PBS-Tween Phosphate Buffered Saline with 0.05% Tween
PE    PhycoErythrin
PI    Propidium Iodide
PMA   Phorbol-12polymyristate 13acetate
RA    Rheumatoid Arthritis
RBCs  Red Blood Cells
RF    Rheumatoid Factor
ROS   Reactive Oxidative Species
TMB   Tetra Methyl Benzidine
TNF-α Tumor Necrosis Factor-α
ABSTRACT:

Introduction:
Rheumatoid arthritis (RA) is a chronic disease characterized by inflammation and destruction of joints. Patients with RA have been sub-grouped by type of autoantibodies present in sera and synovial fluid (SF). Rheumatoid factor (RF) is one of the prevalent autoantibodies in RA. Anti-citrullinated protein/peptide antibodies (ACPA) are another class of autoantibodies, which has a higher diagnostic specificity for RA. ACPA along with RF is associated with the severity of the disease. Our research group has found a new subgroup of patients with high levels of collagen type II (CII) autoantibodies. These patients have an acute onset. There are 14 types of collagen known to be present in humans of which CII is present almost only in hyaline cartilage. Anti-CII antibodies are cartilage specific antibodies. These antibodies form immune complexes (IC) with collagen found in cartilage joints, and research in our group has earlier shown that such anti-CII IC induces monocytes to produce pro-inflammatory cytokines e.g. IL-8. We hypothesized that anti-CII IC probably recruit granulocytes to the joint space. The neutrophil granulocytes were chosen as cells of interest, as neutrophils are the cells to arrive first at the site of inflammation.

Methods:
To investigate the effect of anti-CII IC on granulocytes, granulocytes were purified from healthy donors and analyzed after stimulating with anti-CII IC in vitro. Anti-CII IC was prepared from a set of RA patient serum samples with high levels of anti-CII antibodies. A set of anti-CII negative RA samples and another set of 10 healthy sera were used as controls. Activation of granulocytes was measured by in vitro techniques viz. analysis of granulocyte surface markers by flowcytometry (FC), measurement of reactive oxidative species (ROS) by using a fluorescent dye, dihydrorhodamine (DHR) and production of major inflammatory cytokines as measured by cytokine-ELISA (cELISA).

Results:
Anti-CII IC did not induce significant granulocyte activation evaluated by these three methods, whereas control IC induced ROS production and alterations in CD markers, and PBMC showed the awaited responses concerning cytokine production when stimulated with anti-CII IC.

Conclusion: Granulocytes were not stimulated by anti-CII IC and results from all three types of experiments show that there was either no response or if so a very weak response from the granulocytes. Probably the anti-CII antibody density is too low for granulocyte activation in our system, as the cells responded to surface-bound control IC with very high IgG density. It can be concluded that granulocytes respond to other stronger IC (positive IC control). Anti-CII IC might have an arthritis-initiating effect by stimulation of PBMC as had been shown earlier in the group and repeated by me.
INTRODUCTION:

Immune system:
Our immune system protects our body by fighting against infections and certain diseases. It comprises of innate and adaptive immune systems. Innate immunity is non-specific and comprises of anatomical barriers, physiological barriers, inflammatory barrier, natural killer cells and phagocytic cells like granulocytes and macrophages. The complement system plays a role in both innate and adaptive immunity. Adaptive immune system provides specific immune response towards the antigen. The adaptive immunity comprises of T cells and B cells which recognize a wide range of antigens with specificity. It also provides diversity, immunologic memory by which it reacts more rapidly during a second exposure of the same antigen. T cells cannot recognize whole antigen and need antigen presenting cells (APC) to process and present the antigen as small linear peptides. B cell recognizes epitopes present on antigenic surface based on their conformation. Although the innate and adaptive immunity seems to function independent they often communicate with each other through cytokines and other molecules and operate as a network.

Autoimmunity:
Autoimmunity is a misdirected immune reaction towards self-antigens present in body. It can even be due to immune reactivity towards a non-self antigen by mechanism of molecular mimicry, in which the non-self antigens mimic as self-antigens. Autoimmunity involves T cells autoantibodies and innate immune cells. Specificity of the immune response is provided by tolerance mechanisms, which involve central tolerance, and peripheral tolerance. Central tolerance takes place during the development of B and T cells in the bone marrow and thymus before cells reach the periphery. Self-reactive B cells, which react with self-surface antigens, are deleted by clonal deletion. Mature self-reactive B cells that have escaped central tolerance are mostly not activated in periphery as they express only IgD. T cells undergo positive and negative selection. In positive selection the cells, which cannot bind major histocompatibility complex (MHC) are deleted by apoptosis. In negative selection self-reactive T cells, which binds too tight to MHC are deleted. In humans MHC is referred as human leucocyte antigen (HLA). Polymorphism in the HLA allele can result in increased risk of developing arthritis (1-3). T regulatory cells suppress T cells, which reach the periphery. T regulatory cells function by producing immuno suppressive cytokines like IL-10 and TGF-β. It also functions by blocking the co-stimulatory signal, resulting in anergy.
**Inflammation:**

Inflammation is a non-specific immune response against infection injury or as autoimmunity and involves steps like adhesion to endothelial membrane, transmigration and recruitment of cells responsible for inflammation. The neutrophils are primarily involved in this process. They have certain cell surface markers like CD66b, endothelial cell adhesion marker, CD11b a part of the integrin that mediates cell adhesion, CD35 complement receptor, CD32 Fc gamma receptor II (FcγRII), CD16 Fc gamma receptor III (FcγRIII). Neutrophils and granulocytes get activated during inflammatory processes and up-regulate or down-regulate these markers (4). Once the neutrophils are activated they reach the site of inflammation with the aid of these markers and release ROS that contain toxic peroxides, which kill microbes during infection, but these toxic peroxides destroy site of inflammation in autoimmune diseases. They also produce pro-inflammatory cytokines like IL-8 and chemokine CCL2 and CCL20 to attract inflammatory cells.

**Granulocytes:**

Granulocytes include neutrophil, eosinophil and basophil granulocytes and belong to the non-specific part of the immune system. Their granules and lobed nuclei distinguish them from other cell types. They are short lived, and react rapidly and aid to elevate the immune response during inflammation eg. Injury and infection. Among the granulocytes the neutrophils are of primary importance in context of inflammation. During inflammation neutrophils are the first to arrive and initiate the immune response. They interact with the specific part of the immune system via the CCL2 and CCL20 chemokines and attract other immune cells to the site of inflammation. The recruited immune cells produce CXCL8, which attracts neutrophils (5). There are several ways to measure granulocyte activation. They express cell surface markers and produce ROS and pro-inflammatory cytokines during activation. Migration assay can also be done in order to verify the activation of granulocytes. The production of ROS is dependent upon NADPH oxidase, which is involved in release of toxic substances, like superoxide, which gets converted to hydrogen peroxide by superoxide dismutase. They can kill the trapped antigen or can activate proteases. They also produce lactoferrin, cathelicidin, gelatinase and chemokines, after degranulation. Mutations in the NADPH gene can result in defective production of ROS. An important gene, which regulates the NADPH oxidase, is the Ncf1 gene and a mutation in this gene is also associated with T regulatory cell mediated suppression of CD4 positive cells also modifies the T cell dependent autoimmune response (6). Thus granulocytes communicate with the adaptive immune system.

**Cytokines:**

Cytokines are important for the integrated function of immune system. They help in communication between cells of innate and adaptive immune system. IC
formation can result in cytokine production, which sustains inflammatory response and this has been the major focus of the research performed within the research group. IC in systemic lupus erythematosus (SLE) initiate the production of cytokines (7, 8). Cytokine production has also been reported in cryoglobulinemia (9). Pro-inflammatory cytokine production mediated by immune cells is seen in RA (10, 11). Apart from autoimmune diseases cytokines also play a part in infectious diseases (12). Three cytokines Tumor necrosis factor α (TNF–α) and Interleukin 1β (IL-1β) were chosen based on their ability to initiate the inflammatory process. IL-8 was also chosen as it had been one of the more important chemokines produced by neutrophils, and because IL-8 very specifically activates neutrophil chemotaxis. Recent anti TNF-α therapy with monoclonal antibodies proves the potential role of TNF-α released by immune cells involved in arthritis (13). Granulocytes were tested for release of these pro-inflammatory cytokines.

**Immune complexes:**

IC are formed between an antigen and any of the 5 classes of antibodies. When these IC are not cleared from the circulation it causes problems by depositing on the tissues and organs and eventually leads to injury and damage. IgM IC are often cleared from the system as they are larger than those formed by IgG and IgA. Kupffer cells among the reticuloendothelial cells are essential in clearing away IC from circulation (14). The IC, if not, removed from circulation can activate the complement system. The autoantibodies bind to the antigens present in the body and forms IC that can bind to cell membrane of cells, which contains complement receptors and Fc gamma receptors. The blocking of complement resulted in decrease of inflammatory cytokine showing that the complement system plays a part in arthritis by being activated by IC (9). When the Fc gamma II receptor was blocked with monoclonal antibodies (mab) there was a significant decrease in the production of cytokines (15). There are four types of hypersensitivity reactions as shown in figure 1. IC mediated diseases belong to type III hypersensitivity. The IC can be soluble, insoluble and surface bound. Type II and Type III hypersensitivity reaction results in many of the autoimmune manifestations in inflammatory diseases. In type II reaction, autoantibodies can attach to the cell surface antigens and can mount an immune response with the help of complement by ADCC. Type II reactions also induce cytokine production from monocytes (10). In type III reaction soluble IC formed gets deposited in the tissues and cells and destroy the site of inflammation. Both Type II and Type III reaction not only involves complement, but they also include Fc receptors found on cells. IC bind to Fc receptors and activate them. IC deposited in the tissue initiate inflammatory process mediated by massive neutrophil infiltration.
Rheumatoid Arthritis (RA):

Rheumatoid arthritis is a chronic inflammatory disease characterized by morning stiffness, joint pain and destruction. The ease of life is very much affected and damages incurred can be irreversible if not adequately treated. Women are predominantly affected, but degree of disease is more severe in men. Until recently at least four out of seven criteria framed by the American College of Rheumatology (ACR) had to be present to fulfill the diagnosis or RA. The criteria included morning stiffness for more than 1 h, number of soft tissue and swollen joints, presence of auto antibody rheumatoid factor (RF) and radiographic erosions and duration of disease (16). ACPA are autoantibodies formed against citrullinated proteins. The citrullinated peptide is formed by conversion of the arginine residue to citrulline by peptidyl arginine deiminases (PAD) which is expressed in normal human cells as well in some bacteria as shown in figure 3 (17). With the discovery of ACPA, serology diagnostic test for RA became more specific. RF along with ACPA positivity showed even higher specificity for RA. ACPA positive patients develop more joint erosions over time as compared to ACPA negative patients (18, 19). The discovery of ACPA, together with the understanding that the old RA criteria could lead to RA diagnosis at a late stage when the disease process had already led to tissue destruction have led to new RA classification criteria including ACPA, where the new criteria have the clinical aim to diagnose RA at an earlier stage (20). ACPA positive and negative patients showed no difference in symptoms at the time of RA diagnosis, but ACPA positive RA patients had the worst prognosis with more severe joint erosions appearing many years after diagnosis (21). This means that the ACPA-associated RA phenotype is associated with late signs of inflammation. Colleagues in my research group have found a new RA phenotype associated with high levels of anti-CII autoantibodies. This RA phenotype differs from the ACPA phenotype, as the anti-CII-associated RA phenotype is associated with increased signs of inflammation (11) and joint erosions (22) at the time of diagnosis but not later on, as shown in figure 4. Treatment of arthritis involves administration of steroids into joints as well as usage of disease modifying anti-rheumatic drugs (DMARDS). Recent therapy involves usage of mabs against pro-inflammatory cytokine TNF-\(\alpha\). Studies have also shown that RA is associated with environmental factors as well. For instance smoking is associated with increased risk of RA development (23).
Figure: 3. Citrullination of protein and development of ACPA by PAD enzymes (17).
Collagen type II (CII):

Collagen is an essential for the formation of extracellular matrix and joints. There are four types of collagen subclasses: fibrillar collagens, basement membrane associated collagens, fibril associated collagens and short chain collagens. CII is cartilage specific collagen. More than half the dry weight of cartilage is attributed to CII. CII has triple helical confirmation arranged as shown in figure 5. CII is a homotrimer made up of three identical a chains. Each a chains have glycine in third position and is highly repetitive. Therefore the sequence of a could be (Gly-X-Y) where X and Y are more often occupied by proline and hydroxyproline. Since, arthritis is characterized by cartilage and joint destruction, CII immunity and tolerance is an important aspect in RA.
Humoral immunity against CII:

Mice immunized with rat CII resulted in chronic relapsing arthritis. This CII induced arthritis (CIA) in mice was mediated by both T cells and B cells. B cells recognition of CII differed among mice. Antibodies were produced against major epitopes U1, J1 and C1 and the antibody levels were correlated with chronic relapsing arthritis in mice (24). The significance of anti-CII antibodies against conformational epitopes in inducing arthritis lead to development of collagen antibody induced arthritis (CAIA) model. In CAIA model specific antibodies against certain CII conformational epitopes associated with arthritis were passively transferred into healthy mice. Anti-CII antibodies were able to induce an arthritis in healthy mice (25). This shows that anti-CII antibodies specific to cartilage were able to induce arthritis. Anti-CII antibodies were able to induce arthritis in mice lacking adaptive immune system (26). Similar to the CAIA model in vivo my research group working with the functional role of anti-CII in vitro have shown that anti-CII antibodies in humans are associated with early joint erosions (22), as well as that human IC containing human anti-CII antibodies induced cytokines from PBMC via FcγIIa receptor in vitro (11). The effect was almost totally abrogated when monocytes were depleted, showing that monocytes were the cells responding to IC stimulation (10). We therefore assume that high levels of CII antibodies and acute onset of RA in humans follow pathology similar to what characterize CAIA in mice. From findings by the group of Hugo Jasin (27) performed in normal bovine, human and rabbit cartilages it is clear that the binding of anti-CII is low to the surface of intact cartilage but higher after cartilage damage. The destruction of cartilage was done by a brief exposure of the cartilage to 4M-guanidine solution or neutrophil elastase. Controls made by exposing the cartilage to NaCl showed minimum binding of CII antibodies. Anti-CII antibodies are produced by cells in RA synovial fluid (28).We therefore assume that activated granulocytes might enhance the possibility for anti-CII in the synovial fluid to bind to cartilage and produce surface-bound anti-CII IC. If the anti-CII IC themself can stimulate granulocyte activation, a vicious cycle might appear. To investigate this was the first scope of my thesis work.

In another paper where my colleagues investigated CII antibodies in patients with early synovitis before any diagnosis was made (29), they found that elevated levels of anti-CII were not only found in patients who subsequently developed RA, but also patients who subsequently developed other diagnoses with acute arthritis onset: gouty arthritis and reactive arthritis. Hence they developed the hypothesis that anti-CII antibodies may be associated with acute onset arthritis in general instead of being a specific RA marker. The earlier study from our group (29) contained however very few patients developing gouty arthritis and reactive arthritis, and more patients with these diagnoses must be investigated to test this hypothesis. To do this was the second scope of this thesis work.
**AIM:** To investigate the potential role of anti-CII IC on granulocytes by various in vitro techniques

- To evaluate the kinetics of expression of CD markers by FC the surface of purified granulocytes stimulated with anti-CII IC.
- To deduce the time kinetics of ROS release after anti-CII IC stimulation of purified granulocytes by means of FC.
- To find the kinetics and measure the amount of pro-inflammatory cytokines released by anti-CII IC stimulated granulocytes.
- To evaluate concentration of anti-CII needed for granulocyte stimulation in these assays.
- To analyze a set of clinically well-characterized RA samples after having estimated the optimum time kinetics and anti-CII antibody concentrations, and to correlate the results with the anti-CII antibody levels to the degree of disease in patients with RA.
- To investigate a group of patients with other acute onset arthritis diagnoses concerning levels of anti-CII antibodies.

**MATERIALS AND METHODS:**

**Patient serum samples:**

Peripheral blood was collected from healthy donors at Uppsala Academic hospital. The serum HP was obtained from a rheumatoid arthritis patient with high levels of anti-collagen antibodies stored at -20°C. This is the serum used in most of the experiments in my thesis. A set of 10 RA patient serum samples was investigated in the final experiments. Nine of these patients with high collagen antibodies were picked from the cohort of 274 RA patients, from Karolinska University Hospital used in the previous study (11). These patients fulfilled the American College of Rheumatology classification Criteria (16). One of the patients did not fulfill the RA criteria but was included as the serum contained high levels of anti-CII antibodies. Serum samples from patients with acute onset of arthritis, which includes gout, remitting seronegative polyarthritis with pitting edema (RS3PE), bacterial and viral reactive arthritis were obtained from professor René Toes, Department of Rheumatology, Leiden, and were analyzed by collagen ELISA. All patients included in the study gave informed consent to take part in the cohort study that had been ethically approved by the relevant hospitals and by Uppsala University.

**Purification of granulocytes:**

Granulocytes were purified from human blood by using Ficoll-Paque™Plus (GE Healthcare, Uppsala, Sweden) density gradient medium. Blood from healthy donor was taken in sodium-heparinized tubes, 6ml (Greiner bio-
one GmbH, Kremsmünster, Austria) to prevent coagulation of the blood. The blood was diluted with equal volume of phosphate buffer saline (PBS) pH 7.2 in a 50ml Falcon tube (BD, Franklin Lakes, NJ, USA). The diluted blood was layered carefully on the top of the density gradient medium and subjected to centrifugation at 1000 g for 30 minutes. Plasma at the top was removed. Sometimes, mononuclear cells in the middle were separated from medium by carefully sucking out the PBMC layer without any medium and the medium was thereafter removed using a Pasteur pipette. Then the granulocytes at the top of the red blood cells (RBCs) were taken using a Pasteur pipette and suspended in 3 ml of PBS in a Falcon tube. Remaining RBCs mixed in the granulocyte population were lysed by adding 30ml of ice-cold Milli-Q (MQ) water and tilting the tube gently for 45 seconds. Cells were brought back to isotonic state by adding four times concentrated PBS (4X PBS). After the red blood cells had been lysed the granulocytes at the bottom were suspended in PBS for performing assays. Initially another density gradient medium, Percoll (GE Healthcare) was also used to purify granulocytes and purity between the granulocytes separated by two procedures was compared.

**Purity determination of granulocytes:**

The purity of the granulocytes was determined by staining with Türk’s solution (Merck, Darmstadt, Germany) and counting the number of granulocytes on the Bürker chamber (Merck Eurolab, Stockholm, Sweden). The purity was determined based upon their cell morphology. The purity percentage was calculated by counting the number of polymorphonuclear cells to the mononuclear cells.

**Viability assay:**

Neutrophils are short lived and hence it is essential to check the cells for their viability before starting an assay. The viability test was done using propidium iodide (PI) dye (BD Biosciences, Stockholm, Sweden). 20ng/ml of the PI was used to stain approximately 200,000 granulocytes. Another tube containing 200,000 granulocytes was left unstained. Both the tubes were incubated for 25 min at 4°C and fixed using 0.1% paraformaldehyde. The stained cells and the unstained cells were analyzed by FC in the PE range (620nm) of absorbance. Quadrant gating was used to gate where quadrants Q1 and Q3 are hidden. Gate for living cells was defined by MFI of unstained cells at time 0 h in the PE range (620nm) as shown in figure 6a. Cells with higher fluorescence intensity above the gate were considered dead as shown in Figure 6b. Two distinct populations of living cells were seen in Q4, with eosinophils at the top and neutrophils below them as eosinophils have higher relative auto fluorescence as in figure 6a.
Preparation of anti-CII IC:

This procedure was done as described in previous studies (10, 11). Pure ELISA grade human CII antigen (Chondrex Inc., Redmond, WA, USA) was coated in 96well, ELISA plates (Nunc, Roskilde Denmark) and incubated for overnight at 4°C. ELISA grade CII antigen is used as it retains the triple helical structure of collagen in contrast to denatured CII which might be dominated by non-helical linear peptides. As we are looking for autoantibodies against conformational epitopes it is important that CII antigen is in correct conformation. Collagen buffer (Chondrex Inc.) was used to dissolve collagen to prevent stacking of CII antigen into fibrils. After blocking with PBS-1% HSA for 1 h, serum containing anti-CII antibodies was added to ELISA well plate and left on a shaker for 3 hours for anti-CII IC formation. Initially HP sera of 1:10 dilution were used. Later it was concentrated four times 1:2.5 dilution was used and finally the experiments were tried with undiluted sera. Normal human serum (NHS) or sera from patients, negative for anti-CII antibodies were used as controls (11). In some assays the stimulation by IC were augmented with several factors like RF and or complements. In these experiments the anti-CII IC was prepared as mentioned before and then high level RF positive sera (494 IU/ml) and complements were added to the anti-CII IC and incubated for 1 h at 37°C in a cell incubator.

ELISA for detecting anti-CII-IgG antibodies:

The procedure was done as described in previous studies (11, 22, 29). IgG antibodies against CII was detected by ELISA by coating 2.5µg/ml of human CII antigen (Chondrex Inc.) on 96 well ELISA plate (Nunc) at 4°C overnight. The CII antigen was diluted in 1 parts of collagen buffer (Chondrex Inc.) and 9 parts of MQ water to a final concentration of 2.5µg/ml. After coating the plates were blocked with 150µl of PBS-1%BSA. Bovine serum albumin (BSA) was bought from Sigma-Aldrich, St.Louis, MO, USA. The serum samples were diluted to 1:100 in PBS and added to the wells coated with CII along with two in-house internal controls,
standards and negative control and left for 2 h on a shaker at room temperature (RT). 100µl of IgG CII secondary antibody (Jackson Immunoresearch Laboratories, West Grove, PA, USA) was added to the wells at a dilution of 1:10000 after washing the plate with PBS-0.05%Tween. Tween was bought from Sigma-Aldrich. The plate was left for 1 h on the shaker at RT. Substrate solution was prepared by adding P-nitrophenyl phosphate tablets (Sigma-Aldrich) to diethanolamine buffer. One tablet was added to 5ml of buffer. 100µl of substrate was added after washing with PBS-0.05%Tween. The plates were read at 450nm in an ELISA reader. The results were interpreted by using Deltasoft software (BioMetallics Incorporated, Princeton, NJ, USA). The cut off value for the CII antibody positive serum was determined as 29 Arbitrary Units (AU/ml), which was the 95th percentile for 100 healthy blood donors, as defined by previous studies (11). The in-house controls consist of 2 sera one with high Anti-CII antibodies and the other close to the cut off value for being CII antibody positive.

Activation and CD marker staining of granulocytes:
The granulocytes isolated from healthy donors were subjected to anti-CII IC stimulation at different time points from 1 hour, 3 hours, 6 hours, and 18 hours in an ELISA plate (Nunc) in 37°C in CO2 incubator with 150ml of 200,000 cells/well. The granulocytes were transferred to FACS tubes (BD Biosciences) and washed with PBS- 0.1%HSA. Cells were pelleted down to bottom of FACS tubes by centrifuging at 250g forces. A list of CD markers analyzed on the granulocytes and their significances are given in table1. Antibodies against the CD markers were all are of IgG1 isotype as unspecific binding used to be lower with mouse IgG1 antibodies than other IgG subclasses. Antibodies were added to the tubes and kept for 30 minutes at 4°C. CD9 antibody was added to every tube as they are expressed mostly by eosinophils and was used as a marker to separate eosinophils from that of the neutrophils, which are mostly CD16 positive, but have a lower expression of CD9 than the eosinophils. FC analysis was carried out to find the expression of activation markers like CD11b, CD66b, CD35, CD16, which are conjugated with fluorescence iso thiocyanate (FITC), (BD Biosciences). CD9 and CD32 were conjugated with phyco erythrin (PE), Beckman Coulter (Bromma, Sweden). Colour compensation of PE and FITC were done using beads (BD Biosciences). After washing with 0.1% PBS- HSA, the cells are fixed using 0.1% paraformaldehyde and kept in dark at 4°C until analysis. Non-specific binding was kept in check by using IgG1 isotype controls (BD Biosciences).
Cell surface markers | Present on | Function
--- | --- | ---
CD9 | Found more in eosinophils | Stains positive for granulocytes except neutrophils. Used for separating neutrophils from eosinophil population. Two rather distinct populations of cells with CD9+eosinophils and CD9-neutrophils were expected.
CD11b | Neutrophils, macrophages | Mediates adhesion by opsonisation and helps in phagocytosis, neutrophil aggregation and chemotaxis. CD11b should be upregulated by activating stimuli.
Cd16 (FcγRIII) | Neutrophils, macrophages | Low affinity receptor for Fc region of gamma Ig. It mediates phagocytosis by antibody dependent mechanism. CD16 should be downregulated by activating stimuli.
Cd32 (FcγRII) | Neutrophils, macrophages | Binds Fc region of IgG–IC. CD32 should be downregulated by activating stimuli.
CD66b | Neutrophils, eosinophils, macrophages | It is a cell adhesion molecule that can activate neutrophils. It is seen in high number in RA patients. Expected to be upregulated by activating stimuli (30).

Table: 1. Various granulocyte cell surface markers and their significance.

**Flow Cytometry:**

FC was done using flow cytometer FACS Calibur (BD Biosciences). After the samples were ready to be analyzed, fluidic start up was done to avoid air bubbles. Colour compensation was run the first time and the same compensation set up was linked to all future experimental analysis, provided when using the same fluorochrome antibodies. Voltage was adjusted to get the cell populations positioned correct in the worksheet and was set constant. After run the data was exported to a nearby analysis computer with BD FACS DIVA analysis software (BD Biosciences). Using this analysis software gating was done so that granulocytes were separated from rest of the contaminant cell population. The gating was done based on the forward scatter (FSC) and side scatter (SCC). Mean fluorescence intensity (MFI) was compared between NHS IC and anti-CII IC from histograms. The CD markers were analyzed for their up-regulation and down-regulation after stimulation with anti-CII IC, positive control IC or negative control sera.

**ROS Assay:**

The neutrophils produce ROS when they are activated by stimuli. IC bind to the Fc receptors and complement receptors present on the surface of granulocytes and activate them to produce ROS. DHR was used for measuring the ROS produced by granulocytes. DHR was added to the granulocyte in the plate where
the granulocytes were stimulated with anti-CII IC. The concentration of DHR used in
the assay was 2µg/ml and was diluted in PBS. DHR is a cell permeable substance and
gets into the cell. Activated neutrophils produce ROS, these ROS reacts with DHR
and converts it into rhodamine, a fluorescent dye that cannot pass out the membrane
and gets trapped within the cell. Negative IC controls made with NHS and two
positive controls one made with intravenous immunoglobulin coated on ELISA plate
(Endobulin5µg/ml) and Phorbol-12polymyristate 13acetate (PMA) diluted in solution
were used in most of the investigations.

ROS production was initially tried with flat-bottomed 96 well ELISA plate (Nunc) by
measuring the ROS response directly from the plate with a fluorescent scanner
Fluoroscan Ascent®, Thermo Electron (Thermofisher Scientific, The kinetics of ROS
response was measured following the time points from 0 h, 15 min, 30min, 1 h, 2 h, 3
h. The ROS was thereafter measured using flow cytometer. The cells along with DHR
dye were transferred to FACS tubes (BD Biosciences). The absorbance range was set
to FITC range 488nm for DHR, as FITC has the closest absorbance range for DHR.
The MFI values of the cells stimulated with IC and the cells, which are not stimulated,
were compared.

**Priming of granulocytes:**

In some experiments the granulocytes separated from human
blood were subjected to cytokines like TNF-α (10ng/ml), GM-CSF (100ng/ml) and
both TNF-α (10ng/ml) and GM-CSF (100ng/ml). These cytokines were from R&D
Biosciences, UK. This procedure was done prior to the anti-CII IC stimulation for 1h
at 37°C in Co2 incubator.

**Measurement of cytokine production by IC-stimulated granulocytes with cELISA:**

The granulocytes were cultured with anti-CII IC stimulation in
cell culture medium in ELISA plate (Nunc) with 300,000cells/well. The activated
granulocytes produce cytokines out to the cell culture medium and the supernatants
from different time points from 0 h, 6 h, 24 h and 48 h were harvested and stored for
analyzing amount of cytokine. They were measured by sandwich ELISA. The
cELISA was done following the protocols used in pervious studies (10, 11). Anti-
TNF-α (MAB610)(5mg/ml), anti-IL-1β (MAB601) (0.2mg/ml) and anti-IL-8
(2mg/ml) (R&D Biosciences) were coated on three different plates and 50µl of
supernatant were added to wells in the plate and left for 2h incubation on a shaker. As
the neutrophils produce a lot of IL-8 cytokine the samples for IL-8 ELISA were
prediluted to a dilution of 1:200 whereas the supernatants were analyzed undiluted for
TNF-α and IL-1β. Then the detection antibody for Anti-TNF-α (BAF210)
(0.5mg/ml), anti-IL-1β (BAF201) (0.125mg/ml) and anti-IL-8 (0.2mg/ml) (R&D
Biosciences) were added and kept on the shaker for 2h. The substrate TMB (Dako
Sweden AB, S-114 79 Stockholm) is added and left until bright blue colour develops.
The reaction was stopped by adding 1M sulphuric acid. The plates were read by
ELISA reader. The data was interpreted with Deltasoft software (BioMetallics Incorporated) and compared between the cytokine released from wells coated with NHS and CII antigen.

Statistical Analysis:

Statistical analyses were done using the Mann-Whitney non-parametric test that does not assume normal distribution of data. Non-parametric test was used as the anti-Anti-CII antibodies are dichotomously distributed among patient populations and it had been used in our previous studies. The calculations were done with the JMP 9.0 software.
RESULTS:
Purity determination of granulocytes:
The cells stained with Türk solution showed distinct multi-lobed nuclei and few cells with mononucleus. The percentage of purity obtained by this method was 98%.

Viability of granulocytes:
The viability assay showed that 91% of the cells did not take up the dye and 9% of the cells were stained. This shows that there was cell membrane destruction in the 9% of the cells. Hence the purified granulocytes by gradient centrifugation had 91% of viable cells.

Purification of granulocytes by Percoll and Ficoll:
Evaluation of purity between the granulocytes purified by 2 procedures using percoll and ficol showed that percoll was a better medium for purifying granulocytes. The percentage of purity obtained by using ficoll was 96% and the percentage of purity obtained by using percoll medium was 98%.

Evaluation of CD marker expression on anti-CII IC-stimulated granulocytes:
No real significant difference in the CD marker expression was found between NHS+CII and anti-CII IC when using the anti-CII antibody concentrations used before (figure 7), which have proven to stimulate cytokine production from monocytes efficiently in our previous studies (10, 11). There was no further effect by using a concentration of anti-CII antibodies that was four times higher (figure 7) than what had been found to be optimal for monocyte stimulation (figure 18) The addition of complement and RF to the HP sera showed a minimal trend to increase the response but was not as strong as the positive IC control (figure 7). The changes in CD marker expression were optimal after 1 h of incubation and thereafter gradually decreased as shown in figure 7.

We then performed a cohort study of 20 samples from same group with anti-CII positive RA and anti-CII negative RA samples as we had earlier investigated concerning monocyte stimulation by anti-CII IC (11). The results were found to be contradictory to what was expected as the anti-CII negative RA samples showed a slight up-regulation of CD11b and significant down-regulation of CD16 (lower than positive IC control) as compared to the anti-CII positive samples. There was no significant difference in CD32 and CD66b between the 2 groups. Positive IC control showed a down-regulation of CD16 but was not lower than CII antibody negative samples. The positive IC control showed a marked down-regulation for CD32 and up-regulation of CD11b. CD66b was stable for all 3 IC as in figure 8.
The anti-CII positive and anti-CII negative sera used in the previous experiment had been treated differently during the years after sampling during the years 1995-2000, where the anti-CII negative sera had been repeatedly thawed and frozen in numerous studies. Due to earlier anti-CII studies, the anti-CII positive sera treated the same way had been used up, and we had obtained new and well-preserved aliquots of the anti-CII positive RA sera to perform this study. We suspected that this difference in treatment might be the cause of the unexpected results. We therefore performed another proof-of-concept study with ten negative healthy control sera treated like the anti-CII positive RA sera. The study with 10 patient serum samples with high anti-CII antibody levels and 10 normal human sera showed no significant differences between the groups. When granulocytes were further refined by CD9 positivity and negativity we were able to find a better response as expected and there was a tendency that anti-CII IC induce granulocytes. Figure 9e and figure 9f (CD9 negative neutrophils) show a better response when compared to figure 9a and figure 9b (all granulocytes).
Evaluation of CD marker expression by granulocytes with IC stimulation by FC:

Figure 7. MFI of 5 CD markers on granulocyte sample treated with CII antibody containing sera and the addition of RF and complement. HP, HP+RF, complement sera+ HP (HP+C), 4 times concentrated HP (4x HP), RA serum from two more RA patients with high anti-CII levels (RA001 and RA002), negative controls (NHS+CII) and positive IC control.
Evaluation of CD marker expression by granulocytes with IC stimulation by FC:

Figure 8. MFI of CD markers on granulocytes incubated with IC formed with 10 anti-CII antibody positive RA (anti-CII positive RA) sera, 10 anti-CII antibody negative RA (anti-CII negative RA) sera from the same RA patients cohort along with positive IC control stimulated cells run by FC. The dots represent individual samples.
Figure 9. MFI of CD markers on granulocytes treated with 10 anti-CII positive RA and 10 healthy control sera (healthy ctrl) by FC. CD9 negative cells and CD9 positive cells represented in figure 9b, 9c, 9d, and 9e. The dots represent individual samples.
Measurement of ROS released from granulocytes by FC with IC stimulation:

Measurement of ROS by plate method was not optimal and showed high background reactivity; hence FC method was used thereafter.

In early experiments, the anti-CII IC showed a gradual increase in ROS production at 2 h time point and the response was higher at 4 h (figure 10). The scatter picture showed aggregated cells as in figure10, which lead to a refinement of our granulocyte purification protocol.

After refining our purification protocol the scatter pictures showed less of granulocyte aggregation. Granulocytes stimulated with HP sera showed no significant difference in ROS production as compared to negative controls. The positive IC controls induced in increase in ROS production. Addition of RF and/or complement containing sera to the HP serum did not change ROS production (figure 11).

We thereafter tried to increase the concentration of anti-CII on our IC. Granulocytes stimulated with 4xHP sera showed no difference to the negative control IC neither used directly, or after addition of RF and fresh serum as a source of complement (figure 12).

Priming of granulocytes by the cytokines TNF-α and GM-CSF at concentrations reported to activate neutrophil granulocytes optimally before anti-CII IC stimulation increased the ROS production in general, but did not contribute specifically to the ROS induction by anti-CII IC (figure 13). Of the cytokines used TNF-α dramatically changed the kinetics of response, both when used alone (figure 13b) and in combination with GM-CSF (figure 13d).

We then performed a cohort study of 20 samples from same group with anti-CII positive RA and anti-CII negative RA samples as we had earlier investigated concerning monocyte stimulation by anti-CII IC (11). The results were found to be contradictory to what was expected, with a significant decrease in ROS production among the anti-CII positive samples when compared to anti-CII negative serum samples. The positive IC control and PMA showed a high production of ROS (figure 14).

When the ten anti-CII negative RA sera were exchanged with ten healthy control sera that before the experiment had been stored as rigorously as the anti-CII positive sera, no difference was found between the MFI values of anti-CII positive and normal human serum. Positive IC control and PMA showed very high ROS production (figure 15).
Measurement of ROS released from granulocytes by FC with IC stimulation:

Figure 10. Production of ROS by PMA and anti-CII IC in comparison to NHS+CII by FC. The scatter picture of granulocytes on the right side.

Figure 11. Time kinetics of ROS production with RF and complement added to HP sera by FC. One serum containing high anti-CII levels (HP), with addition of a high level RF positive serum (HP+RF), complement (HP+C) or both RF and complement (HP+RF+C), negative control (NHS+CII) and positive IC control.
Figure 12. Time kinetics of ROS production with RF and complement added to the RA serum containing high anti-CII levels, and where the serum had been concentrated four times more than in earlier experiments either alone (4xHP), with subsequent addition of RF-containing serum (4xHP+RF), complement (4xHP+C), or the combination of RF and complement (4xHP+C+RF), negative control (NHS+CII) and positive IC control.

Production of ROS by anti-CII IC stimulation from granulocytes primed with cytokines

13a)  

13b)  

13c)  

13d)
Figure 13. Production of ROS by anti-CII IC stimulation from granulocytes primed with cytokines. 13a) Control experiment for ROS production without cytokine priming. 13b) ROS production of granulocytes primed with TNF-α (10 pg/ml), 13c) GM-CSF (100 pg/ml) and 13d) the combination of GM-CSF and TNF-α.

Figure 14. ROS induction by IC made with ten anti-CII positive RA sera and 10 anti-CII negative RA sera from the same cohort along with positive IC control (control IC) and PMA run by FC. The dots represent individual investigations.

Figure 15. ROS induction by IC made with ten anti-CII positive RA sera (anti-CII+ RA) and 10 normal human sera (healthy ctrl), a positive IC control (IgG control) and PMA were run by FC. The dots represent individual investigations.
Measurement of pro-inflammatory cytokine production from granulocytes by cELISA at 6 h and 24 h.

Initially we performed studies on the kinetics of cytokine production (for TNF-α, IL-1β and IL-8) from purified granulocytes stimulated with anti-CII IC made with our anti-CII reference serum HP. As shown in figure 15, anti-CII IC seemed to induce higher levels of all the three investigated cytokines as compared to the negative control at all investigated time points, but the ratio between IC and control were generally highest at 6 hours.

When ten anti-CII positive RA were compared with ten anti-CII negative RA sera, the OD values were very low, below the standard curve and showed very low production for all pro-inflammatory cytokines (data not shown). There was however a tendency for lower cytokine production from anti-CII IC produced with anti-CII positive sera as compared to the controls with anti-CII negative RA sera. This tendency was the opposite to what we had expected, in parallel to the changes in CD markers in figures 8a and 8b, and the induction of ROS (figure 14).

As we presumed that the pre-analytic difference in handling of the anti-CII positive and the anti-CII negative RA sera might have influenced cytokine production in the same way as we had presumed for the induction of CD markers and ROS, we exchanged the anti-CII negative RA sera for 10 healthy control sera. In this experiment we found no difference in induction of IL-8, TNF-α or IL-1β as in figure 17.

As none of the two experiments with the ten high level anti-CII sera showed any positive effect of anti-CII IC on granulocyte cytokine production, we suspected that there might be some general flaw in our preparation of cells. I therefore repeated the stimulation of PBMC with anti-CII IC exactly as was the starting point of earlier studies on anti-CII IC in the group (10, 11). Supernatants from PBMC cultured on IC produced with anti-CII positive RA sera showed significantly higher levels of all the three investigated pro-inflammatory cytokines than the supernatant from control wells (figure 18).
Measurement of Cytokine released from granulocyte and PBMC cell cultures by cELISA.

16a) 16b)

![Graphs showing cytokine production](image)

**Figure 16.** Time kinetics of IL-1β, IL-8, TNF-α production by granulocytes treated with HPsera+ CII (CII IC) against Normal human sera+ CII (NHS+CII) at 6 h, 24 h, 48 h. This experiment is representative for three kinetic investigations performed with the same setup.
Figure 17. Comparison between levels of IL-8 (figure 17a) IL-1β (figure 17b) and TNF-α (figure 17c) produced by granulocytes stimulated with IC made with anti-CII positive RA sera and controls with NHS at 6 h and 24 h. Dots represent individual serum samples.
Figure: 18. Cytokine levels in supernatants from PBMC cultured on IC made with anti-CII positive RA sera or with normal human sera (healthy ctrl). Dots represent individual cell cultures.
Clinical study involving patients with acute onset:

From the clinical study of 95 patients with three other diagnoses (not RA) with acute onset arthritis, anti-CII antibodies were found to be positive in 8.4% of the total patients investigated by anti-CII IgG ELISA. The fractions of anti-CII positive patients were very similar in all the investigated diagnoses, and also comparable to what has earlier been described from our group concerning patients with recent diagnosis of RA (11). Details of the data are shown in table 2.

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>No of patients investigated</th>
<th>No of anti-CII positive patients</th>
<th>% of patients with elevated levels of anti-CII antibodies</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gout</td>
<td>23</td>
<td>2</td>
<td>8.7</td>
</tr>
<tr>
<td>Bacterial reactive arthritis</td>
<td>24</td>
<td>1</td>
<td>4.2</td>
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<tr>
<td>Viral reactive arthritis</td>
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<td>2</td>
<td>14.3</td>
</tr>
<tr>
<td>All patients with reactive arthritis</td>
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<td>3</td>
<td>7.9</td>
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<td>(bacterial and viral)</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>RS3PE</td>
<td>34</td>
<td>3</td>
<td>8.8</td>
</tr>
<tr>
<td>All patients in my investigation</td>
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<td>8</td>
<td>8.4</td>
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<tr>
<td>Early RA data from Mullazehi (11)</td>
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<td>8.8</td>
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<tr>
<td>Healthy controls data from Mullazehi (11)</td>
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</tr>
</tbody>
</table>

Table 2. Results from the measurement of anti-CII antibodies present in patient samples with acute onset.
DISCUSSION:

Granulocytes are involved in inflammatory diseases and are seen in high numbers in the synovial fluid of RA patients. Enzymes from granulocytes can also erode the thin layer of small proteoglycans covering the intact cartilage surface (27), and thereby allowing anti-CII antibodies to get access to cartilage CII to create surface-bound IC, and thereby induce an inflammatory response. Reduction of blood granulocytes alleviate RA symptoms (31). We therefore chose to investigate the role of granulocytes in inflammatory responses induced by anti-CII antibodies. The results from our analyses were essentially negative. The results from the experiments show that positive IC controls stimulate granulocytes and the experimental set-up works well, but anti-CII IC do not stimulate granulocytes. In the ROS experiments and in the evaluation of CD markers on granulocytes, control IC consisting of high-density IgG-coated surfaces induced ROS production and changed CD marker expression in the expected way, and for the cytokine experiments, parallel investigations of production by PBMC in the same experimental systems worked as expected from earlier investigations in the group (11, 32). Therefore the results from the experiments show that positive IC controls stimulate granulocytes and the experiment set-up works well, but anti-CII IC do not stimulate granulocytes. Investigations with four times more concentrated anti-CII antibodies, well above the stimulus necessary for the PBMC activation, also yielded no results. We thereafter tried to augment the signal by addition of RF, as has been reported to work for the induction of cytokines from macrophages stimulated with ACPA-containing IC (33, 34) and we also tried to let the anti-CII IC activate the classical complement pathway, as granulocyte activation might be induced by cell surface complement receptors (35). Neither RF, nor complement activation or the combination of RF and complement could augment the granulocyte responses to anti-CII IC. Priming with cytokines with the cytokines TNF-α and GM-CSF resulted in increase in background signal, but did not increase the specific response to anti-CII IC. In the final experiments we used concentrated sera, with ten times higher anti-CII levels as compared to what has been used in earlier test systems in the group, and also here there were no specific responses to anti-CII IC measured as changes in CD marker expression, ROS induction or cytokine production. The only trace of a positive response was for CD markers after specific gating for CD9 positive eosinophils and CD9 negative neutrophils. The gated CD9 negative cells showed a mild positive response in the expected direction for CD11b and CD16 (figure 9d and 9f), the response was better than whole population (figure 9a) but the response was not very high. The results from these figures suggest that there is a mild tendency for the anti-CII IC to initiate a response in granulocytes. Our results were thus essentially totally negative, and imply that either do the granulocytes not respond to anti-CII IC, or that PBMC need much lower stimuli than granulocytes.

In one of our final set of experiments we compared the effect of anti-CII positive and anti-CII negative sera obtained from the same RA cohort, and obtained results inverse
to what we had expected concerning CD marker expression and ROS induction (figures 11 d and 9). We believe that these results contradicting our hypothesis were due to the fact that the anti-CII negative RA sera used were not equally treated as the positive sera. We had obtained new and never thawed vials with the anti-CII positive sera from Karolinska Institute (as we had used up earlier aliquots), whereas the anti-CII negative samples had been used in many studies here in Uppsala, and had been frozen and thawed at multiple occasions. The latter may contain IC and aggregates, and may be contaminated with bacterial LPS as these were frozen and thawed many times. As we lacked control RA sera treated in the same way as the anti-CII positive RA sera, NHS controls were used in the final experiments, although the validity of the experiment would be stronger having used equally treated anti-CII negative RA sera from the same cohort.

The development of the project was delayed by early positive responses concerning cytokine induction and ROS induction by anti-CII IC, both of which we could not repeat later, and where not data are shown in this report. These earlier responses might be due to aggregation of granulocytes for ROS induction, and contamination with mononuclear cells concerning the induction of cytokine responses by anti-CII IC. Granulocytes purified without aggregation and contamination showed no response, neither for the induction of reactive oxidative species nor for production of cytokines.

Glycosylation of IgG antibodies play an important role in binding to FcγR. IgG lacking galactosyl residues had defects in binding to the Fc receptors on cell surfaces (36-38). Other researchers have however shown results implying that there is no significant difference in binding between IgG with and without galactosyl residues to the Fc receptors (39). Whether human anti-collagen antibodies express different levels of galactosylation as compared to IgG with other specificities has not been investigated. Pathogenic mouse anti-CII antibodies that can induce chronic arthritis do however express glycosyl residues, as treatment with the enzyme Endo-S that rips glycosyl residues from IgG molecules alleviated CAIA symptoms (38).

The CAIA method involves mab against major CII epitopes and they induce arthritis by binding to CII cartilage (40). The importance of the innate immune system in this experimental model have been shown to be extensive in the mouse CAIA model, as mice lacking the adaptive immune system (T and B cells) also develop arthritis after anti-CII injection (26). The CAIA model induced arthritis in healthy mice with normal cartilage. From the findings from Hugo Jasin it seems that the binding of anti-CII antibodies to cartilage is dependent on cartilage damage. The healthy joints had a low binding of anti-CII antibodies when compared to joints where the covering protein surface had been destroyed by neutrophil elastase (41). This implies that other immune cells might recruit neutrophil to the joint space and destroy joints prior to CII antibody binding.
Anti-CII antibodies are involved in acute onset of RA (11, 22). In an earlier study from our group where we investigated very early synovitis patients and looked for an association between anti-CII antibodies appearing pre-diagnosis and the establishment of RA diagnosis within one year, we found no such association. There was however a rather high percentage of patients with elevated anti-CII levels who subsequently developed gouty arthritis and reactive arthritis, two diagnoses often accompanied with acute disease onset (29). The patient group contained however very few patients developing gouty arthritis and reactive arthritis, and more patients with these diagnoses must be investigated to test this hypothesis. A set of 95 patients with acute onset arthritis was now tested for anti-CII antibodies. All the three diagnostic groups showed very similar results as Mohammed Mullazehi’s earlier findings (11) that only a low percentage (8.4%) of early RA patients were anti-CII antibody positive. However, none of the anti-CII positive patients investigated in my thesis work had such high levels of anti-CII antibodies as found earlier to be associated with cytokine production in vitro (>450 AU/ml) (11). My results thus indicate that anti-CII antibodies not are associated with acute onset arthritis in general, irrespective of diagnosis.

There are other ways to try to enhance any granulocyte responses to anti-CII IC, which we have not yet investigated. The complement fragment C5a might be used to prime granulocytes (35). Granulocytes from RA patients, either from joints or peripheral blood might be used, as RA granulocytes have another phenotype concerning CD markers compared to healthy control granulocytes (30). Probably other assays like migration assays and phagocytosis activity measurements could have been made. Neutrophils react to stimuli in different ways during infection and with IC, as the IC response has been shown to be mediated by CD16 (FcγRIIIb), whereas blocking of FcγRIIIb did not affect the phagocytosis of microbes (42). IC induces ROS via FcγRII and FcγRIIb (43). The anti-CII IC used in our experiment is a surface-bound insoluble IC and the generation of ROS takes place within the cells (42, 44), and might not respond to surface-bound anti-CII IC; our control IC that repeatedly induced ROS responses were however also surface-bound, a finding arguing against this possibility. Another not yet investigated approach would be to co-culture granulocytes with monocytes to see whether monocyte activation induced by anti-CII IC secondarily might activate the granulocytes. Previous investigations in the group have shown that synovial fibroblasts that individually do not respond to anti-CII IC will be activated in such co-culture systems (45).
CONCLUSION:

Granulocytes were not stimulated by anti-CII IC and results from all three types of experiments show that there is no response or if so a very weak response from the granulocytes. Probably the anti-CII density is too low for granulocyte activation in our system, as the cells responded to surface-bound control IC with very high IgG density. It can be concluded that granulocytes respond to other stronger IC signals (positive IC control). Anti-CII IC might have an arthritis-initiating effect by stimulation of PBMC as has been shown earlier in the group and repeated by me.

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