Identification of T cells undergoing/escaping tolerance to self-type II collagen in an autoimmune animal model for rheumatoid arthritis

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Degree project in biology, Master of science (2 years), 2011
Examensarbete i biologi 45 hp till masterexamen, 2011
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Abstract

Objectives: To investigate T cells under tolerance mechanisms to self collagen type II (CII) and behavior of T cells that escaped from those mechanisms by using different transgenic mice.

Methods: T cell receptor transgenic mice that we called Vb12-transgenic (Vb12-tg), HCQ3-tg mice and DR4/DR1 transgenic mice have been bred. CIA has been induced in Vb12-tg and DR4 transgenic mice. HCQ3-tg strain is used as a source of naïve T cells with a TCR specific for CII. Cells from HCQ3-tg mice are labeled with CFSE and then transferred to naïve MMC mice (express heterologous CII) which are known to be tolerant to some extent.

Vb12tg mice have been crossed with mice which have congenic TCRα fragment (so called DBA mice). By taking into consideration that the number of copies of TCRα chain may have an impact on activation of antigen specific T cells, we analyzed activation patterns of Vb12tg mice. For this purpose, cells from immunized mice were cultured with naked and galactosylated form of CII. CD40L upregulation was investigated.

Collagen induced arthritis (CIA) susceptibility has been compared in different DR4 mice expressing human MHCII molecule instead of mouse MHCII, which enables us to study human molecules in mouse models of arthritis.

Results: Firstly, numbers of copies of TCRα chain from DBA/1 strain was important in response to post-modified collagen II peptides in Vb12-tg mice. However arthritis development did not depend on how many TCRα chain locus the mice had.

Secondly, we transferred labeled T cells from naïve HCQ3tg mice to MMC mice which expressed rCII in the cartilage. This means the antigen was already expressed in the mice and T cells were tolerized up to a point. We found that, arthritogenic peptides in this case rat CII (rCII) that leaked from cartilage activated T cells in the periphery within three days.

Lastly, expression patterns of DR4 and DR1 molecules in transgenic mice thymus were analyzed and CIA susceptibility together with anti-CII antibody production was determined. According to results, no arthritis was observed in different DR4 transgenic mouse founders while anti-CII antibody titers were significant.
**LIST OF ABBREVIATIONS**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>APC</td>
<td>antigen presenting cell</td>
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<tr>
<td>APRIL</td>
<td>a proliferation inducing ligand</td>
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<tr>
<td>BFA</td>
<td>Brefeldin A</td>
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<tr>
<td>CFA</td>
<td>complete Freund adjuvant</td>
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<tr>
<td>CFSE</td>
<td>Carboxyfluorescein succinimidyl ester</td>
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<tr>
<td>CIA</td>
<td>collagen induced arthritis</td>
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<tr>
<td>CII</td>
<td>collagen type II</td>
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<tr>
<td>cTEC</td>
<td>cortical thymic epithelial cell</td>
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<tr>
<td>DC</td>
<td>dendritic cell</td>
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<tr>
<td>EBV</td>
<td>Ebstein-Barr virus</td>
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<tr>
<td>HLA</td>
<td>human leukocyte antigen</td>
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<tr>
<td>IFA</td>
<td>incomplete Freund adjuvant</td>
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<tr>
<td>IFN</td>
<td>Interferon</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
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<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
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<td>MMC</td>
<td>mutated mouse collagen</td>
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<tr>
<td>MMP</td>
<td>matric metalloproteinase</td>
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<tr>
<td>MQ</td>
<td>Macrophages</td>
</tr>
<tr>
<td>mTEC</td>
<td>medullary thymic epithelial cell</td>
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<tr>
<td>NSAIDs</td>
<td>non-steroid anti-inflammatory drugs</td>
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<tr>
<td>PTPN22</td>
<td>Protein tyrosine phosphatase, non-receptor type 22</td>
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<tr>
<td>RA</td>
<td>rheumatoid arthritis</td>
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<tr>
<td>RANKL</td>
<td>receptor activator for nuclear κ B ligand</td>
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<tr>
<td>RF</td>
<td>rheumatoid factor</td>
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<tr>
<td>TCR</td>
<td>T cell receptor</td>
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<td>TNF</td>
<td>tumor necrosis factor</td>
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Introduction

Rheumatoid arthritis (RA) is a chronic autoimmune disease, a result of inflammation response that mostly affects synovial joints leading to cartilage and bone destruction. RA affects 1% of the world population in which women are more prone to disease than men. Synovial inflammation is often the initial clinical event in the arthritis and occurred by the secretion of inflammatory cytokines such as TNF-α, IL-1, and IL-6 from stimulated macrophages like synoviocytes. However, the mechanism of the activation and how the disease starts are yet not clear.

Symptoms and treatment

Hallmarks of RA are given as pain, swelling of at least three of the joints, morning stiffness for more than one hour, redness and reduction in the function, rheumatoid nodules, and rheumatoid factor (RF) in the serum. Symptoms can start at different rates and intensities in different joints. Criteria that are listed above can be used for diagnosis. Additionally, antibodies against citrullinated peptides or RF can be listed in these biomarkers. However it should be noted that not all RA patients carry all these symptoms or biomarkers.

Pain management is mostly done with non-steroid anti-inflammatory drugs (NSAIDs). There are some drugs that have been used in the clinic over 50 years to control the disease, like corticosteroids and antimalarial drugs. However, new therapeutic targets are continuing to be discovered. These newly designed therapies are mostly on inhibiting the cytokine actions. For example, anti-TNFα and IL-1 receptor antagonist are licensed to be used clinically.\[^{14}\]

Risk factors

Environmental factors

For a given environmental factor, such as smoking, microorganisms, stress and age, it has not been concluded that they are the only reason why people get RA. However it has been suggested that they may have an impact on the onset, progress or the rate of the disease. For example, in genetically predisposed individuals, citrullination of self-proteins can more often be found in smokers than non-smokers, implying smoking can increase the auto-antigen responses.\[^{4}\] Infectious agents are another non-genetic factors that may contribute to the disease. There are some bacteria or virus that has been identified as potential arthritogenic agents. However none of the microorganisms has been found as a causative agent. Among the microorganisms mycoplasma and Ebstein-Barr virus (EBV) are the most striking examples, since high titers of the anti EBV antibodies have been found in RA patients.\[^{8}\]. Additionally, in animal models immunological crossreactivity between cartilage protein and mycoplasma protein has been observed.\[^{9}\].
Genetic factors

Genetic predisposition has a great role in the development of RA. The biggest association has been shown in human leukocyte antigen (HLA) locus; specifically DR genes. Even though there are a vast number of DR4 alleles, only some of them are associated with RA. There is a significant increase in the expression of DRB1*0101, DRB1*0401, DRB1*0404 compared to healthy controls. These DR1/DR4 subtypes vary at hypervariable region with one to three amino acid and they share a pattern which makes it prone to RA. The similarity between amino acid is observed at 67th, 70th and 71st positions of the β chain of MHC II which gives difference between size and charge from other molecules. Polymorphic parts of the protein such as negative charge on 70th and 71st positions coming from aspartic acid and glutamic acid give functional difference to disease associated MHC II molecules because αβ T cell receptor (TCR) binds both the peptide to be presented and the polymorphic parts of the MHCII molecule. The most often suggested mechanism is the selection of auto reactive T cells by predisposed MHCII molecules in the thymus as well as in the periphery. MHCII molecules help auto reactive T cell clones to survive by presenting arthritogenic peptide to T cells.

MHC genes are not the only cluster of genes that can influence the disease. There are other loci that have been confirmed that also may be important. PTPN22 is a T-cell activation gene and a missense mutation, arginine to tryptophane at the 620 position of the gene, is correlated to RA. The mutation is a gain of function mutation and it makes the T cell activation pathway stronger and T cells produce more IL-2 than the wild type counterparts.

Another gene which shows significance in disease association is STAT4, a transcription factor that converts naïve T cell into Th1 helper cell. However, a direct role of STAT4 in RA has not been clearly established.

Additionally, genome wide studies have spotted some more additional genes. Single nucleotide polymorphisms in 6q23 and chromosome 9 which maps to genes coding for tumor necrosis factor receptor associated factor 1 (TRAF1) and complement component 5 (C5) have been identified as risk factors in RA.

Pathogenesis of RA

Inflamed joint synovium contains large numbers of infiltrated cells, including activated T cells, B cells, macrophages, neutrophils and mast cells. Except for the environmental factors, most of the genetically related factors show the significance of T cell contribution in RA. It is known that T cells should be activated in order to function as an effector T cell. HLA-DR1/DR4, PTPN22 and STAT4 are all related with T cell immunology; either in selection of auto-reactive T cells, presentation of arthritogenic peptides or in the downstream cascade to keep them activated. It has been considered that RA is a Th-1 driven response as interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α), which belongs to the classical cytokines of Th1 responses are the prominent cytokines as the disease progresses. On the other hand, IFN-γ−/− mice develop more severe arthritis than the wild type mice and, IL17−/− mice were unable to develop collagen induced arthritis (CIA) whereas over expression of IL-17 may exaggerate development of disease, suggesting that TH17 cells may also participate in RA. In addition to these T cell subsets, FOXP3+ CD4+CD25+ regulatory T cells (Tregs) are present in the synovium but with spoiled functions. Synovial T cells express early activation markers CD69 and
CD40L which promote B cell proliferation and immunoglobulin production. Synovial CD4 T cells also express CD45RO, a memory cell marker. Cytokines are active players in each part of the disease and responsible for the continuation of the disease and the damage in the joints. TNFα, IL-6, IL-12, IL23, TGF β, IL15 and IL-18 are secreted by myeloid dendritic cells and stimulate different T cell subsets. As response to stimulation, T cells expand and produce TNFα, IFN-γ, and IL17 and activate macrophages. Macrophages are believed to be a first-class source of pro-inflammatory cytokines namely IL-6, RANKL and TNFα in big amounts. They attract inflammatory cells to the site of inflammation by secreting chemokines. They secrete matrix degradation enzymes and they activate osteoclasts in the bone. Therefore, macrophages as well as osteoclasts are responsible from the bone degradation to a certain extent. They activate dendritic cells and help them maturate to become antigen presenting cells (APCs). Then in turn APRIL is produced by dendritic cells and makes B cells accumulate to the joints. B cells have been suggested that they have a role in disease progress. They clonally expand upon activation in an antigen specific manner and differentiate into antibody producing cells like RF producing IgMs and IgGs as well as antibodies against citrullinated peptides. These can then promote immune complex formation and production of cytokines and chemokines. They also act like APCs and stimulate T cells.

Neutrophils are attracted to the site of inflammation and activated by complement proteins and immune complexes. Neutrophils hence contribute to disease by producing cytokines and creating a hypoxic environment in the joints. This change in homeostasis and production of cytokines make “aggrecanases and matrix metalloproteinase” produced. These enzymes are responsible for the cartilage destruction (Figure 1).

CIA

CIA is a mouse model of RA. CIA is dependent on both B and T cells and susceptibility is, like RA, linked to the expression of specific MHCII. Induction of the arthritis mostly requires a strong adjuvant like Freund complete adjuvant (CFA). In this kind of arthritis, Th1 response would be observed with IFN-γ and IL-12 production together with antibody secretion. It should be noted that, when incomplete Freund adjuvant (IFA) is used, IL-17 production may increase and response may be shifted to a more TH2 response with more IgG1 antibody being produced.

Collagen type II (CII) is a candidate auto-antigen because it constitutes the main protein of hyaline cartilage and because of the fact that B cells and T cells give response to CII in RA patients. CII bears an immunodominant T cell epitope at position 259-273 and also has an ability to bind both HLA-DR4 and HLA-DR1. Even though animal studies demonstrate that immunization of animals with CII can lead to a RA-like autoimmune disease, it is not the sole factor that promotes the disease. Initiation is directly connected to expression of certain class II molecules, I-A^q and I-A^r. Both of the molecules are capable of binding to CII and present the peptide to T cells even though they do not contain sequence homology in the polymorphic regions like in the DR molecules in human. While 259-270 part of CII is the immunodominant epitope for A^q mice, it is 442-456 in A^r mice. This means that the response against CII can differ between different strains.

CIA is generally induced by intradermal injection of heterologous CII and H-2q mice can develop CIA upon immunization with human CII (hCII), rat CII (rCII) or bovine CII (bCII). The only difference
between heterologous CII and the mouse collagen occurs at position 266 within the 259-273 epitope. Mouse collagen bears an aspartic acid while heterologous collagen has a glutamic acid. Mouse strains are generally resistant to arthritis upon immunization with homologous CII molecule and T cell response against self-collagen is hard to detect. Furthermore, affinity of I-A\(^q\) molecule binding to self-collagen is lower than heterologous CII. In order to investigate this, a mouse model called MMC has been developed in which mice express a mutated mouse CII with the human/rat version of the CII259-270 immunodominant epitope in a cartilage restricted fashion. In this mouse model, aspartic acid in position 266 is replaced with glutamic acid. It has been observed that CIA is partly reduced in MMC mice and T cell response is shown to be decreased as well, but not totally diminished. This shows that T cells are tolerant to heterologous CII in MMC mice therefore have a decreased response. However tolerance can be broken down with an unknown mechanism so that mice can still develop some arthritis\(^{[18]}\).

Both heterologous and homologous CII display lysine residues at position 264 and 270 within 259-273 epitope. One important feature with these lysine residues are that they may be posttranslationally modified by hydroxylation and subsequent glycosylation. This may change the affinity of the binding of the peptide to the MHC II molecule and each modified form also seems to be recognized by distinct T cell clones. Removal of carbohydrate residues from the collagen molecule makes it less arthritogenic; hence the modifications are accepted to be significant for the disease. There are four different options in posttranslational modifications of CII in 259-273 epitope; lysine can stay as it is (K), it can be hydroxylated (HyK), can be galactosylated (GalHyK) and finally both glycosylated and galactosylated (GlcGalHyK) \(^{[19]}\).

These modifications are done by chondrocytes before triple helix structure of collagen is completed. It is not known to what extend these modifications are done however it is possible that the levels of modification may vary, depending on the status of the chondrocytes. Each of these modifications is thought to be recognized by polyclonal T cell sub-populations even if they all recognize the CII259-273epitope in a sequence restricted manner. In 2000, Malmström \textit{et al.} showed that T cell response is mainly directed towards galactosylated form of the peptide.\(^{[18]}\)

\textbf{Figure 1.} Immunodominant CII\textsubscript{256-270} peptide. Post-translational modifications which are hydroxylation and galactosylation as well as the naked form of the peptide at 264\textsuperscript{th} lysine residue are
shown in the figure. One amino acid difference between mouse collagen and heterologous collagen at 266th position is also depicted\(^\text{(19)}\).

**Mouse genetics**

In murine models of arthritis, it has been shown that genetic mechanisms play a significant role. Throughout this study, transgenic mice, which were previously established, have been used. By means of transgenic mice, it was possible to mimic the clinical autoimmune arthritis and to study how T cells and MHCII molecules affect disease susceptibility and regulation.

To begin with Vβ12-tg mouse, it has higher susceptibility to arthritis than a B10Q mouse and strongly biased T cell response to galactosylated form of CII (259-270) epitope. However only the CII-specific β-chain of the T cell receptor is transgenically expressed in the Vb12-tg mouse and it can therefore combine with different endogenous α-chains resulting in different T cell clones. However, the transgenic β-chain was originally cloned from the DBA/1 mouse and fails to form a functional CII-specific TCR when expressed on the B10Q background. In order to increase the number of CII specific T cells, Vβ12-tg mice was crossed with B10Q mice expressing a congenic TCRα locus fragment originating from DBA/1 mice\(^\text{(20)}\).

![TCR of wild type, Vβ12-tg and Vβ12.DBA mice.](image)

HCQ3-tg mouse is a newer transgenic model which expresses T cell specific for galactosylated form of CII epitope. Difference between HCQ3-tg and Vβ12-tg mice is that the T cells in HCQ3 express CII specific TCR α and β chain together. As a result, HCQ3 mice have T cells for galactosylated CII epitope in higher frequencies.
MMC mice as described earlier express heterologus CII in a cartilage specific manner. In this mouse model, aspartic acid in position 266 within 259-273 epitope is replaced with glutamic acid so that mice have heterologous sequence of the CII259-273 peptide in their cartilage.

Humanized mouse models have also been used to study human MHC II molecules (DR4 and DR1) in mice. The first model, DR4fug mice and DR1 mice express complete human α and β chains as transgene whereas, in the second model, DR4tac, exon2 of the murine MHC class II molecule was replaced with corresponding sequence of the HLA-DR4 molecules in a chimeric molecule. They may or may not express mouse Eβ molecule. However they are knocked out for mouse class II molecules (Fig. 4).
Purpose

The aim of this project was to determine how T cells acquire tolerance to self-CII and once they acquire the tolerance how it can be broken and develop CIA in MMC mice. By means of several different T cell receptor transgenic mice, we also tried to follow collagen specific T cells \textit{in vivo} as well as \textit{in vitro} with in the response to collagen peptides to analyze the importance of post-translational modifications of collagen.

Materials and Methods

Mice between 7 to 12 weeks in age were used in the arthritis experiment and transfer experiments. Mice were kept in an animal house and experiments were conducted with the approval of the Swedish laboratory animal ethics committee.

Table 1. Mice that have been used in the experiments

<table>
<thead>
<tr>
<th>Mice</th>
<th>Summary of the properties</th>
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<tbody>
<tr>
<td>B10Q</td>
<td>control mice,</td>
</tr>
<tr>
<td>B10RIII</td>
<td>control mice(expressing Eb)</td>
</tr>
<tr>
<td>Vb12-tg</td>
<td>transgenic mice expressing TCR-β specific for galactosylated CII</td>
</tr>
<tr>
<td>Vb12.DBA</td>
<td>transgenic mice expressing TCR-β specific for galactosylated CII and congeneric TCR-α from DBA/1 mice</td>
</tr>
<tr>
<td>HCQ-3</td>
<td>transgenic mice expressing TCR specific for galactosylated CII immunodominant epitope</td>
</tr>
<tr>
<td>MMC</td>
<td>mice expressing heterologous CII in cartilage restricted fashion</td>
</tr>
<tr>
<td>DR1</td>
<td>mice expressing human DR1 molecule</td>
</tr>
<tr>
<td>DR4tac</td>
<td>mice expressing MHCII chimerically (exon 2 of mouse MHCII is replaced with human DR4)</td>
</tr>
<tr>
<td>DR4fug</td>
<td>mice expressing complete human DR4 as a transgene</td>
</tr>
<tr>
<td>DR4.Ncf1+</td>
<td>DR4 mice expressing mutated Ncf1 protein which is the part of neutrophil NADPH dehydrogenase complex, and responsible from reactive oxygen species</td>
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</table>
**Induction of CIA and assessment of disease**

Immunizations were done in the base of tail with 100 µg of rCII emulsified with 100 µl complete Freund adjuvant (CFA). rCII was obtained from SWARM sarcoma with pepsin digestion. Clinical disease was followed three times per week with scoring and weighing. Maximum score per animal was 60 in which each paw could be given maximum score of 15. Each toe and knuckle got 1 point and an ankle got 5 point if it was arthritic.

**Antigens**

rCII with 259-273 immunodominant epitopes, non-modified lysine at 264th position (K264), with [beta]-D-galactopyranosyl residue at 264th position (GalHyK264) were produced as described before [21].

**FACS staining**

All staining for FACS analysis were carried out on FACS LSRII (BD Biosciences, San Jose, CA, USA) and results were analyzed with Flow-jo and GraphPad Prism softwares. [20]

Lymphocytes obtained from mice immunized with rCII in CFA were cultured in DMEM with %5 heat inactivated fetal calf serum (FCS), penicillin/streptomycin. For CD40L detection, cultures were treated in U-bottomed 96 well plates (NUNC, Thermo Fisher Scientific, Roskilde, Denmark) with 20 µg/ml of Brefeldin A (BFA), 2 mg/ml of DNase, 2 µg/ml of α-CD28, 10 µg/ml antigens (K264, GalHyK264, ConA or nothing) at 37 C°. Monoclonal antibody, B22a1, which is specific for TCR of galactosylated CII epitope of Vb12 chain, was used to detect galactosylated CII specific T cells. After 6 hours of culture, cells were collected and extracellular staining was done with B22a1-biotinylated antibody (homestock), streptavidin-APC, anti-CD4-PE Cy5.5, Pacific Blue to remove dead cells, B cells and macrophages (with LIVE/DEAD Fixable Violet Dead Cell Stain, anti-M1-17-PB, anti-B220-PB). After that, cells were incubated for 30 min in dark with Cytofix/Cytoperm solution (BD Biosciences, San Jose, CA, USA) to fix the cells. Then they were washed with Perm/Wash buffer and intracellular staining was performed with anti-CD40L –PE, anti-IFN-γ- PE Cy7 and anti-IL-2 AF700.

For the staining of thymic cells, 2x10^4 cells/sample were collected by percoll centrifugation and stained with anti-Ly51-FITC (for detection of thymic dendritic cells), antiHLA-DR4-PE, anti-F4/80-PerCp-Cy5.5 (for detection of macrophages), anti-EpCAM-APC-Cy7 (anti epithelial cell adhesion molecule), M1/70-APC (for detection of monocytes), anti-CD11c-PeCy7, and B220-PB. For staining of I-A^q as control, cells from B10Q mice were stained with biotinlated PCQ6 antibody and streptavidin PE. Fluorescence minus one (FMO) controls were carried out with leftover cells.
**B cell depletion**

Cells were collected from spleen and lymph nodes of naïve HCQ3 mice. A single cell suspension was made and $2 \times 10^7$ cells per ml were obtained. The cells were incubated with 1:100 diluted biotinylated anti-CD45R (RA3-6B2;BD Pharmigen, San Diego, CA, USA) antibody in Dynabead buffer (sterile PBS + 0.1% BSA + 2mM EDTA) for 10 minutes on ice. After the cells were washed and diluted in Dynabead buffer as before. Dynabeads coated with streptavidin (Invitrogen, Dynal, Oslo, Norway) were added to sample and incubated on shaker at 4°C for 30 minutes. Samples were put in a magnet and after 5 minutes supernatant was taken.

**CFSE labeling**

Enriched T cells were resuspend in $1 \times 10^7$ cells per ml in PBS with 0.1% BSA for fluorescent dye labelling with cell trace CFSE (Invitrogen, Eugene, Oregano, USA) prior to transfer. Cells were incubated at room temperature for 10 minutes with 3 µM CFSE. In order to quench the reaction, PBS containing 5% FCS was added to the cells in 5 more volumes. Cells were washed three times with PBS and resuspended in PBS to $5 \times 10^7$ cells/ml. Transfer of $2 \times 10^7$ cells per recipient was done intravenously to BQ mice (as negative control), BQ.MMC mice and BQ mice immunized with 100 µg of rCII in emulsion with 100 µl CFA on the day of immunization (as a positive control). Three days later; cells from the inguinal, axillary and mesenteric lymph nodes as well as from the spleen were collected and incubated with 2.4G2 (homemade anti-Fc RIII antibody) for 5 minutes. Then, cells were stained with LIVE/DEAD Fixable Violet Dead Cell Stain Kit (Invitrogen, Eugene, Oregon, USA), anti-B220-Pacific Blue (ebioscience , San Diego,CA,USA) for the exclusion of B cells and dead cells, anti-CD4-PeCy5.5 (ebioscience , San Diego,CA,USA), anti-Vb8-biotin and streptavidin APC (BD bioscience, San Diego,CA,USA). Cells were analyzed with FACS LSRII (BD Biosciences, San Jose, CA, USA) and analysis was performed with Flow-jo software.

**Anti-CII ELISA**

Flat bottom 96 well bottom plates were coated with 10 µg/ml rCII in PBS and incubated overnight at 4 °C. Plates were washed and sera were serially diluted and samples were prepared in duplicates. Serum from each sample was pooled as standard on each plate for interpolate comparison. In order to calculate the concentration of total anti-CII IgG, a standard of affinity purified polyclonal anti-CII antibodies (1mg/ml) was used in one of the plates and compared to pooled serum. The plate was incubated for one hour at room temperature. Plates were washed and secondary antibody for total IgG, biotinylated 187.1aK antibody was diluted as 1 µg/ml in Elisa buffer. For detection, ExtrAvidin-Peroxidase (Sigma E2886) was used and color was developed with ABTS-substrate buffer mix (Roche) (0, 5 ml 10X ABTS buffer, 4,5 ml ddH2O and 1 ABTS tablet). Plates read at 405 nm with Synergy-2 (Bio-tek Instruments).

**Eβ detection**

Blood was taken from B10Q mice (as a negative control), B10RII (as a positive control of 17.3.3 antibody staining), DR1, DR4tac and DR4 fug mice. Red blood cells were lysed with ACK lysing buffer. Cells were stained with anti HLA-DR-PE and 17.3.3-biotin and SA-FITC.
Isolation of non-thymocytes

Thymi were collected from different DR4 founders and DR1 mice at weaning age (4 weeks old). Minced thymi were incubated with 500 µl DMEM (Gibco, Life technologies, Grand Island, NY, USA) per thymus and 0.2 mg/ml collagenase (ROCHE Diagnostics, Mannheim, Germany), 0.2 mg/ml dispase I (ROCHE Diagnostics, Mannheim, Germany) and 0.2 mg/ml DNase (ROCHE Diagnostics) were added and samples were incubated at 37 °C for 30 minutes. EDTA was added to final concentration of 10 µM and incubated for 5 minutes. Cells were resuspended in 4 ml of 1.115 Percoll (GE Healthcare Biosciences, Uppsala, Sweden) gradients and put in FCS coated tubes. Two ml of 1.065 gradient percoll was added on it and 2 ml of PBS added on top. Cells were centrifuged without a break, at 2700 rpm for 30 min at 4 °C. Cells between the PBS and 1.065 Percoll layer were collected.

RESULTS

Influence of TCRα locus gene dosage on frequency of B22a1 T cells in naïve and primed mice

Vb12-tg mice have been used in this experiment in order to determine T cell response in mouse model of CIA. In Vb12-tg mice the β chain of T cell receptor is specific for galactosylated form of CII. However specific β chain can combine with any endogenous α chain and can create a lot of different functional TCRs, decreasing the frequency of T cells specific for CII. To increase the number of T cells recognizing collagen, Vb12-tg mice have been crossed with DBA-TCRα-locus congenic B10Q mice. It has been shown that these congenic mice have an increased susceptibility to CIA. A clonotypic antibody, B22a1 has earlier been generated which recognizes the TCR specific for galactosylated form of the CII 259-273 epitope in VB12tg mice. In this experiment, the effect of numbers of TCRα locus on frequency of B22a1+ T cells was determined.

For this purpose, frequency of B22a1+CD4+ T cells from mice with either heterozygote or homozygote expression of the DBA/1 TCRα chain congenic fragment was investigated. Additionally, CD40L upregulation, IL-2 and IFN-γ production upon stimulation with the non-modified (K264) and galactosylated (GalHyk264) form of rCII 259-273 peptide were examined.

In naïve mice, frequency of B22a1+CD4+ T cells from mice with either heterozygote or homozygote expression of the DBA/1 TCRα chain congenic fragment was investigated. Additionally, CD40L upregulation, IL-2 and IFN-γ production upon stimulation with the non-modified (K264) and galactosylated (GalHyk264) form of rCII 259-273 peptide were examined.

In order to determine the frequency of activated B22a1+CD4+ T cells, we cultured the cells with antigens (GalHyk264 and K264) and BFA for 6 hours and we checked early activation marker CD154 (CD40L) and inflammatory cytokine production (IFN-γ and IL-2). There was not a significant difference upon stimulation with K264. However, when cells were cultured with GalHyk264 peptide, CD40L expression and IFN-γ production was higher in Vb12.DBA+/+ than Vb12.DBA+/− mice. In addition to this, cells expressing CD40L mainly consisted of B22a1+ CD4 cells. (Figure 6).
We also investigated the effect of the number of TCRα copies on arthritis development. Mice were immunized and monitored for the clinical disease for 10 weeks for the first experiment and 7 weeks for the second experiment. Moreover, we kept arthritis scores on males and females separately and anti-CII antibody has been measured at day 35 and day 70 (Figure 7). According to the first arthritis experiment, there was no significant difference in arthritis incidence and anti-CII antibody concentrations between the groups, neither in females nor in males. In the second experiment, significant differences in incidence and disease severity was only observed between Vb12 positive and negative mice, but not between Vb12.DBA\(^{+/+}\) and Vb12.DBA\(^{+/−}\) mice.

**Figure 5.** Gene dose effect of TCRα locus in Vb12tg mice. Figure shows effect of one copy or two copies of congenic TCRα locus on B22a1 cell frequency in naïve and immunized mice.
GalHyk264 stimulation

Bb12(+)DBA

Bb12(+)DBAhet

Bb12(-)DBA

Bb12(-)DBAhet

0

2

4

6

% of CD4+B22a1+ cells

GalHyk264 stimulation

Vb12(+)DBA++

Vb12(+)DBA+-

Vb12(-)DBA++

Vb12(-)DBA+-

0

2

4

6

CD40+ B22a1 + T cells

CD40L

CD40L

B22a1

B22a1
Figure 6. It shows CD40L upregulation, IFN-γ and IL-2 production in K264 and GalHyK264 stimulated cultures.

Figure 7. It shows arthritis susceptibility and anti-CII ELISA in Vb12tg mice with different copies of TCRα locus.
Maintenance and breaking of tolerance to self-CII in HCQ.3 mice

Another animal model (HCQ3-tg mice) for studying T cell behavior in CIA has been established in which mice express a transgenic TCR specific for galactosylated form of immunodominant T cell epitope. Unlike Vb12-tg mice, the HCQ3-tg mice express both the CII specific TCR α and β chain as transgene. By this way, it is possible to isolate naïve CII-specific T cells. In this experiment, we aimed to determine how T cells interact with the antigen in vivo under physiological conditions. In order to achieve this, we took naïve T cells from HCQ3 mice, labeled them with a fluorescent dye called CFSE that enabled us to track the cells and their activation status. CFSE is a dye in which the intensity decreases by half in each daughter cell and therefore one can determine the proliferation status of the cell.

We transferred the labeled cells intravenously to a) B10Q mice, B) B10Q mice immunized with rCII and c) MMC mice, which express the immunodominant T cell epitope cartilage specific manner. Three days later, we obtained spleen, mesenteric lymph nodes and inguinal and axillary lymph nodes and tried to visualize labeled cells via FACS. Since we only transferred T cells, we excluded dead cells and B cells (Figure 8A). After we obtained CFSE labeled donor cells, we determined how many of these CFSE positive donors that had a diluted CFSE-profile, and would therefore correspond to responding HCQ3+CD4 cells. In our positive control, where we immunized B10Q mice on the day of transfer, around 20 percent of the labeled cells had proliferated. In MMC mice, around 8% of the cells had divided, compared to only 2% of the cells had proliferated in naïve B10Q mice (Figure 8B). Additionally, we could detect CFSE labeled cells in mesenteric lymph nodes, spleen and inguinal lymph nodes yet only the ones who met with the antigen at the draining lymph nodes (inguinal) divided (Figure 8C).
Figure 8. Adoptive transfer of CFSE labeled HCQ3 cells to recipients. B cells and dead cells were excluded from the analysis and CFSE positive HCQ3 cells chosen for further analysis (A). Histogram of the divided CFSE labeled cells in B10Q mice immunized with rCII as positive control, in naïve B10Q mice as negative control and in MMC mice at day 3 (B). Percentage of dividing cells in different organs (C).

Comparison of tolerance to self-CII and susceptibility to CIA in different DR4-transgenic founders

Two CIA experiments were conducted with different DR4 strains with or without MMC transgene and Ncf1. Ncf1 is a protein in neutrophil NAPH oxidase complex and it takes part in production of reactive oxygen species. It has been shown that Ncf1 mutation increases the susceptibility to CIA. However, none of the animals used in the experiment developed significant arthritis (Table 1). Anti-CII antibody levels were measured at day 35 and at the end of experiment at day 57. Anti-CII antibodies were found to be significantly higher in the DR4tacNcf+/− strains than DR4fugNcf−/− strain. In addition to this, DR4tac.MMC−Ncf+/− mice had lower titers than DR4tac.MMC−Ncf−/−, and DR4tacNcf−/− mice had higher titers than DR4tacNcf+/− (Figure 9).

Another experiment regarding DR4 mouse was aimed to investigate the expression of I-ß molecules. DR1 and DR4 transgenic mice bred on B10 background that had been knocked out for the Ab molecule. The B10 background naturally lacks a functional Eα molecule but they may still, at least theoretically produce Aα and Eß molecules. This means, if mice express the β chain of I-E, it may combine with the α chain of DR4 molecule and create a functional MHCIIR molecule.

To investigate this we used the 17.3.3 antibody which recognizes the I-E and in the FACS analysis. For positive control, we used B10RIII since they express Eß. For negative control, BQ mice have been used. As a result, DR1 mice were found not to express any Eß molecule. In contrast, Eß chain was found to be expressed in DR4fug mice and even more so in DR4tac mice (Figure 10).

Last experiment regarding the DR4 project was to investigate DR4 expression in different cell populations in the thymus. Macrophages (MQ), monocytes, dendritic cells (DC), medullary thymic epithelial cells (mTECs) and cortical thymic epithelial cells (cTECs) from EPCAM+ cells (Epithelial cell adhesion molecule) were analyzed and compared to Aα expression in B10Q mice. Even though the percentage of cells are similar in DR1, BQ, DR4tac and DR4fug mice, the level of expression of DR4 and DR1 was different from the expression of the Aα molecule. Except for the expression on cTECs, DR4 and DR1 expression expression was always lower than that Aα in B10Q mice (Figure 11).
Table 2. CIA susceptibility in DR4 mice

<table>
<thead>
<tr>
<th>Experiment 1</th>
<th>DR4tac Ncf1 +/- MMC</th>
<th>DR4tac Ncf1 +/- MMC</th>
<th>DR4tac Ncf1 +/- MMC</th>
<th>Dr4fug Ncf1/- MMC</th>
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Figure 9. Total anti-CII IgG amount at day 35 and 57 in different DR4 mice is shown. DR4tac.Ncf1+/− mice had more anti-CII than DR4fug at day 35 and at day 57. Difference was statistically significant between DR4tac.Ncf1+/− and DR4tac and DR4fug.Ncf1−/− for day 35. For day 57, anti-CII IgGs differed between DR4tac.Ncf1+/− and DR4tac.Ncf1+/−.MMC and DR4fug.Ncf1−/−.

Figure 10. Histogram of Eβ molecule measured with 17.3.3 antibody in DR1, DR4tac and DR4fug mice. Negative controls were represented in green and dark blue in color and have geometric mean 5.43 whereas positive controls were shown in red and orange and had geometric mean 22.4. The experimental samples (DR1, DR4tac and DR4fug) were shown in light blue and pink histograms. DR1 mice had geometric mean 5.43, DR4tac have 11.9 and DR4fug mice had 6.82.
Figure 11. Percentage of dendritic cells, macrophages, EPCAM+ cells (cTECs and mTECs) and monocytes in thymus in DR1 (shown as blue), DR4tac (shown as red and light orange) and DR4fug (green) mice has been shown together with the DR4 expressions in these cell populations compared to A^q in B10Q (shown as purple).

Discussion
In this study, the aim was to study how T cells attain tolerance to self CII, how they break tolerance and develop arthritis, how they behave in response to collagen II and post-modifications on CII.

In the first experiment, the purpose was to investigate the influence of TCRα locus gene dosage on arthritis susceptibility and frequency of B22a1^+ T cells in naive and immunized mice. For this purpose, Vb12-tg mice with one or two copies of DBA/1 derived TCRα locus have been immunized and frequency of B22a1 T cells, CD40L upregulation, IFNγ and IL-2 production have been determined. To begin with, in naive mice the frequency of B22a1 cells in Vb12-tg.TCRαDBA^+/+ were nearly the same as the Vb12-tg.TCRαDBA^+/− and the non-Vb12 -tg littermates. After 10 days of immunization, frequency of cells increased to 4.56% in TCRαDBA^+/+ and 3.09% TCRαDBA^+/− whereas the frequency remained around 0.24 and 0.19 in the non-Vb12-tg counterparts. There is a trend in gene dose dependent manner that means that as the numbers of TCRα locus increases, frequency of B22a1^+ (galactosylated CII specific) T cells increases as well. However one should note that there was not enough numbers of mice to make a statistical comparison.
Same trend was observed in CD40L upregulation and intracellular IFN-γ production with response to galactosylated peptide stimulation. T cells did respond more to the galactosylated CII epitope than to the naked peptide in gene dose dependent manner. Besides, the majority of the cells that express CD40L were B22a1+CD4+ T cells, which argue for that the direction of the response, was against galactosylated epitope. The first experiment done on susceptibility of arthritis in Vb12-tg mice showed no obvious effect coming from number of TCRα locus because of the low incidence of arthritis in Vb12-tg.TCRαDBA+/+. Anti-CII antibody titers were not significant between the groups as well. We therefore repeated the experiments with males only to increase the susceptibility to CIA. However, preliminary data suggest that the number of the copies DBA-TCRα copies did not affect the disease since only difference occurs between Vb12-tg and non-Vb12-tg mice.

In the second experiment, we determined where and how fast the tolerization occurs in the mice. We adoptively transferred naïve T cells from HCQ3-tg mice to MMC mice. T cells in HCQ3 mice express TCR specific for the galactosylated version of the immunodominant T cell epitope. Since these mice only carry homologous collagen, T cells are not tolerized to heterologous CII like in MMC mice therefore they react to it. Cells were labeled with a fluorescent dye called CFSE that enables one to determine where and how many times cells get divided. According to the results, CFSE labeled cells were possible to obtain from the spleen, mesenteric lymph nodes and inguinal lymph nodes. This means that once T cells were injected, they were distributed throughout the lymphoid organs. However, dividing cells i.e activated cells were only observed in inguinal lymph nodes of positive control (BQ mice immunized with rCII) and MMC mice at day 3. Additionally, some of the cells already divided more than once in the mice that indicates a fast activation. Together, the data suggests that epitope presentation occurred within three days in the periphery owing to continuous leakage of heterologous CII expressed in cartilage to the periphery.

Two CIA experiments have been performed including different combinations of genes, however none of the mice got clinical arthritis. Previous data from the same group shows that DR4tac mice get significant arthritis even though the present anti-CII antibody data shows a significant difference between DR4 fug and DR4tac mice. However experiments were done in different animal house suggesting differences in environmental factors may affect the disease initiation or progression. All of the DR4 strains were knocked out for mouse class II genes that mean they do not express Aβ molecule and they also lack a functional Eβ molecule. However, they might still express α chain of I-A and β chain of I-E. This means, if mice express the Eβ chain, it may combine with α chain of DR4 molecule and may create a chimeric but functional MHCII molecule. Moreover, data showed that DR4fug mice did express intermediate levels of Eβ whereas DR1 mice did not express any. There was a slight expression of Eβ in DR4tac mice which might not be a concern since the expression levels were too low compared to positive control. One of the other concern regarding to these strains was whether they express the human DR molecules in thymus in a physiological manner like Aα expression in B10Q mice. Hence the question was how normal these mice are in comparison to “normal” mice. So we investigated the different cell populations in the thymus and their DR expression. Results were indicating that both DR1 and DR4 strains had more or less same percentage of macrophages, monocytes, EPCAM+ cells (mTECs and cTECs) and slightly more dendritic cells compared to B10Q mice. However, when one compared with Aα expression in B10Q mice, DR1 and DR4 levels were lower than the Aα mice except for the cTECs. According to previous studies of thymic MHCII expression and thymic microenvironment on central tolerance, it is important that, cTECs,mTECs and DC cells do express MHCII. Exact roles of these cells in thymus has not been fully
elucidated. However, the experiments suggest that cTECs which are expressing self antigen and MHCII take part in positive selection while mTECs that are expressing self antigen together with MHCII are responsible for negative selection of T cell populations. Additionally, DCs in thymus may participate and activate the pathway for the clearance of T cells which are responsive to self peptides. Therefore, it may be important to have MHCII expression in thymus together with the enough number of cells for the selection process.

By and large, using the transgenic animal models, the behavior of collagen specific T cells has been characterized. With this report, the effect of TCR genes to the development of CIA, activation pattern of collagen specific T cells and how human MHCII molecule reacts to antigen have been shown. However, a lot of things about how T cells gain tolerance, at which point tolerance is broken and they develop clinical arthritis, at which extent post-translational modifications do have role in the disease are still remained to be elucidated. These informations may enlighten the disease mechanism in the future.
Acknowledgements

I firstly thank my supervisor Johan Bäcklund for his patience to my endless questions and for guiding me for one year and then to Prof. Rikard Holmdahl for giving me the opportunity to work in Medical Inflammation Research with a wonderful group. I would like to thank to best flatmate ever Canan, to Egemen and Nejla, for being there for me listening my complaints and never complaint back about it, to Pyti, for bringing joy in my life. Isvece gelmemi sağlayan herkese, hayatım boyunca size minnettar olacagım. Ailem, soyadı Sener olan yeni eski herkes, her zaman yanında olduğunuz, binlerce kilometre mesafeden bana destek olup, beni güldürmeye basardığınız kendimi hiç bir zaman yalnız hissettirmediniz için... mukemmel insanlar topluluğu... Hepinizi çok çok seviyorum, iyi varsınız, iyi ki benimsiniz...
REFERENCES:

