# Role of environmental toxins in chronic experimental arthritis

## - in search of anti-inflammatory pathways for ethanol and nicotine

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# PER ASPERA AD ASTRA

Till mina älskade föräldrar

Utan er - *inget*

# ABSTRACT

Rheumatoid arthritis (RA) is a common systemic autoimmune disorder and a debilitating disease affecting 1% of the world population. The etiology of RA is an unresolved issue. Environmental factors such as alcohol intake and cigarette smoking have been described as contributing to the pathogenesis of RA. These assumptions are based on epidemiological studies, while experimental proof on this issue is limited. This thesis studies the effect of common environmental toxins on experimental arthritis induced by collagen type II (collagen-induced arthritis, CIA), an established murine model closely resembling human RA. We propose biological mechanisms behind the anti-inflammatory properties of environmental stimuli such as ethanol and nicotine, and provide new insights into the pathogenesis of RA.

**Paper I** shows that a continuous intake of ethanol delays the onset and halts the progression of CIA in mice. This anti-arthritic effect is mediated by increased testosterone secretion leading to (i) decreased activation of transcription factor NF-κB, (ii) down-regulation of pro-inflammatory cyto- and chemokines and (iii) down-regulation of leukocyte migration.

**Paper II** studies the effect of cigarette smoking and nicotine exposure in CIA mice. Results show that mice exposed to cigarette smoke develop a significantly milder arthritis with reduced destruction of joints. Nicotine-exposed mice show a tendency to decreased inflammation. Notably, exposure to cigarette smoke reduces antigen response and decreases the level of CII-specific antibodies.

**Paper III** handles intervention with ethanol-sensitive glutamate receptors. CIA mice subjected to the NMDA receptor antagonist memantine showed significantly decreased severity of arthritis and reduced destructive disease. We show that memantine upregulates transcription factor Foxp3 and enhances formation of $CD4^+CD25^+Foxp3^+$ regulatory T cells, which may be a potential reason for the anti-arthritic properties of the NMDA receptor blockade.

In conclusion, our results provide new insights into the anti-inflammatory properties of environmental toxins such as ethanol and nicotine, as well as of blockade of the ethanol-sensitive NMDA receptor. Our findings from experimental studies need further validation in the population of RA patients.

# LIST OF PUBLICATIONS

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals (I-III):

I. Ing-Marie Jonsson, Margareta Verdrengh, Mikael Brisslert, Sofia S. Lindblad, Maria Bokarewa, Ulrika Islander, Hans Carlsten, Claes Ohlsson, Kutty Selva Nandakumar, Rikard Holmdahl, Andrej Tarkowski.
**Ethanol prevents development of destructive arthritis**
*Proc Natl Acad Sci U S A. 2007 Jan 2;104(1):258-63**

II. Sofia S. Lindblad, Piotr Mydel, Ing-Marie Jonsson, Robert Senior M, Andrej Tarkowski, Maria Bokarewa
**Smoking and nicotine exposure delay development of collagen-induced arthritis in mice**
*Arthritis Research & Therapy 2009;11(3):R88**

III. Sofia S. Lindblad, Piotr Mydel, Annelie Hellvard, Ing-Marie Jonsson, Maria Bokarewa
**The NMDA receptor antagonist memantine ameliorates and delays development of collagen-induced arthritis by intensifying development of regulatory T cells**
*Arthritis & Rheumatism, revision submitted**

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# AIM

I. Evaluate the role of ethanol and its active metabolite acetaldehyde in development and progression of CIA in mice.

II. Evaluate the role of smoking and exposure to nicotine on development and progression of arthritis in the CIA model.

III. Elucidate the anti-inflammatory properties of NMDA receptor-blockade in CIA.

# ABBREVIATIONS

| | |
|---|---|
| **aCCP** | anti-cyclic citrullinated peptide |
| **AIDA** | 1-Aminoindan-1, 5-dicarboxylic acid |
| **AP-1** | activator protein 1 |
| **BMD** | bone mineral density |
| **CAIA** | collagen antibody-induced arthritis |
| **CD** | cluster of differentiation |
| **CIA** | collagen-induced arthritis |
| **CII** | collagen type II |
| **Con A** | concanavalin A |
| **Foxp3** | Forkhead box p3 |
| **GABA** | gamma amino butyric acid |
| **HLA** | human leukocyte antigen |
| **Ig** | immunoglobulin |
| **IGF-1** | insulin-like growth factor 1 |
| **IL** | interleukin |
| **IQR** | interquartile range |
| **LPS** | lipopolysaccharide |
| **MCP-1** | monocyte chemoattractant protein 1 |
| **MHC** | major histocompatibility complex |
| **MIP-1α** | macrophage inflammatory protein 1α |
| **NF-κB** | nuclear factor kappa-light-chain-enhancer of activated B cells |
| **NK** | natural killer |
| **NMDA** | N-methyl-D-aspartate |
| **PMN** | polymorphonuclear cell |
| **pQCT** | peripheral quantitative computed tomography |
| **PRR** | pattern recognition receptor |
| **RA** | rheumatoid arthritis |
| **RF** | rheumatoid factor |
| **TLR** | toll-like receptor |
| **TNF-α** | tumor necrosis factor α |

# THE IMMUNE SYSTEM

The immune system is defined as a system of biological structures and processes within the human body that **protects** against disease by identifying and destroying pathogens and tumor cells. It has evolved over time to protect man aginst various possible threats in best possible way. These threats can come as pathogenic infections (bacteria, viruses, parasites, prions), physical injury or tumors. The immune system is divided into **innate** and **adaptive immunity** (Fig 1). The innate system, also called the native immunity since being in place from birth, is a **fast-working** system. It reacts non-specifically and in the same way for every challenge and does not improve over time. The innate part of the immune system is composed of (i) the physical and chemical barriers of the body such as **the skin**, **the gastro intestinal tract epithelium** and the **anti-microbial substances** produced there, (ii) phagocytic cells (**neutrophils** and **macrophages**) and natural killer (**NK**) cells, (iii) blood proteins, including defensins and members of the **complement system** and (iv) **cytokines**. If the body is infected or injured, the innate part of the immune system reacts within minutes or hours. Recruitment of inflammatory cells from the blood is initiated when the endothelium of blood vessels surrounding the affected area expresses signals elicited by injury or presence of a microbe. **Monocytes** (later developing into **macrophages**) and **granulocytes** (neutrophils, eosinophils and basophils) migrate through the endothelium of the blood vessels towards these signals. Once in the tissue, the cells will try to combat the inflammation/infection.

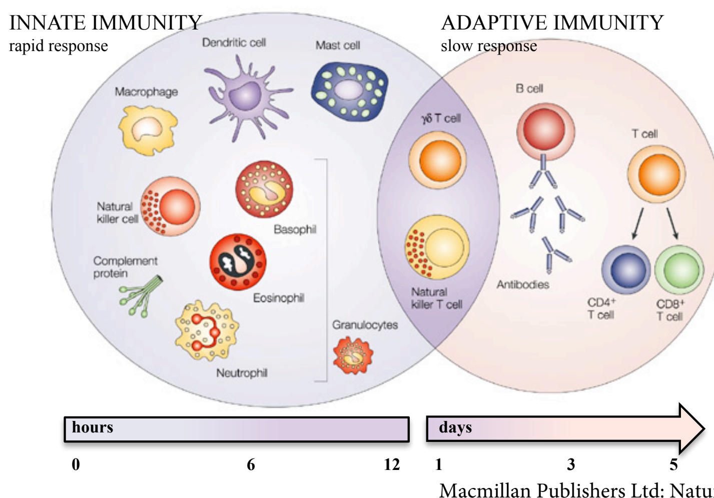



**Figure 1 Innate and adaptive immunity.** In case of infection or injury, the innate part of the immune system will elicit a rapid response of defense. Dendritic cells, NK cells, complement proteins and granulocytes present in the skin, intestine and blood will very fast react to protect the body. While this occurs, the adaptive part of the immune system is being activated. Within a couple of days, T cells, B cells and antibodies produced by B cells will join in the battle. Figure adapted with permission from Macmillan Publishers Ltd: Nature Reviews Cancer(Dranoff, 2004).

The adaptive immune system, undergoes development and maturation during the entire lifetime, adjusting its reactions in response to exogenous pathogens and endogenous stimuli. It takes longer to activate than the innate immune system but is more specific and generates a **memory** resulting in **quicker** and more **vigorous** response to repeated infection. Adaptive immunity was historically divided into two parts; **humoral** and **cell-mediated immunity**. The humoral immune response is mediated by serum **antibodies** secreted by activated **B lymphocytes (B cells)**. The antibodies recognize antigens and elicit various effector mechanisms participating in deletion of the antigen. Cell-mediated immunity is mediated by T lymphocytes (**T** cells). Activated T cells produce and secrete cytokines that activate macrophages, NK cells, cytotoxic T cells, regulatory T cells and B cells, enhancing their antibody-production. The various different functional subgroups of T cells are discussed below.

For a T cell to be able to carry out its principle functions, defense against microbes and activation of other immune cells, it has to interact with an **antigen**. The antigen receptor on a T cell, the T cell receptor (**TcR**), can only recognize an antigen when presented on specialized antigen presenting cells, so called **APC**s. Additionally, the APC has to display the antigen on a certain **peptide display molecule** named the major histocompatibility complex (**MHC**), required for proper T cell recognition (Fig 2).

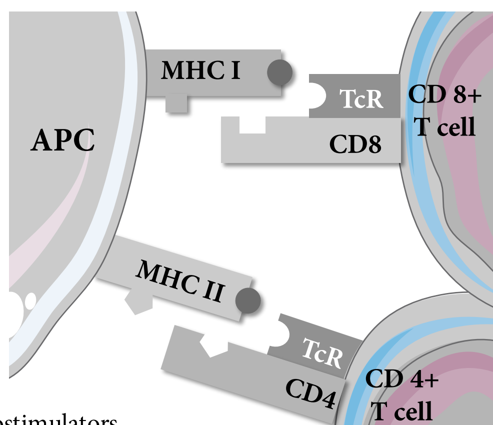



**Figure 2 Antigen presentation to T cells.** For a T cell to recognize its antigen, it has to be presented on an APC via MHC. MHC I is present on all nucleated cells and presents antigens to CD8$^{+}$ T cells. MHC II is present on professional APCs and presents antigens to CD4$^{+}$ T cells. Figure illustrated by the author using Medical Art picture disc kindly provided by Proteolos®

Yet another factor required for T cell proliferation, differentation and cytokine secretion is the expression of costimulatory molecules on the APCs. These bind to co-receptors on the naïve T cell. The best defined costimulators for T cells are CD80 and CD86 (formerly called B7-1 and B7-2) expressed on professional APCs. These bind to the co-receptor CD28 on the T cell (CTLA-4 in activated T cells, see p. 22). There are several different types of APCs; **dendritic cells (DCs)**, **macrophages** and **B cells** are called professional APCs due to their expression of MHC II, efficiency in **internalizing** and **presenting**

the antigen to a T cell and expression of costimulatory molecules. Examples of non-professional APCs are fibroblasts of the skin and thymic epithelial cells. The antigen-MHC complex is presented to the T cell receptor in combination with the signaling and adhesion co-receptors CD8 or CD4. There are two general classes of MHC; MHC class I (**MHC I**), which is present on essentially all nucleated cells and presents antigen to $CD8^{+}$ T cells and MHC class II (**MHC II)** which is present on all professional APCs. They present antigen to $CD4^{+}$ T cells. The interaction between APC and T cell takes place in the **lymph nodes**, to which the cells travel via the lymphatic system from the thymus. The migration from thymus to the lymph nodes is dependent on a chemical concentration gradient of chemokines along the way. This migratory process is hence termed **chemotaxis**. During the journey, the APC processes the internalized antigen into peptide fragments, synthesises the MHC, associates the processed peptides with the MHC and finally expresses it on the cell surface for interaction with the T cell receptor (**TcR)**.

In the thymus, T cells are initially **double-positive** cells, expressing both CD4 and CD8 **co-receptors** on their cell surface. T cells are rendered single-positive in the thymic cortex; T cells recognizing self-antigens presented on **MHC I** interacting with the co-receptor **CD8**, will lose expression of CD4 and thus develop into **$CD8^{+}$ T cells**. Most $CD8^{+}$ T cells are **cytolytic** T cells and serve to **protect against viral infections and tumors**, doing this by inflicting **cytotoxic damage** on target cells. $CD4^{+}$ T cells develop in an analogous process where MHC II interacts with the CD4 co-receptor. Most **$CD4^{+}$ T cells** are cytokine-producing helper cells and function in **host defense against extracellular microbes**. The described processes make the distinction between the two major functional classes of T cells: **T-helper cells** ($CD4^{+}$ cells) and **cytotoxic T cells** ($CD8^{+}$ cells). During further development, the effector $CD4^{+}$ T cells are separated into different **T helper lineages** according to which cytokines they produce (Bottomly, 1988). These are T helper types 1 (Th1), 2 (Th2) or 17 (Th17**)**. **Th1 cells** develop during infections with intracellular bacteria and secrete the cytokines interferon gamma (**IFN-γ)**, interleukin (**IL**)-**2**, tumor necrosis factor (**TNF**) and **lymphotoxin-α** when activated. Th1 cells activate macrophages and promote complement fixing and opsonization - all processes involved in cell-mediated immunity. **Th2 cells** are activated following extracellular parasitic infections with, for example, nematodes and helminths. They secrete cytokines **IL-4**, **IL-5**, **IL-6** and **IL-10** and promote **B cell activation** and other functions important in humoral immunity (Skapenko, et al., 2005). The **Th17** subtype, first described by Harrington in 2005 (Harrington, et al., 2005), secrete the cytokines **IL-17A**, **IL-17F**, **IL-21** and **IL-22,** and is important in host defense against gram

negative extracellular bacterial infections (Infante-Duarte, et al., 2000). Another important population of T cells are the regulatory T cells (**Tregs**), which regulate the activation of other T cells and are important in controlling self-tolerance, discussed below.

One remarkable and important feature of the immune system is its capability to discriminate between foreign antigens (non-self) and the individual´s own antigens (self). Immunological unresponsiveness to self is termed **self-tolerance**. Disturbances in the maintenance of self-tolerance lead to immune responses to the body's own proteins - self-antigens - resulting in **autoimmune diseases** such as rheumatoid arthritis.

### SELF-TOLERANCE

The immune system uses several mechanisms to preserve self-tolerance and prevent autoimmunity. Self-tolerance is induced early in the maturation of the immune system: (i) during lymphopoiesis in thymus and bone marrow (central tolerance) and (ii) in mature lymphocytes in the periphery (peripheral tolerance). **Central tolerance** (Fig 3, p.13, upper box) is the deletion of self-reactive T cells in primary lymphoid organs (thymus and bone marrow). **Positive selection** ensures that only cells capable of interacting with self MHC will proliferate. This process also distinguishes between class I and class II MHC restriction of T cell subsets, ensuring that $CD8^{+}$ T cells are specific for peptides presented on MHC I and $CD4^{+}$ T cells for peptides on MHC II, as discussed above. The process of **negative selection** follows next. This process serves to eliminate cells with self-reactive TcRs and is determined by the interaction between the TcR and the antigen-MHC complex. T cells that express TcR with low/intermediate affinity for self-antigens are selected to survive and will continue to mature and migrate to the peripheral lymphoid tissues where they will encounter foreign antigens. T cells with receptors that bind to the antigen-MHC complex with strong/high-affinity-binding are detected as auto-reactive and killed. This process is part of the **negative selection** which is mediated by: (a) elimination by apoptosis (**clonal deletion**), (b) change of the receptor to no longer recognizing self-antigens (**receptor editing**, for immature B-cells in bone marrow) or (c) **development of regulatory lymphocytes** (**Tregs**, only $CD4^{+}$ T cells). The net effect of these selection processes are that the mature T cells that leave the thymus are **self-MHC restricted** and **tolerant** to many **self-antigens**. **Peripheral tolerance** (Fig 3, lower box) is the mechanism by which mature lymphocytes that recognize self-antigens in the periphery are rendered unresponsive. Peripheral tolerance for T cells is obtained through **anergy**, **deletion** or **suppression** of self-reactive T cells. A cell becomes anergic (i.e inactive)

following exposure to antigen without co-stimulation. This reduces the ability of the lymphocyte to respond to self-antigens. Deletion of a self-reactive T cell takes place after recognition of a self-antigen without inflammation or after repeated stimulations. Deletion is achieved through apoptosis (i.e programmed cell death). Suppression of self-reactive T cells is performed by regulatory T cells.

The **Tregs** formed during negative selection will migrate to the periphery, where they actively suppress activation and expansion of self-reactive T cells. Tregs express CD4 and high levels of the IL-2 receptor α chain, also termed **CD25**. $CD25^{+}$ T cells constitute around 5-10% of all $CD4^{+}$ T cells found in peripheral lymphoid organs and peripheral blood of healthy adults (Gavin, et al., 2003;Dieckmann, et al., 2001). The expression of transcription factor Forkhead box p3 (**Foxp3)** is critical for the development and function of Tregs (discussed below).

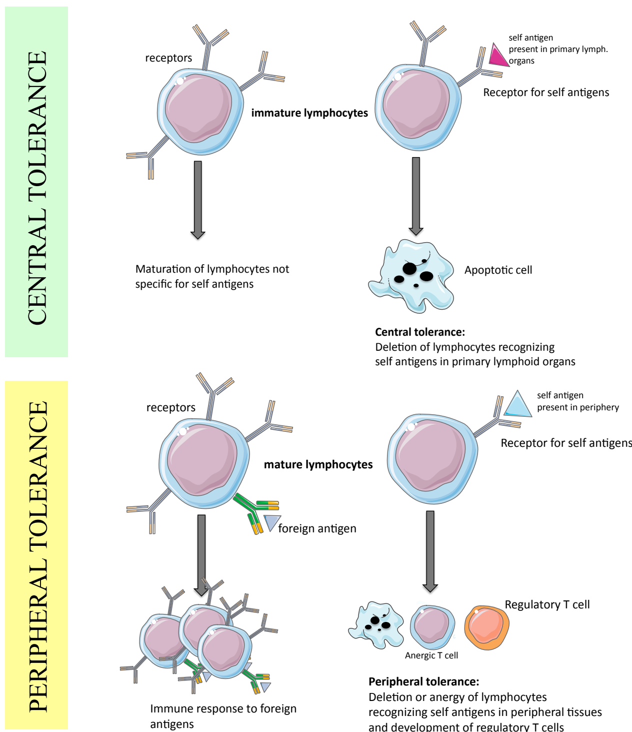



**Figure 3 Central and peripheral tolerance.** Induction of central tolerance takes place in the primary lymphoid organs; thymus and bone marrow. Lymphocytes carrying receptors recognizing self-antigens are deleted, whereas lymphocytes not specific for self-antigens are allowed to survive, let to mature and migrate to peripheral tissues. Peripheral tolerance is an ongoing process in the peripery where cells with receptors recognizing self-antigen in the periphery are rendered anergic, deleted or suppressed by Tregs. Figure illustrated by the author using Medical Art picture disc kindly provided by Proteolos®

## Break of self-tolerance

Failure or loss of self-tolerance results in autoimmunity. The cause of the broken self-tolerance and the identity of the self-antigen in RA is unknown but highly speculated on and researched into. The resulting inflammation and destruction seen in the RA-joint is mediated by various immunocompetent cells, such as T and B cells.

Loss of tolerance in a B cell can be incuced in a TLR-dependent fashion when a B cell recognizes self-antigens that carry ligands for **toll-like receptors (TLRs)**. TLRs are receptors that recognize structurally conserved molecules derived from microbes but also some human ligands. These endogenous ligands being able to bind to TLRs are DNA, RNA, fibrinogen and heat shock proteins. A number of different TLRs are present on a multitude of cells in both humans and mice. The activated B cell with lost tolerance can then present the auto-antigen to self-reactive T cells inducing joint-destruction and full-blown autoimmunity (Shlomchik, 2009). TLRs have also been proposed to activate T cell-driven autoimmunity. In the presence of an infection and high concentrations of TLR-agonists, Treg functions might be abrogated and self-reactive T cells expanded (Marsland, et al., 2007). Structural similarity between microbial and self-antigens - **molecular mimicry** - can activate auto-reactive T cells, leading to development of autoimmune disease. Other ways in which infection can lead to autoimmunity include release of sequestered antigens resulting from tissue damage, activation of multiple T cells from superantigens and induction of inflammatory cytokines and co-stimulatory molecules (Kamradt, et al., 2001).

Why and by what cells are the self-antigens presented? A number of studies report that DCs are the main APCs responsible for the presentation of self-antigens to auto-reactive T cells. Benson et al proposed that conventional DCs (cDCs) are the cells responsible for initiating breach of self-tolerance. cDCs, in contrast to other APCs, can mature and present antigen to auto-reactive T cells, in this way initiating breach of self-tolerance. Limiting maturation of cDCs, for example through TNF-$\alpha$ blockade, prevents cDC-increased MHC class II expression leading to a decrease in CII-specific T cells and antibody responses. This is proposed to prevent breach of self-tolerance (Benson, et al., 2010).

The process of **post-translational modification** give rise to new self-antigens, so called **neo-antigens**, not recognized by the immune system. Post-translational modifications, such as **citrullination**, glycosylation and phosphorylation, are estimated to take place in 50-90% of the proteins in the human body (Doyle, et al., 2001). Though necessary for biological functions, these

modifications can give rise to autoimmunity. It is postulated that human leukocyte antigen (HLA) alleles can select potential auto-reactive T cells in the thymus and present arthritogenic peptides to these auto-reactive T cells in the periphery. These arthritogenic peptides might be citrullinated peptides which have increased affinity for MHC molecules and facilitate their presentation to T cells (Imboden, 2009; Taneja, et al., 2010). T and B cells encountering these neo-antigens consider them as foreign, and intramolecular epitope spreading might then account for the response found in a number of autoimmune diseases (Doyle, et al., 2005; Mamula, 1998).

## Immunological mechanisms and cells participating in the development of RA

Rheumatoid arthritis (**RA**) is a chronic, systemic inflammatory disease characterized by inflammation of the lining of the joint, the **synovia**, followed by destruction of cartilage and bone. The inflammatory process in RA expresses all of the true hallmarks of inflammation; rubor, calor, tumor, dolor and functio laesa (redness, heat, swelling, pain and loss of function).

RA is believed to be an **antigen-driven disease**. The antigen driving the process is, however, not yet identified. The cells involved in the early, presymptomatic phase, of such an immune-mediated condition are **antigen presenting cells** (APCs), **T cells** and **B cells**. These respond to the as yet unknown antigen by mounting an inflammatory response trying to combat the possible threat. When the disease becomes symptomatic, additional cell types are recruited and become involved in the destructive process. These include **neutrophils**, **fibroblasts**, **osteoclasts**, **chondrocytes**, **mast cells** and others (Harle, et al., 2005; Tak, et al., 1997). Even later, **synoviocytes** in the joint will proliferate rapidly and the synovial tissue will become **invasive**, forming a **pannus**, ultimately **destroying cartilage** and **bone of the joint** (Strand, et al., 2007)(Fig 4).

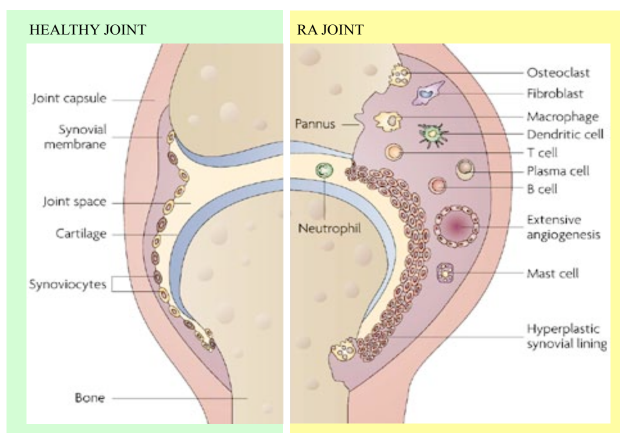



**Figure 4 Healthy versus RA joint**. A normal, healthy joint has a thin synovial membrane with a single layer of synoviocytes and a smooth surface of cartilage. The RA joint is invaded by inflammatory cells such as neutrophils, macrophages, dendritic cells, T and B cells. The synovial membrane becomes hyperplastic, migrating into the articular cartilage forming a so called pannus. The cartilage and bone is eventually eroded by osteoclasts. Figure adapted with permission from Macmillan Publishers Ltd: Nature Reviews Drug(Strand, et al., 2007), 2007.

Pro-inflammatory cytokines such as TNF-α and IL-1 are abundant in the synovium of patients with various types of chronic arthritis, disrupting normal tissue homeostasis in cartilage and bone (Luyten, et al., 2006; Rengel,

et al., 2007). This causes the involved joint to lose its shape and alignment, leading to pain and disability. Systemic involvement can also manifest in RA, including diffuse inflammation of the lungs, heart, pericardium, pleura and sclera (Triggiani, et al., 2003; Turiel, et al., 2009).

### T CELLS AND B CELLS IN PATHOGENESIS OF RA

RA was previously believed to be driven by pro-inflammatory Th1 cells and was therefore characterized as a Th1-driven disease. This type of categorization has now been abandoned and RA is today considered as a disease involving T cells of lineages Th1, Th2, Th17 and Tregs, as well as B cells. Both T and B cells are found in synovium, synovial fluid and peripheral blood of RA patients (Marston, et al., 2010; Panayi, et al., 1992). $CD4^+$ and $CD8^+$ T cells are found in synovial infiltrates in RA patients where they can lead to activation of macrophages and synovial fibroblasts. Recent evidence indicates a role for auto-reactive $CD8^+$ T cells in rheumatoid inflammation: a specific subgroup co-expressing CD57 (NK cell marker) accumulates in peripheral blood and synovial fluid as the disease progresses (Arai, et al., 1998). Activated $CD8^+$ cells can produce very high levels of **IFNγ** and **TNF,** which contribute to tissue damage in RA. The precise location of differentiation of naïve T cells into pathogenic effector T cells in RA is not known. The synovial milieu, at least in established disease, contains various macrophage- and synovial fibroblast-derived cytokines such as IL-1β, IL-6, IL-12, IL-15 and transforming growth factor (TGF) β, that support the expansion and differentiation of Th1 cells and/or Th17 cells. Synovial T cells contribute to synovitis directly via production of inflammatory cytokines or by interaction with neighboring macrophages and synovial fibroblasts that promote their activation (McInnes, et al., 2007). **Th17 cells** are implicated in autoimmune diseases such as RA and the cytokine IL-17A has been shown in experimental arthritis models to contribute to the pathogenesis (Lubberts, 2010). Clinical trials with anti-IL-17A-antibody treatment in RA patients have shown promising results (Genovese, et al., 2010). Naturally occurring **Tregs** have been detected in synovium of RA patients with active disease, but these Tregs seem to have impaired regulatory functions. Treatment with anti-TNF-α therapy restores the capacity of the Tregs to inhibit cytokine production and function as suppressive, conventional Tregs (Ehrenstein, et al., 2004). **B cells** in RA are responsible for the production of autoantibodies such as the rheumatoid factor (**RF**), antibodies towards collagen II (**aCII**) and anti-cyclic citrullinated peptides (**aCCP**). The presence of autoantibodies produced by B cells is considered a hallmark of patients with RA (Aletaha, et al., 2010), and B cell targeted therapy depleting $CD20^+$ B cells has been shown to

ameliorate inflammation in RA (Edwards, et al., 2004). B cells also contribute to the pathogenesis of RA by producing cytokines and chemokines such as IL-6 and IL-10 (McInnes, et al., 2007). The lymphocyte infiltrate in the synovium of RA patients can form ectopic germinal centers and this occurrs in around 20% of patient. This may give rise to auto-reactive B cells. B cells can also present antigen to T cells initiating a feedback loop between T and B cells: T cells help promote clonal expansion, isotype switching and differentiation of B cells into antibody secreting cells, which then activate T cells by presenting antigens to them. What initiates the positive feedback cycle is at present not clear (Shlomchik, 2009).

### *AUTOANTIBODIES*

**The rheumatoid factor**, found in approximately 80% of RA patients, have originally been described as **autoantibodies** (IgM) toward the Fc part of the IgG antibody. RF can form **immune complexes** in the inflamed joint being able to fix **complement** and release chemotactic factors, which in turn attract neutrophils. RF is, however, also present in healthy individuals and in other chronic inflammatory conditions. It is for that reason not a specific marker for RA. Whether the presence of RF is a consequence of inflammation, or part of the initiating process is not clear.

The presence of aCII antibodies (aCII ab) in sera from RA patients was first shown in 1963 by Steffen et al (Steffen, et al., 1963). The presence of such antibodies is reported in 3-27% of all RA patients (Beard, et al., 1980). Early detection of aCII ab in combination with RA susceptibility motif (the shared epitope, discussed below) seems to be **predictive** of a rapidly **progressive** and **erosive** disease (Cook, et al., 1996). The antibody level and frequency decrease over time, usually with the development of erosive disease. Anti-CII antibody levels thus appear to peak around the time of diagnosis of RA, when they are associated with active inflammation.

In 1998 Schellekens et al found autoantibodies reactive with synthetic peptides containing the unusual amino acid **citrulline** in sera from RA patients (Schellekens, et al., 1998). Citrulline is not encoded for in the human DNA directly, but is present in many proteins due to post-translational modifications. Conversion of the amino acid arginine into citrulline is done in a process known as **citrullination** or deimination, and it changes the chemical nature of the amino acid; arginine is positively charged at neutral pH, whereas citrulline is uncharged. Citrullination takes place during cell-death and tissue inflammation and several citrullinated proteins, such as fibrinogen, has been identified in the rheumatic joint. Further studies after Schellekens finding, showed that these **aCCP antibodies** can be detected in sera from RA patients several years before symptoms

arise, and they are currently used as both prognostic markers and as measure of disease activity for RA (Markatseli, et al., 2010; Predeteanu, et al., 2009). Antibodies to the citrullinated proteins are speculated to mediate inflammation by the formation of immune complexes (Wegner, et al., 2010).

Since 2010, the presence of aCCP antibodies is included in the ACR/EULAR criteria for RA (Aletaha, et al., 2010).

### *CYTOKINES*

Cytokines regulate many different inflammatory processes implicated in the pathogenesis of RA. It is known that an **imbalance** between pro- and anti-inflammatory cytokines exists in the RA joint, which leads to induction of autoimmunity, chronic inflammation and eventually joint damage (McInnes, et al., 2007). Cytokines involved in RA are, for example, IL-1, IL-6, IL-10, IL-12, IL-15, IL-17A, IL-18, TNF-α and TGF-β. Many of the cytokines mentioned above activate T cell differentiation and proliferation resulting in further cytokine release. Therapies directed towards various cytokines involved in RA have been developed over the past few years, and today there are substances in use directed towards cytokines TNF, IL-1 and IL-6 and clinical trials ongoing for IL-15, IL-17 and IL-18 (McInnes, et al., 2007).

### *TRANSCRIPTION FACTORS*

A transcription factors is a protein that **regulate gene expression** by binding to specific DNA sequences and control the transcription of DNA to mRNA. A transcription factor can either activate or repress the trancription of a gene and in this way both increase and decrease the expression of a specific protein. The three transcription factors mentioned in this thesis are **NF-κB**, **AP-1** and **Foxp3**, all being important in immune responses and RA.

**NF-κB** is found in almost all eukaryotic cells and regulates genes responsible for both the innate and adaptive immune responses. It is one of the most important mediators of inflammation and has a central role in many inflammatory conditions such as RA. A wide range of stimuli can activate NF-κB, including pathogens (via TLRs), stress signals and pro-inflammatory cytokines such as TNF-α, IL-1, and IL-17 (Q. Li, et al., 2002). Products of genes regulated by NF-κB, also cause activation of NF-κB, in a positive feed-back loop. Proteins regulated by NF-κB are, to name some, TNF-α, IL-1, IL-6, MIP-1α and GM-CSF (granulocyte macrophage colony-stimulating factor). This type of positive regulatory loop amplifies and perpetuate local inflammation (Barnes,

et al., 1997) resulting in chronic conditions, as seen in RA. While in an inactivated state, NF-κB is located in the cytosol of the cell, complexed with the inhibitory protein IκB (Fig 5). Extra-cellular signals can activate dissociation of IκB from NF-κB which, when activated, translocates into the nucleus. In the nucleus, it binds to DNA and induces transcription of the DNA into mRNA which, later, will be translated into a functional protein. The NK-κB family of genes has been reported to be highly
expressed and activated in RA-affected tissues and contribute to the hyperproliferation of synovial cells, playing an important role in the pathogenesis of RA (Okamoto, et al., 2008).

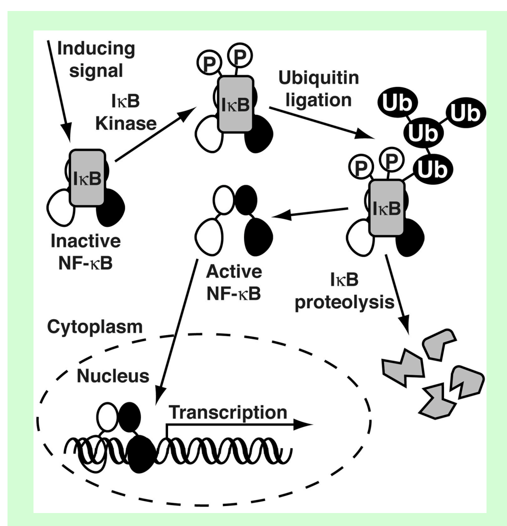



**Figure 5 The NF-κB signaling pathway** The prototypical NF-κB is a heterodimer of two subunits, p50 and p65. NF-κB is present in the cytoplasm of all cells as an inactive factor in complex with a member of the IκB inhibitor protein family. Diverse NF-κB-inducing stimuli lead to activation of the IκB kinase complex. The IKK phosphorylates IκB and further enzymatic process will eventually lead to proteolysis of IκB. Removal of IκB renders NF-κB active. It rapidly translocates from the cytoplasm to the nucleus where it binds specifically to DNA elements within the promoter regions of target genes and activates their transcription. Figure adapted with permission from Tom Huxford at Structural Biochemistry Laboratory, Department of Chemistry and Biochemistry, San Diego State University (www-rohan.sdsu.edu/~thuxford/)

**AP-1** is a family of DNA-binding factors composed of dimers, of which the best known are the Fos and Jun factor. AP-1 is found in many cell types and is activated in response to a variety of physiological and pathological stimuli; cytokines, growth factors, stress signals, bacterial and viral infections, as well as oncogenic stimuli (Hess, et al., 2004). The activated AP-1 transduces these extra-cellular signals to immune cells and mediates gene regulation of processes such as proliferation, differentiation, apoptosis and transformation. In RA, AP-1 is known to control the expression of inflammatory cytokines and matrix-degrading matrix metalloproteinases (MMPs), involved in the cartilage- and bone eroding processes (Okamoto, et al., 2008). In CIA mice, activation of AP-1 and NF-κB precede both clinical arthritis and MMP gene expression (Han, et al., 1998). The signalling pathways leading to NF-κB and AP-1 activation are overlapping, the

signal transduction pathways and following inflammatory cytokine induction by these transcription factors are, however, not yet completely understood (Khalaf, et al., 2010).

**Foxp3** is a key control molecule for the development and function of natural $CD4^+CD25^+$ Tregs. In mice, foxp3 is exclusively expressed by Tregs and is believed to be the responsible for the supppressor phenotype of the Treg population (Hori, et al., 2003). Foxp3 is currently the most specific and reliable molecular marker for natural Tregs in both rodents and humans (Sakaguchi, et al., 2006).

## INCIDENCE AND PREVALENCE OF RA

About 1% of the world's population is affected by RA. Women are more likely to develop the disease than men: the female to male incidence ratio is around 3:l overall, but decreases with increasing age. At premenopausal age, the sex ratio is 4-5:1. After the age of 60 the incidence ratio is lower than 2:1 (Kvien, et al., 2006). The overall incidence rate of RA is 20-40 cases/100.000/year in women and 10-20/100.000/year in men. Inheritance of RA, is estimated from twin studies to account for 50-60% (MacGregor, et al., 2000) while the remaining 30-40% are believed to be accounted for by environmental and hormonal influences.

## PATHOGENESIS OF RA

The pathogenesis of RA is not yet completely understood. Several contributing components have, however, been identified over the years. **Genetic**, **environmetal** and **hormonal** factors are all believed to take part in the pathogenic processes leading to RA and these are presented below.

### *GENETICS AND RA*

The most definitive genetic association for RA is with the human leukocyte antigen (HLA) alleles of the major histocompatibility complex (MHC) on chromosome 6. **The shared epitope hypothesis**, described in the 1980's, postulates that susceptibility to RA is carried by a discrete part of HLA-DR4, the third hypervariable region of the DRB1 chain. People bearing certain epitopes (a conserved amino acid sequence at positions 70-74 in the third hypervariable region) within the class II MHC complex have an increased risk of developing RA (Gregersen, et al., 1987). The mechanism by which HLA confer susceptibility to RA is not yet fully understood. Several other genetic susceptibility loci have in recent years been associated with RA, HLA-DRB1

though remaining the strongest. Protein tyrosine phosphatase non-receptor type 22 (**PTPN22**) expression have been associated to RA. PTPN22 is a gene that codes for a tyrosine phosphatase with key regulatory functions in signal transduction pathways and regulation of T and B cell activation. Association with RA has also been found with the cytotoxic T lymphocyte antigen 4 (**CTLA4**) gene which encodes a protein essential for the regulatory functions of Tregs. The CTLA4 protein is expressed on the surface of the Treg and binds to ligands on APCs.

### *ENVIRONMENTAL FACTORS IN THE DEVELOPMENT OF RA*

Studies concerning RA and environmental influences are most often epidemiological studies based on retrospective cohort studies. These types of studies can show **association** between a disease and an environmental parameter, but no true causative relation or biological mechanism is proven. In this thesis we have focused our attention on **environmental toxins** commonly used by the general population. We have studied the effects of **ethanol** (alcohol) and **nicotine** on the development of arthritis in CIA mice and tried to elucidate the mechanistical pathways behind the anti-inflammatory actions observed.

**Alcohol** intake has been coupled to a decreased risk of developing RA in several studies (Kallberg, et al., 2009; Lu, et al., 2010; Voigt, et al., 1994). Alcohol consumption has also been shown to be inversely and dose-dependently related to the severity of disease; RA severity measures such as CRP and DAS-28 acore are inversely associated with increasing frequency of alcohol consumption (Maxwell, et al., 2010). Recent data also show that occasional and daily alcohol intake is associated with reduced radiographic progression in alcohol drinkers compared to non-drinkers (Nissen, et al., 2010). Additionally, a study of women with pre-clinical RA has confirmed that light to moderate drinking is associated with decreased levels of IL-6 and sTNFRII (measured as a proxy for TNFα) (Lu, et al., 2010). These data all come from epidemiological studies and present no mechanistical explanation of the decreased risk for RA following alcohol use.

In **Paper I,** we present data from our experimental study with ethanol supplemented to CIA mice. In this study, we show that a chronic intake of ethanol prevents the development of destructive arthritis in mice. We also show that this is mediated through a significant reduction in IL-6, macrophage inflammatory protein (MIP) 1α and TNF-α, as well as through down-

regulation of leukocyte migration. These anti-inflammatory effects are most probably mediated through an increase in testosterone and a reduction in NF-κB translocation (see Results: Paper I).

**Cigarette smoking** is a common environmental factor described as increasing the risk of developing RA (Vessey, et al., 1987). Epidemiological studies have showed that smoking increases the risk of developing RA, but only the aCCP positive phenotype in patients bearing the HLA-DR shared epitope genes (Klareskog, et al., 2006). Since no experimental studies concerning smoking and arthritis had previously been performed, we sought to determine if **exposure to cigarette smoke** (CS) had any impact on development of arthritis in the CIA model, presented in **Paper II**. A possible link between smoking and development of RA is the presence of citrullinated peptides found in lungs of smokers. This has been implied as a possible initiating event for the development of RA, as it could trigger formation of antibodies towards aCCP (Makrygiannakis, et al., 2008). Such antibodies are found in around 60% of RA patients (van Gaalen, et al., 2004). Smokers show a lower response to vaccine than do non-smokers (Struve, 1992), and the reduction in antibodies is not related to a decrease in number of B cells but rather with suppression of their function and ability to produce antibodies. Smoking has, though, been shown to have beneficial effects on autoimmune disorders. Cigarette smoking is shown to be negatively associated with focal inflammation in patients with primary Sjögrens syndrome (Manthorpe, et al., 2000). The tobacco decreases inflammation in a dose dependent manner by reducing the accumulation of lymphocytes/plasma cells in salivary glands. Smoking has also been shown to exhibit protective effects on cartilage in patients with osteoarthritis (OA). Epidemiological studies show a modest but statistically significant protective association between smoking and OA (Felson, et al., 1989). The protective effect of smoking on bone is hypothesized to be mediated by nicotine.

**Nicotine,** at the physiological levels found in smokers, has been shown in *in vitro* studies to be a potent stimulator of collagen synthesis in chondrocytes and bone (Gullahorn, et al., 2005). Tobacco smoke contains more than 4,500 chemical compounds besides nicotine. The association between smoking and increased risk of developing various inflammatory disorders, such as RA, might therefore be independent of nicotine and perhaps instead driven by other, non-nicotinic components, present in cigarette smoke. The use of moist snuff and risk of RA was studied by Carlens et al (Carlens, et al., 2010) in a cohort of Swedish construction workers from 1978-1993. The study showed no association between use of snuff and risk of developing RA, indicating that the increased risk of RA from cigarette smoking is mediated through components other than

nicotine. These could possibly be silica and charcoal, previously identified as risk factors for RA (Stolt, et al., 2005). To examine the effect of pure nicotine on arthritic inflammation in experimental model of RA, we subjected CIA mice to nicotine in drinking water and assessed the effect on development and severity of arthritis. Results from the study are presented in **Paper II**.

### *HORMONES AND RA*

The majority of autoimmune diseases are predominant in women (Oliver, et al., 2009). The mechanism behind this phenomenon is not clear, but much focus has turned to the **hormonal differences** in men and women. Immune reactivity in general is more enhanced in females than in males. Estrogens have been shown to suppress T and B lymphopoiesis (Medina, et al., 1994; Rijhsinghani, et al., 1996), but are generally considered to be enhancers of cell proliferation and the humoral immune response (Cutolo, et al., 2010); lymphocytes and monocytes from females show higher antigen presenting activity, women have higher levels of immunoglobulins than men, and they exhibit higher antibody production to primary and secondary antigen stimulation (Zandman-Goddard, et al., 2007). Estrogen affects both innate and adaptive immune responses. It inhibits neutrophil function and adhesion to endothelial cells, decreases NK cell activity, induces apoptosis in monocytes and modulates the release of pro-inflammatory cytokines from activated monocytes and macrophages (Islander, et al., 2010). It is not clear wheter or not this fact is due to sex hormones specifically, but it is known that androgens have a more suppressive, anti-inflammatory effect on humoral and cellular responses in comparison to estrogens. In fact, both male and female RA patients express lower androgen and higher estrogen levels in body fluids (blood, synovial fluid, smears, saliva). This supports the possibility of a pathogenic role for low levels of androgens (Cutolo, et al., 2002). The fact that women have a higher incidence rate of RA than men implicates estrogen as a possible mediator of disease. The complexity of the effects of estrogens on RA, is evident when considering the fact that many female RA patients experience remission during pregnancy (75%), when estrogen levels are high. Epidemiological, immunological and clinical studies show that estrogen in normal conditions enhances immune responses, mainly humoral. Any definite pro- or anti-inflammatory effects of estrogen can, however, not be demonstrated; variations in estrogen receptor composition and diversity, hormone concentration, aromatase activity and types of estrogen metabolites are all factors having an impact on the final effect that estrogen exercises on RA (Cutolo, et al., 2010).

## Sympathetic regulation in inflammation and RA

All primary and secondary immune organs are innervated by sympathetic postganglionic neurons. The **sympathetic nervous system** (SNS) and its main neurotransmitter norepinephrine (NE), primarily inhibit the innate immune system, whilst either inhibiting or activating the adaptive system. This difference is probably due to a diversity in receptor expression; innate cells express both $\alpha$- and $\beta$-adrenergic receptor subtypes while T and B cells exclusively express beta2 receptors (Nance, et al., 2007). Both CIA mice and RA patients have significantly decreased density of sympathetic nerve fibers in synovial tissue, indicating impairment of the anti-inflammatory effects of SNS (Miller, et al., 2000). The effect of the SNS on innate immunity is demonstrated by its effect on macrophages (being central in the early immune responses); *in vivo* activation by **stress** or inflammatory stimuli **inhibits** splenic **macrophage function**. In mice with CIA, an opposing time-dependent effect of the SNS has been demonstrated by Härle et al (Harle, et al., 2005) by performing sympathectomy on CIA mice at different time points; sympathectomy **before** immunization **decreased** CIA in mice while sympathectomy **after** immunization increased CIA (Harle, et al., 2008). This shows that in the **early, asymptomatic, phase** of arthritis, **SNS is pro-inflammatory** whereas it is **anti-inflammatory** in the **chronic, symptomatic, phase**. The same group also showed that activation of the SNS ameliorates CIA in the late phase, but aggravates the presymptomatic phase of the disease. This is believed to be mediated through stimulation of the pro-inflammatory properties of regulatory $CD4^{+}CD25^{+}$ T cells (Harle, et al., 2008).

## Parasympathetic regulation in inflammation – the cholinergic/nicotinic anti-inflammatory pathway

There is no neuroanatomical evidence for a parasympathetic or vagal nerve supply to the immune organs (Nance, et al., 2007). The connection between the parasympaticus and the immune system is rather conveyed as neurotransmission through the **vagus nerve**. Acetylcholine released by the vagus nerve endings binds to the **alpha7 subunit of nicotinic acetylcholine receptors** ($\alpha$7nAChR) (see also p. 35) on cytokine-producing cells such as macrophages, and in this way regulates immunity by **inhibiting pro-inflammatory cytokine production**. Activation of this cholinergic pathway, either by electrical stimulation of the vagus nerve, or by administration of

$\alpha$7nACh receptor agonists, **ameliorates inflammation** and improves survival in experimental sepsis models (Rosas-Ballina, et al., 2009). The $\alpha$7nACh receptor is proven to be essential for inhibition of cytokine-production via the cholinergic anti-inflammatory pathway, as stimulation of the vagus nerve in wild-type mice inhibits TNF synthesis, but fails to do so in $\alpha$7nACh-knockout mice (H. Wang, et al., 2003). In conclusion, the cholinergic anti-inflammatory pathway is a neural mechanism that suppresses the innate inflammatory response, i.e. preventing excessive inflammation. When it comes to parasympathetic regulation in RA and CIA, studies show that vagotomy exarcerbates arthritis, while stimulation (via nicotine administration) ameliorates the disease (van Maanen, et al., 2009). Epidemiological studies in humans have, however, shown that vagotomy has no specific effect on the risk of developing RA (Carlens, et al., 2007).

# ANIMAL MODELS OF RA

The search for a valid animal model of human RA has been a quest for many years. After the demonstration of serum antibodies towards collagen in RA-patients (Steffen, et al., 1963), immunization experiments with collagen type II in various animal species was initiated. In 1977, Trentham and colleaues found that an intradermal injection of collagen type II from human, chick or rat cartilage emulsified in incomplete or complete Freund's adjuvant elicited an inflammatory arthritis termed **collagen-induced arthritis** (CIA) in rats (Trentham, et al., 1977). In 1980, a mouse-model of CIA was developed by a similar protocol (Courtenay, et al., 1980).
The similarities in clinical manifestations between CIA and human RA are evident: symmetrical swelling of joints and engagement of small joints in the extremities, followed by cartilage and bone erosions seen on histological examination. When it comes to certain predictive and prognostic serum markers, there is still debated as to wheter or not they exist in the murine models of RA. There are contradictory reports on auto-antibodies towards IgG (RF), collagen II and aCCP (Kuhn, et al., 2006; Vossenaar, et al., 2003). The CIA model is nonetheless widely used in pathological and pharmacological RA-research. It was, for example, applied when first developing the successful anti-rheumatic drugs targeting TNF-$\alpha$ (Wooley, et al., 1993).

## COLLAGEN-INDUCED ARTHRITIS (CIA) IN MICE

All three papers presented in this thesis employes the collagen-induced arthritis model. Induction of CIA is based on the principle of immunizing the animal with collagen type II emulsified in complete or incomplete Freund's adjuvant through an intradermal or subcutaneous injection at the base of the tail. A booster injection is given about three weeks later, and clinical signs of joint inflammation follows within a few days. A schematic drawing of the mechanism of CIA is depicted in figure 6. The incidence of arthritis is typically around 80%. Susceptibility to CIA is strongly linked to MHC class II, primarily to expression of genes homologous to human DQ and DR (the classical susceptibility-genes for human RA), namely H2 I-A and H2 I-E, and more specifically the H2 I-A q and r alleles (HolmdahlJansson, et al., 1988; Wooley, et al., 1985). The I-Aq allele is found in the widely used DBA/1J strain. In contrast, C57BL/6 mice carrying the I-Ab allele are less susceptible to CIA, and develop only a mild form of the disease (Rosloniec, et al., 2010).

## PASSIVE TRANSFER

The involvement of T and B cells in CIA is considered proven, as mice lacking T (Ehinger, et al., 2001; Tada, et al., 1996) or B-cells (Svensson, et al., 1998) fail to develop the disease. Passive transfer of CII-specific T cells to susceptible naïve strains induces only minor histological changes in the synovium of the recipient. CII-specific T cells are, however, present in the arthritic joints of CIA mice, which indicates a pathogenic role for these cells (HolmdahlJonsson, et al., 1988). Transfer of CII-specific antibodies from arthritic mice or RA patients (Wooley, et al., 1984) to naïve mice results in severe inflammation (Brand, et al., 2003). The passive transfer of CII-antibodies, described below under *CAIA*, results in arthritis not only in strains susceptible to CIA, but also in CIA-nonsusceptible strains (Watson, et al., 1987).

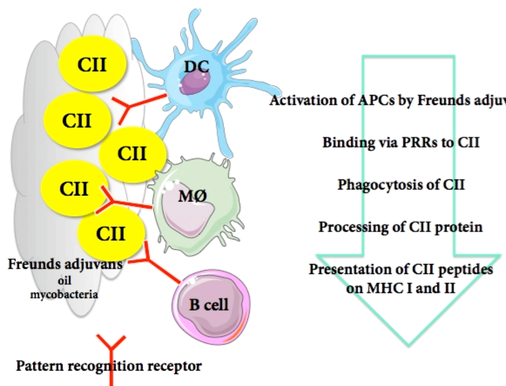



**Figure 6A Mechanism of collagen-induced arthritis. Antigen-recognition and processing by innate immune cells** On day 0 mice are immunized with collagen II emulsified in Freund's complete adjuvant. The adjuvant, containing oil and mycobacteria, activates dendritic cells (DCs) and macrophages (M∅), via pattern recognition receptors such as toll-like receptors (TLRs). The CII is phagocytosed and processed into peptide fragments which are complexed with MHC I and MHC II, eventually presented on the APC to a T cell in the draining lymph node (fig 6 B).

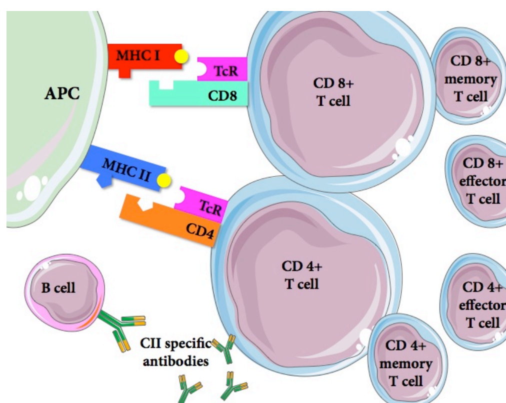



**Figure 6B Presentation of the antigenic peptide via MHC to the T cell.** Processed collagen peptides are presented on MHC I to $CD8^+$ T cells, binding to the T cell receptor (TcR) in co-ordination with the CD8 co-receptor. Following this antigen-recognition, the T cells are activated and differentiate into effector cells and memory cells. The corresponding process takes place with MHC II binding to the TcR on the $CD4^+$ T cell

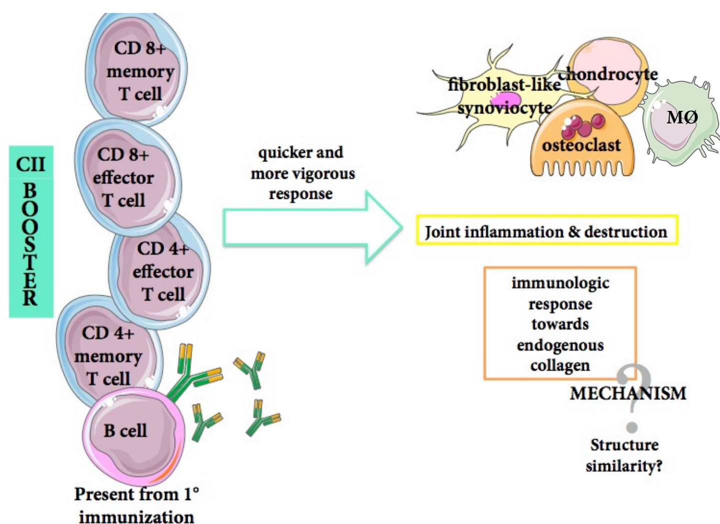



Figure 6C Booster immunization and resulting joint engagement. On day 21 mice receive a booster dose of CII in Freund's incomplete adjuvant. CD4+ and CD8+ effector and memory T cells present from the primary immunization are easily re-activated by the booster dose of CII. A quicker and more vigorous response follows and resident cells such as synoviocytes, chondrocytes, macrophages and osteoclasts will release cartilage- and bone degrading enzymes. The antibodies formed by B cells, bind to cartilage and activate complement, inducing chemotaxis of neutrophils and macrophages. All these factors contribute to inflammation and destruction of the joint. The mechanism behind the attack on endogenous collagen is as yet unclear, but structural similarity between exogenous CII used in immunization and endogenous CII might be a possible explanation. When the antigen is eliminated by the CD8+ cells, the immune response will decline and return to homeostasis.

## Collagen Antibody-Induced Arthritis (CAIA)

The CAIA model of experimental arthritis, is a mouse model based on the passive transfer of CII antibodies. The CAIA model resembles the CIA model but is an **acute form** of arthritis with a rapid onset which completely subsides after about a month. It is mostly employed in studies evaluating the **effector phase** in arthritis (rather than the priming, initiation phase as in CIA). CAIA is MHC-independent and can be induced in T and B-cell deficient mice aswell as SCID mice (Nandakumar, et al., 2006). In this model, a complex of **monoclonal antibodies** towards different epitopes of CII is administered intravenously on day 0, followed by an i.p. LPS injection 3-7 days later. LPS enhances the incidence and severity of arthritis through bypassing epitope specificity and activating complement and pro-inflammatory mediators. Arthritis will develop a few days after LPS injection. CAIA resembles human RA in that there is an influx of neutrophils and deposition of IgG and complement on the articular cartilage, with subsequent bone erosions (Rowley, et al., 2008). It has been proposed that immune complexes, as in the CAIA-model,

initiate inflammation either via activation of the complement system (Colten, 1994) or by engagement of Fc receptor-bearing cells, such as NK-cells and macrophages (Ravetch, et al., 1998). Binding of complement fragments to the immune complex in combination with FcR cross-linking, activates lymphocytes to release pro-inflammatory cytokines, recruiting neutrophils and macrophages. Further activation will affect the resident cell population of synoviocytes and chondrocytes and release cartilage-degrading enzymes, leading to joint destruction (Rowley, et al., 2008).

# ETHANOL

Ethanol, ethyl alcohol ($C_2H_5OH$) is a straight-chain alcohol, a volatile, flammabale liquid, and a common recreational drug. Ethanol disseminates from the blood into all tissues and fluids in proportion to their relative content of water. Ethanol does not bind to any plasma protein, but passes readily over cellmembranes due to its small size. Metabolism of ethanol occurs primarily in the kidneys and liver and proceeds as depicted in Figure 7. The first step is oxidization to **acetaldehyde,** followed by conversion to acetic acid. This second step is catalyzed by the enzyme acetaldehyde dehydrogenase (ALDH), an enzyme with two different isoforms: a mitochondrial (ALDH I) and a cytosolic (ALDH II) one. An inherited deficiency in the ALDH I isozyme has been identified in the Asian population. The structural mutation in the gene causes loss in catalytic activity, leading to an accumulation of acetaldehyde. This results in acute alcohol sensitivity symptoms such as flushing. The deficiency in ALDH I is believed to be a possible explanation for the lower incidence of alcoholism in the Asian population (Goedde, et al., 1987).

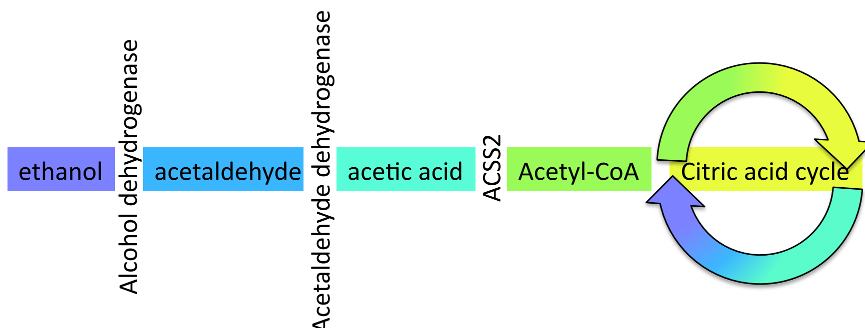



**Figure 7 Ethanol metabolism** The kidneys and liver are the primary sites for ethanol metabolism. Ethanol is oxidized to acetaldehyde via the enzyme alcohol dehydrogenase. Acetaldehyde is highly unstable and forms toxic free radicals if not taken care of by antioxidants. The conversion of acetaldehyde to acetic acid is performed by the enzyme acetaldehyde dehydrogenase. Conversion of acetic acid to acetyl-CoA is performed by ACSS2 (acyl-CoA synthetase short-chain family member 2). The acetyl-CoA produced enters the citric acid cycle.

## ETHANOL (ALCOHOL) AND THE IMMUNE SYSTEM

Chronic alcohol abuse is associated with immunosuppression and increased morbidity and mortality. Moderate alcohol intake, though, appears to exert positive effects on health. In multiple epidemiological studies, subjects drinking moderately (1-3 drinks per day) prove to have lower

mortality rates than non- and heavy-drinkers (Power, et al., 1998; Thun, et al., 1997). Extensive evidence indicates that alcohol possesses dose-dependent immunomodulatory properties. Acute and moderate use is associated with a milder inflammatory response while heavy consumption augments the inflammatory response. Heavy drinkers express high levels of pro-inflammatory cytokines in the circulation, such as TNF-α, IL-1 and IL-6, while acute exposure to ethanol leads to a decrease in cytokine-production (Goral, et al., 2008). Long-term intake of low to moderate amounts of alcohol has been shown to reduce the inflammatory response by downregulating TNF, IL-1, IL-6 and NF-κB activation (Imhof, et al., 2001; Imhof, et al., 2004; Szabo, et al., 1995; Szabo, et al., 1996; Verma, et al., 1993) and by increasing levels of the anti-inflammatory cytokine IL-10 and TGF-β (Mandrekar, et al., 1996; Szabo, et al., 1996). Moreover, both acute and chronic alcohol exposure have been shown to affect phagocytosis, one of the main components of innate immunity. Acute alcohol exposure reduces the expression of phagocytic receptors and co-stimulatory molecules on the cell surface as well as reducing the formation of lysosomal vesicles, in all decreasing the phagocytotic potential in alveolar PMNs and macrophages (Goral, et al., 2008). An increase is, however, seen in the phagocytotic potential of liver PMNs and Kupffer cells after acute ethanol exposure. Chronic alcohol exposure decreases overall phagocytosis and the ability of antigen-presenting cells to express antigen and co-stimulatory molecules on their surface. It decreases chemotaxis and phagocytosis by Kupffer cells as well as complement receptor-mediated phagocytosis. Splenic macrophages, however, have increased phagocytotic potential after chronic ethanol exposure (Goral, et al., 2008). In conclusion, ethanol has various effects on a number of different cell types and the effects are dependent on the timeframe of the exposure. Acute exposure to ethanol has been reported to reduce expression of some TLRs (Nishiyama, et al., 2002), whereas chronic exposure seem to increase the levels (Zuo, et al., 2003). These effects of acute versus chronic alcohol exposure support the theory of a biphasic nature of ethanol.

### ETHANOL RECEPTORS

Ethanol is readily taken up into the blood stream and distributed to all tissues in the body, including the brain. It binds to a number of different receptors in the brain as well as in the periphery. Alcohol-binding receptors in the brain have been extensively studied and several target

receptors have been identified. These are the NMDA (N-methyl-D-aspartate), GABAA (gamma amino butyric acid), glycine, serotonin and nicotinic acetylcholine (nACh) receptors, as well as ion channels of L-type Ca2+ and G-protein-activated inwardly rectifying K+ channels (GIRKs) (Vengeliene, et al., 2008). Importantly, though, after the first hit of ethanol on specific targets in the brain, a more unspecific second wave of action will follow. The subsequent cascade of synaptic events involves multiple neurotransmitter systems. Modulation of excitatory receptors on inhibitory interneurons, and vice versa, gives an example of how the effects of ethanol can vary. For instance, expression of the excitatory serotonin receptor, on inhibitory GABAergic interneurons will contribute to an overall inhibitory action of alcohol (Lovinger, 1999).

# NICOTINE

## NICOTINE AND INFLAMMATION

Nicotine is an alkaloid found in the nightshade family of plants (*Solanaceae*), especially in the tobacco plant. Nicotine is quickly distributed in the body through the blood to the brain, where it passes the blood-brain barrier. Nicotine in its pure form is not a toxic substance. This is, as for all pharmacologically active substances, depending on the dose administered. The common miscomprehension that nicotine is harmful, most probably comes from associations to cigarette smoking and its detrimental effects on health. Cigarette smoke, however, contains thousands of different potent substances besides nicotine, such as tar, carbon monoxide, nitrogen oxide, hydrogen cyanide and metals. Nicotine is, though, believed to be the major addictive component. Nicotine has over the past few years been shown to possess potent **anti-inflammatory effects** and are now studied in several different inflammatory conditions. In experimental ulcerative colitis, the addition of transdermal nicotine to conventional therapy significantly improves symptoms in patients (Pullan, et al., 1994). The mechanism for the anti-inflammatory effect observed, is proposed to be a nicotine-induced reduction in TNF-$\alpha$ (Sykes, et al., 2000). Nicotine has also been shown to improve outcome of sepsis by affecting inflammatory mediators (H. Wang, et al., 2004) and to suppress dendritic cell functions leading to Th1 priming. More recently, the anti-inflammatory properties of nicotine has been proposed to be mediated by the **nicotinic acetylcholine receptor $\alpha$7** ($\alpha$7nAChR) (H. Wang, et al., 2003). This nicotinic anti-inflammatory pathway is part of the parasympathetic regulation in inflammation and RA described previously.

## NICOTINE RECEPTORS

Nicotine binds to nicotinic acetylcholine receptors (nAChRs) located on **neurons** (neuronal-type) in CNS and PNS and on the postsynaptic side of the **neuromuscular junction** (muscular-type) of somatic muscles (when stimulated causing muscle contraction). The nAChR is a ligand-gated ionotropic receptor consisting of a pentamer of five subunnits forming an ion channel (Fig 8). Endogenous agonists opening the channel are acetylcholine, nicotine, epibatidine and choline. Binding of a ligand to the receptor causes a conformational change which opens the ion channel, permitting influx of cations such as Na+ and K+. To date, 17 different nAChR subunits have

been identified and the variation in assembly of subunits results in a large array of possible nAChRs with different physiological functions and ligand affinity. The neuronal-type α7nACh receptor, consisting of five α7 subunits has received most attention regarding inflammation (see Nicotine receptors and RA/CIA below).

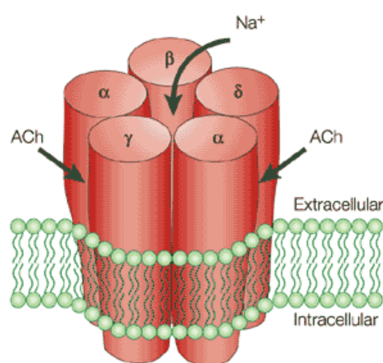



**Figure 8 The nicotinic acetylcholine receptor**
A schematic representation of the quaternary structure of a muscle-type nicotinic receptor. Five subunits arranged as a pentamer form the ion channel where $Na^+$ ions flux when the channel is open and active i.e after binding of agonist such as ACh, as shown in the figure. The α7nACh receptor is made up of exclusively α-subunits arranged in a pentamer, in same way seen in the illustration. Figure adapted by permission from Macmillan Publishers Ltd: Nature Reviews Neuroscience, Emerging structure of the nicotinic acetylcholine receptors, Karlin A, 2002

Recent evidence has also shown expression of nicotinic receptors on **non-neuronal cells** both within and outside the nervous system. In humans, lymphocytes, vascular endothelial cells, keratinocytes and polymorphonuclear cells have been shown to express various subtypes of nAChRs, among these are the α7nAChR (Sharma, et al., 2002). The function of ligand-binding to such non-neuronal nicotinic receptors has not yet been defined.

**Receptor desensitization** in the nACh receptor was demonstrated early on. The phenomenon means that prolonged or repeated exposure to a stimulus (for example nicotine exposure through smoking) can result in decreased responsiveness of the receptor to that stimulus (i.e. nicotine). Chronic administration of nicotine also has the potential to increase the density of nACh receptors in the brain in both rodents and humans (Perry, et al., 1999). It has been implied that the difficulty of smoking cessation is due to this increased receptor expression. nACh receptors are often found on presynaptic terminals of presynaptic cells other than ACh-releasing ones, and can modulate the release of neurotransmitters. α7nAChRs are for example localized on glutamatergic axons, where they can mediate release of glutamate (Marchi, et al., 2002). α7nAChR activation is normally associated with facilitation of glutamatergic transmission in the brain (Hilmas, et al., 2001).

## Nicotine receptors and Rheumatoid artritis and CIA

The expression of nicotinic receptors has been shown in immunocompetent cells such as T and B cells as well as in monocytes, macrophages, neutrophils and dendritic cells. Though the role of the α7nAChR is not yet clearly defined (de Jonge, et al., 2007) the impact of the receptor on CIA and RA has been studied quite extensively: expression of the α7nAChR has been shown to be pronounced in synovium and on synoviocytes from RA patients (van MaanenStoof, et al., 2009; Waldburger, et al., 2008; Westman, et al., 2009), exposure of CIA mice to a specific α7nAChR agonist or nicotine ameliorates arthritis, inhibits bone degradation and reduces TNF-α expression in synovial tissue (van Maanen, et al., 2009), nicotine inhibits HMGB1 release from human macrophages (H. Wang, et al., 2004) through signaling via the α7nACh receptor and blocking activation of NF-κB (Saeed, et al., 2005). Two recent studies proposes that CIA mice lacking the α7nACh receptor show significantly milder arthritis and less cartilage destruction compared to wild-type controls (van Maanen, et al., 2010; Westman, et al., 2010). The α7nAChR knock-outs had fewer $CD3^+$ T cells in their draining lymph nodes, suggesting that the α7nAChR affects basal T cell activity.

In conclusion, the anti-inflammatory effects of cholinergic/nicotinic stimulation on innate immunity are well characterized, while the effects on adaptive immunity are less well-known.

# GLUTAMATE

## GLUTAMATE AND GLUTAMATE RECEPTORS

Glutamate (Glu) is the most abundant excitatory neurotransmitter in the vertebrate nervous system. Glu is essential for many important brain functions and is mostly recognized for its involvement in synaptic plasticity, learning and memory. The neurotransmitter is stored in vesicles in the pre-synaptic cell and released when a nerve impulse is transmitted (Fig 9). The released glutamate binds to glutamate receptors found on the post-synaptic cell. The receptors are **metabotropic** (G protein-activating) or **ionotropic** (ion channel-forming). The two most common receptors are AMPA (α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate) and **NMDA** (N-methyl-D-aspartate) which are both ionotropic glutamate receptors. The NMDA receptor is a ligand-gated, voltage-dependent ion channel. This means that (i) it is opened or closed in response to the binding of its ligand, and (ii) it is activated/opened when the membrane potential is depolarized. At rest, the NMDA receptor is blocked by magnesium ions (Fig 10). When depolarized/activated, the NMDA receptor is opened and an influx of cations, such as $Ca^{2+}$ ions, is permitted through the ion channel. To be fully activated the NMDA receptor requires co-activation by two ligands: glutamate and glycine.

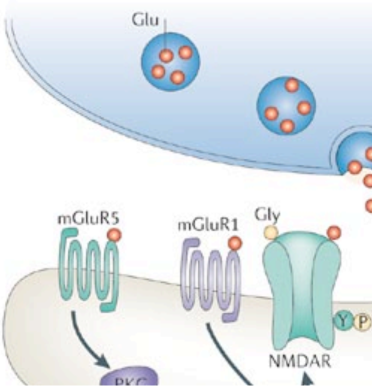



**Figure 9 The glutamatergic synapse**
Vesicles containing glutamate (Glu), in the pre-synaptic cell, are released when a nerve impuls is transmitted. The Glu binds to Glu receptors on the post-synaptic cell which are of metabotropic (mGluR1,5) or ionotropic (NMDAR) sort. When Glu in combintion with glycine (Gly) binds to the NMDAR, the receptor is activated and $Ca^{2+}$ ions will flow through the ion channel. Figure adapted by permission from Macmillan Publishers Ltd: Nature Reviews Neuroscience, Reelin, lipoprotein receptors and synaptic plasticity, Herz et al, 2010

## GLUTAMATE IN INFLAMMATION AND RA

Recent studies implicate glutamate as an important actor in inflammatory conditions such as RA (Hajati, et al., 2010) and multiple sclerosis (Baranzini, et al., 2010). Levels of various aminoacids, including Glu, has been shown to be elevated in plasma (Trang, et al., 1985) and synovial fluid from RA patients (McNearney, et al., 2004), as well as from animal models of arthritis (Hinoi, et

al., 2005; McNearney, et al., 2004; McNearney, et al., 2000). Expression of the glutamate receptor NMDA has been shown in T cells, chondrocytes, osteoblasts, osteocytes, osteoclasts, keratinocytes, fibroblasts and synoviocytes (Flood, et al., 2007; Kvaratskhelia, et al., 2009; Piepoli, et al., 2009) - all cells of importance in RA. Human T cells are also shown to exhibit metabotropic glutamate receptors (Pacheco, et al., 2004). NMDA receptors are also expressed in human cartilage and inhibition of NMDA receptors on fibroblast-like synoviocytes from RA patients increases pro-MMP2 release. Inhibition of the non-NMDA receptor kainate reduces IL-6 production by the same cells. These findings indicate that activation of NMDA and kainate Glu receptors on human synoviocytes may contribute to joint destruction in RA (Flood, et al., 2007).

## MODULATION OF GLU RECEPTORS

The implication of Glu in various inflammatory disorders has raised the question of possible ways to modulate its receptors. In **Paper III** we exposed CIA mice to the NMDA receptor antagonist **memantine**, the metabotropic Glu receptor$_{1a}$ antagonist **AIDA** and the broad spectrum excitatory amino acid antagonist kynurenic acid (**KA**). **Memantine** is an uncompetitive antagonist of glutamatergic NMDA receptors and is used therapeutically to treat moderate and severe Alzheimer´s disease. Alzheimer patients have chronically elevated levels of Glu leading to continuous influx of $Ca^{2+}$, ions which results in neurodegeneration. Memantine blocks the ion channel of the NMDA receptor (Fig 10) and thereby reduces the tonic influx of $Ca^{2+}$ ions (Parsons, et al., 1998) without altering the synaptic receptor activity of the NMDA receptor. Memantine has also been shown to block nicotinic ACh receptors at clinically relevant concentrations. Memantine actually blocks the α7nACh receptor more potently than NMDA receptors in the brain (Aracava, et al., 2005).

**AIDA** is a relatively potent and selective antagonist of group I metabotropic glutamate receptors (mGluR1 and mGluR5). Studies concerning AIDA preferentially concern neuronal interactions but some have been performed on inflammatory nociception and hyperalgesia (Dolan, et al., 2002), showing that development and maintenance of inflammatory hyperalgesia is dependent on activation of group I mGluRs in the spinal cord.

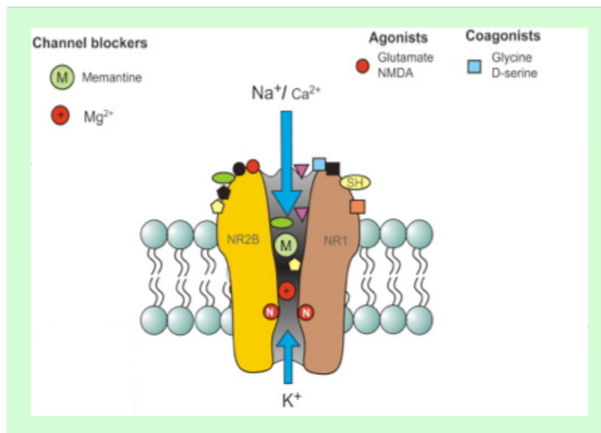



**Figure 10 The memantine-induced blocking of the NMDA receptor.**
NMDA receptors (NMDARs) in the mammalian CNS are formed by tetrameric assemblies of two NR1 and two NR2 subunits which express the glycine and glutamate recognition sites. Binding of agonists glutamate or NMDA and co-agonists glycine and D-serine on the receptor subunit assembly are required for receptor activation. All NMDARs are permeable to cations such as $Ca^{2+}$. At rest, the NMDA channel is blocked by $Mg^{2+}$. When activated, $Mg^{2+}$ leaves and $Ca^{2+}$ ions can flow through the channel leading to neurotransmission. Memantine is an uncompetitive NMDA receptor antagonist with higher affinity to the NMDA receptor than $Mg^{2+}$. It blocks the ion channel, disrupting the influx of $Ca^{2+}$and hence neurotransmission. Reprinted from Neuropharmacology, Nov;53(6), Parsons CG et al, *Memantine: a NMDA receptor antagonist that improves memory by restoration of homeostasis in the glutamatergic system--too little activation is bad, too much is even worse*, p. 699-723, 2007, with permission from Elsevier

**KA** is a broad-spectrum excitatory amino acid antagonist, inhibiting the glycine site of the NMDA receptor (Stone, 1993), the α7nACh receptor (Grilli, et al., 2006) and the orphan G receptor GPR35 (J. Wang, et al., 2006). KA is associated with a number of neurological disorders; a decrease in brain levels in patients with end-stage Parkinson's disease has been observed (Ogawa, et al., 1992), whereas an increase in levels can occur in patients with Alzheimer's disease (Baran, et al., 1999). KA is sparsely studied in the context of inflammation outside the CNS, but a study of experimental colitis in rats shows that KA decreases the motility and increases tonus in the colon, suggesting a possible therapeutic value for bowel disorders (Varga, et al., 2010). KA has been reported to inhibit not only the NMDA receptor, but also, and actually more potently so, the α7nACh receptor. Activation of the α7nACh receptor is normally associated with facilitation of Glu release in the brain. The decrease in Glu transmission observed after KA administration might for this reason be attributed to inhibition of the α7nAChR, rather than to KA acting on the NMDA receptor (Hilmas, et al., 2001 and references therin).

# RESULTS

## PAPER I

### *ETHANOL AMELIORATES EXPERIMENTAL ARTHRITIS IN MICE*

To evaluate the role of ethanol in mice with collagen-induced arthritis, DBA/1 mice were subjected to 10% ethanol as the only liquid from the same day as first immunization with collagen II. These mice showed a significant reduction in both frequency and severity of inflammation in comparison to water-drinking mice (Fig 1A,B). Additionally, ethanol-drinking mice presented significantly less synovitis and erosion (Fig 1C).

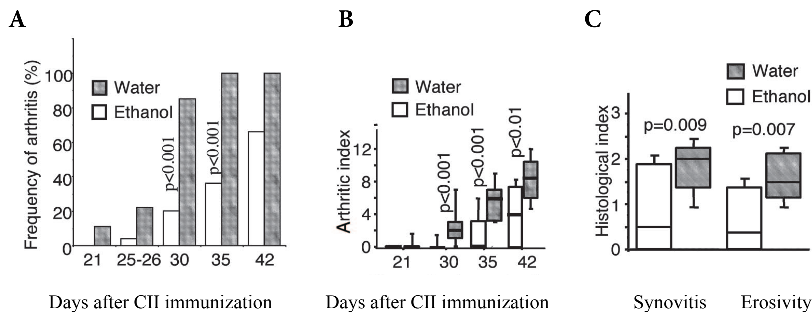



**Figure 1**. Frequency (A) and severity (B) of clinical arthritis and histological index (C) in CIA mice drinking 10% ethanol (open bars) or water (shaded bars).

### *ETHANOL'S METABOLITE ACETALDEHYDE HAS ANTI-INFLAMMATORY PROPERTIES*

Ethanol is oxidized in the body to acetaldehyde. To assess the impact of this metabolite on CIA, we exposed mice to 1% acetaldehyde in water from the same day as immunization. Results showed that the metabolite also possesses anti-inflammatory properties. The acetaldehyde-drinking mice developed a significantly milder arthritis (Fig 2A), continuously staying low until end of experiment. This potent effect was also evident when evaluating histological changes; acetaldehyde proved to significantly decrease synovitis and erosivity in the joints (Fig 2 B).

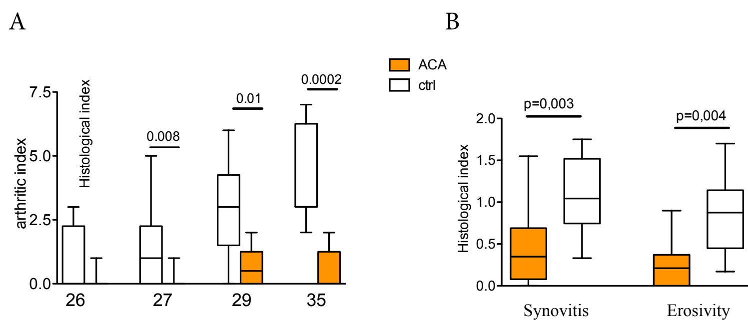



**Figure 2.** Acetaldehyde (ACA) decreases the severity of arthritis from day 27 after the first immunization until the end of the experiment at day 35 (A). Histological evaluation shows a significant decrease in synovitis and erosivity (B).

### *ETHANOL AFFECTS THE INITIATION PHASE, RATHER THAN THE EFFECTOR PHASE, OF CIA*

After establishing an ameliorating effect of ethanol on CIA, we aimed at determining if the effect was obtained during the initiation or the effector phase of the disease. Mice were subjected to 10% ethanol and four weeks later injected with a mixture of monoclonal antibodies towards CII. 14 days after the antibody-injection, 7 of 9 of the ethanol-drinking and 7 of 10 water-drinking mice had developed arthritis of equal severity. These result indicate that ethanol affects the initiation phase of CIA, rather than the effector phase.

### *ETHANOL DOWN-REGULATES EX VIVO PRODUCTION OF MIP-α AND TNF-α BY SPLEEN CELLS*

Spleen cells from NMRI mice subjected to 10% ethanol for two months produced significantly lower levels of the proinflammatory chemokine macrophage inflammatory protein 1 α (MIP-1α) and the pro-inflammatory cytokine TNF-α ex vivo (Fig $3A_1B_1$). When ethanol (0.1% and 0.5%) was added in vitro to spleen cells from water-drinking mice, 0.5% ethanol significantly downregulated levels of MIP1-α (Fig $3A_2$). The cytokines monocyte chemo-attractant protein (MCP) 1 and IL-6 from spleen cells of ethanol-drinking mice showed no differences from control for either *ex vivo* or *in vitro* production after exposure to ethanol (data not shown).

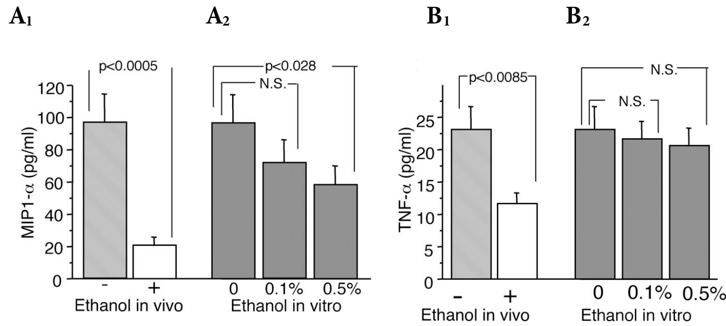



**Figure 3**. *Ex vivo* production of MIP-1 ($A_1$) and TNF-α ($B_1$) by spleen cells from NMRI mice drinking 10% ethanol (open bars) or water (light gray bars) for 2 months. *in vitro* production of MIP-1 ($A_2$) and TNF-α ($B_2$) by spleen cells of naïve NMRI mice after incubation with 0.1% and 0.5% ethanol.

### *ETHANOL DOWN-REGULATES MIGRATION OF PERITONEAL LEUKOCYTES*

To evaluate the migratory capacity of leukocytes after exposure to ethanol, cells from the peritoneal cavity of ethanol-drinking NMRI mice were collected. Cells were put into a migraton chamber and assessed for their ability to migrate toward the chemotactic stimulus hexapeptide WKYMVM. Leukocytes from ethanol-drinking mice migrated significantly less (P=0.026) than did cells from control mice as shown in Fig 4. In addition, cells from water-drinking mice exposed to ethanol *in vitro* (0,5%), also showed significantly weakened migratory capacity (P=0.0006) (Fig 4). We propose that this impaired migratory capacity in leukocytes is one of the mechanisms by which ethanol exercises its anti-inflammatory properties in CIA.

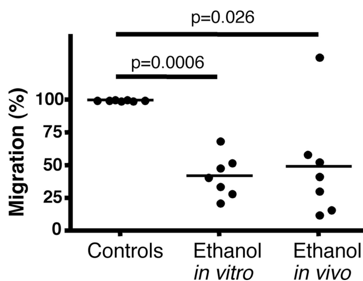



**Figure 4**. Migration of peritoneal leukocytes from controls, water-drinking mice exposed *in vitro* to 0,5% ethanol and mice exposed to 10% ethanol *in vivo* clearly shows the anti-migratory properties that ethanol exerts on leukocytes.

### *ETHANOL DOWN-REGULATES EXPRESSION OF TRANSCRIPTION FACTOR NF-κB AND AP-1*

The pathogenic roles of nuclear transcription factors NF-κB and AP-1 in inflammatory conditions are well known, and previous studies have shown increased expression and higher DNA-binding activity of NF-κB and AP-1 in both human RA and the CIA model (Handel, et al., 1995; Han, et al., 1998). Spleen cells from NMRI mice drinking 10% ethanol for 8 weeks proved to express significantly reduced levels of the nuclear transcription factors NF-κB (Fig 5A) and AP-1 (Fig 5 B) when assessed by EMSA. Addition of Con A overcame the inhibitory effect of ethanol and increased translocation of both NF-κB and AP-1 in control mice (Fig 5).

Our results show that long-term exposure to ethanol *in vivo* inhibits these inflammation-associated signaling pathways and reduces activity of NF-κB and AP-1.

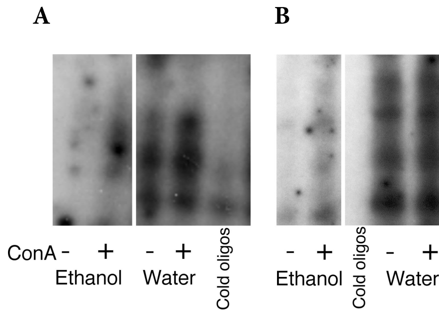



**Figure 5**. EMSA showing nuclear activity of NF-κB (A) and AP-1 (B) in spleen cells from mice drinking 10% ethanol or water for 8 weeks. In the right panel of picture A, expression of NF-κB from water-drinking mice is shown with and without addition of Con A. Spontaneous expression of NF-κB is quite high and addition of Con A potently increases this expression. In comparison, the spleen cells from ethanol-drinking mice (left panel of picture A), show significant reduction in expression of NF-κB in presence or absence of Con A. Same reduction in expression of AP-1 was observed in the ethanol-drinking mice compared to controls, shown in figure B.

### *ETHANOL INCREASES TESTOSTERONE LEVELS*

Five weeks after the start of the CIA-experiment, mice were bled and sera was analysed for levels of the sex hormones testosterone and estradiol, IGF-1 (insulin-like growth factor 1) and cortisol.

Levels of testosterone were significantly higher (almost three times higher) in the ethanol-drinking mice than in the water group (Table 1), levels of IGF1 and cortisol were significantly lower, and estrogen was unaffected.

| Hormone | Drink provided: 10% ethanol | Drink provided: Water | *P* value |
|---|---|---|---|
| Testosterone, ng/ml | 2.05 ± 1.02 | 0.83 ± 0.31 | 0.045 |
| IGF1, ng/ml | 97.6 ± 3.6 | 121.6 ± 7.5 | 0.027 |
| Cortisol, nmol/liter | 4.14 ± 0.54 | 5.61 ± 0.34 | 0.036 |
| Estrogen, pg/ml | 16.13 ± 1.05 | 13.39 ± 1.66 | N.S. |

For each hormone shown, the number of mice observed was 28. Values shown are means ± SEM. *P* values of ≤0.05 were considered N.S.

**Table 1.** Levels of hormones testosterone, IGF-1, cortisol and estrogen in 10% ethanol-drinking group and control.

### *TESTOSTERONE MEDIATES THE ANTI-INFLAMMATORY EFFECTS OF ETHANOL*

The impact of ethanol exposure on levels of testosterone encouraged us to study the effect of ethanol on castrated CIA mice. DBA/1 mice were castrated before induction of CIA and exposure to ethanol. The absence of testosterone in these mice abolished the anti-inflammatory effect of ethanol; mice drinking water had a median score of 2 (IQR 1-5) and mice drinking ethanol had a median score of 4.5 (IQR 2-6) (N.S).

We further evaluated the production of nuclear transcription factors in spleen cells from castrated mice. Interestingly, treatment of splenocytes from these mice with α-hydroxy testosterone down-regulated spontaneous and Con A-induced activation of NF-κB and AP-1 (data not shown).

### *ETHANOL PREVENTS LOSS OF BONE MINERAL DENSITY*

On termination of the CIA experiment, the left femur of very mouse was excised and analysed by peripheral quantitative computed tomography (pQCT) to assess bone mineral density (BMD). The left panel of figure 6 shows that healthy, non-arthritic mice supplied with 10% ethanol do not suffer from bone loss compared to water-drinking ones. Ethanol-drinking arthritic mice did, however, display significantly higher BMD than did water-drinking mice (P=0.029) (Fig 6, right panel). This would indicate that ethanol reduce bone mineral density-loss in inflammatory conditions such as arthritis.

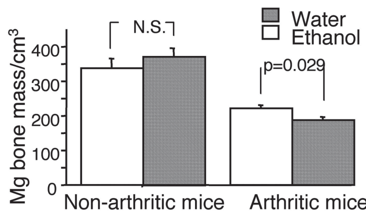



**Figure 6**. Bone mineral density presented as mg/cm3 in non-arthritic (left panel) and arthritic (right panel) mice. The left panel shows non-arthritic mice supplied with water or ethanol and shows that BMD was similar between the groups. In arthritic mice, a significant difference is seen where ethanol-drinking mice preserve their bone mass in comparison to water-drinking ones.

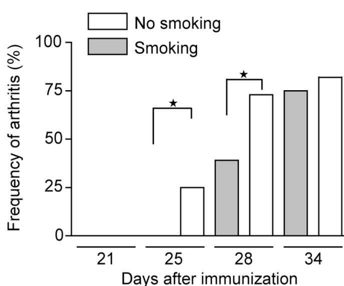
## PAPER II

### *SMOKING AND NICOTINE EXPOSURE DELAY DEVELOPMENT OF CIA IN MICE*

Mice exposed to cigarette smoke (4 unfiltered cigarettes per day, 6 days a week for 16 weeks) developed arthritis later, and to a lesser extent, than did control mice (Fig 7A). At day 25 after immunization, 25% of the control mice had arthritis while none of the smoking mice showed signs of inflammation. This trend persisted to day 28, when around 75% of the control animals were arthritic compared to less than 30% of the smoking animals ($P<0.05$, Fig 7A).

The nicotine-exposed animals showed a reduction in the frequency of arthritis, though not significant (Fig 7B). At day 28, only 10% of nicotine-exposed animals exhibited signs of arthritis compared to 40% in the control group ($P=0.08$).

**A**

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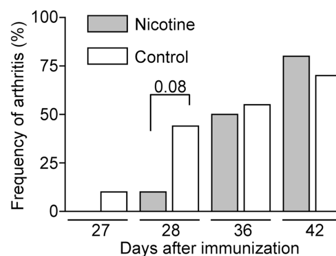



**Figure 7. Frequency of arthritis for smoking (A) and nicotine-exposed (B) CIA mice.** Smoking mice presented significantly less severe arthritis developing later than in non-smoking mice. Nicotine-exposed mice showed the same trend, though not statistically significant.

### *Smoking decreases production of aCCP and aCII antibodies in CIA mice*

When trying to elucidate the potential pathogenic role for smoking in human RA, it has been proposed that production of citrullinated proteins in the lung might be the first step in the pathogenic chain. Healthy smokers have been shown to express increased levels of citrullinated proteins in BAL cells (Makrygiannakis, et al., 2008). Smoking mice expressed lower serum levels of aCCP antibodies; 1 out of 22 animals tested positive for IgG aCCP in the smoking group whereas 5 out of 16 in the control group were positive ($P<0.05$)(Fig 8A). Some reduction in antibody production was seen for aCII antibodies; smoking animals expressed significantly lower levels of IgG against CII than did controls ($P<0.05$)(Fig 8B). Nicotine-exposed animals show no difference in aCCP and aCII antibody levels.

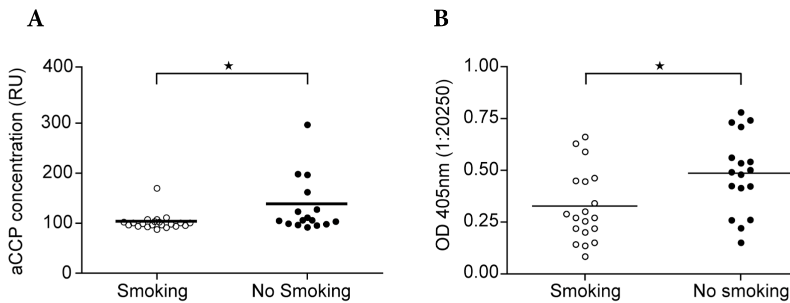



**Figure 8**. Levels of antibodies towards aCCP (A) and aCII (B) in sera from smoking animals at discontinuation of experiments. Smoking mice expressed significantly lower levels of aCCP antibodies compared to non-smoking controls. The same significant decrease was seen for antibodies towards collagen II in smoking mice.

### *Nicotine suppresses production of IL-6 by splenocytes*

Systemic levels of IL-6 showed no differences in between the smoking and non-smoking mice nor between the nicotine-exposed and controls (figure not shown). Splenocytes from healthy NMRI mice were incubated in the presence and absence of nicotine, and stimulated with LPS (10 μg/ml) to evaluate any potential effect on proinflammatory cytokines. Results showed that cells

incubated in the presence of nicotine produced significantly lower levels of IL-6 compared to controls (P<0.005)(Fig 9).

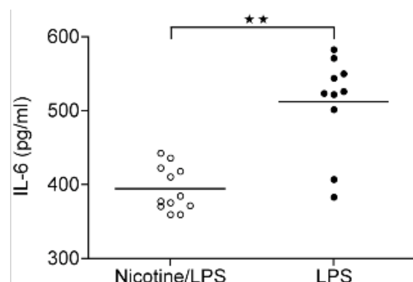



**Figure 9**. Naïve spleen cells were incubated for 24 hours with LPS (10 µg/ml) in the presence or absence of nicotine (10 µg/ml). Results show that ncells grown in presence of nicotine expressed significantly lower levels of IL-6 in supernatants.

## PAPER III

### *THE NMDA RECEPTOR ANTAGONIST MEMANTINE AMELIORATES AND DELAY CIA*

Mice exposed to twice daily subcutaneous injections of the NMDA receptor antagonist memantine (Ebixa®) from day 0 expressed a significantly milder arthritis developing later than controls (Fig 10 A). From nine days after booster and until end of experiments at days 36-41, memantine-treated mice showed significantly lower levels of arthritis. Data are pooled from three separate experiments. From one of the *in vivo* experiments, joints were taken for histological examination, with the results shown in Fig 10 B. Memantine-treated mice suffered from significantly less synovitis and erosion (P<0.05). Hematoxylin/eosin- and safranin O-staining (Fig 10D) shows representative pictures of the histological changes in the two groups; a massive cellular infiltration (CI) with subsequent erosion (E) of cartilage and bone in the joint from the control group is evident. Pictures from the memantine-treated mouse shows an almost unaffected joint cavity (JC) in comparison, with healthy-looking cartilage and bone. This was also demonstrated by significantly lower serum levels of the bone resorption marker CTX-I (P=0.04)(Fig 10C).

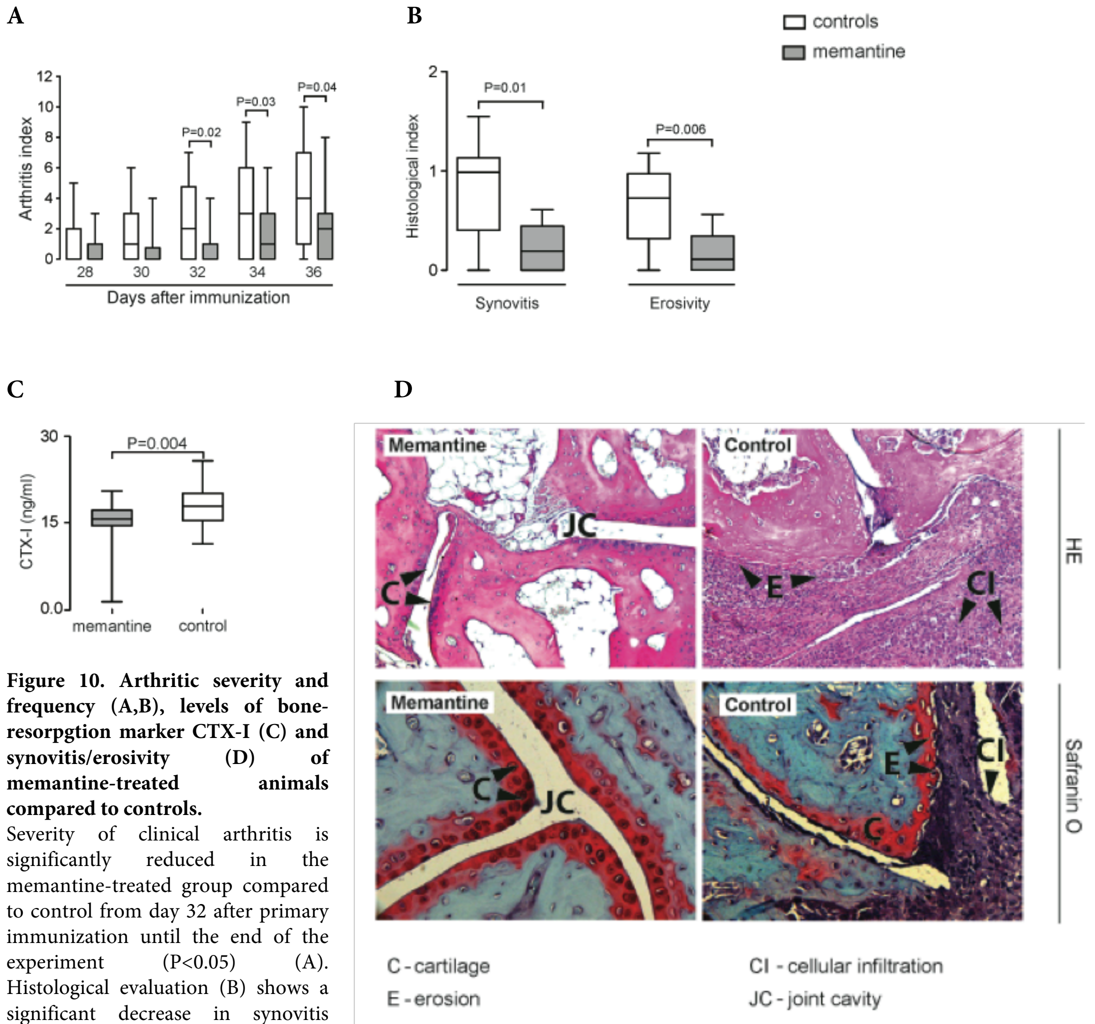
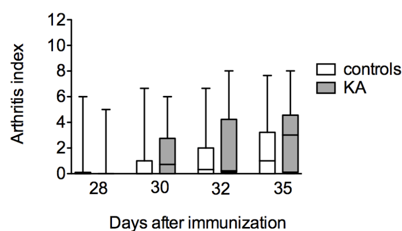



**Figure 10. Arthritic severity and frequency (A,B), levels of bone-resorpgtion marker CTX-I (C) and synovitis/erosivity (D) of memantine-treated animals compared to controls.**
Severity of clinical arthritis is significantly reduced in the memantine-treated group compared to control from day 32 after primary immunization until the end of the experiment ($P<0.05$) (A). Histological evaluation (B) shows a significant decrease in synovitis (P=0.01) and erosion (P=0.006). Serum analysis of bone resorption marker CTX-I (C) shows that memantine-treated mice had significantly lower levels compared to controls). Histological sections (D) in hematoxylin/eosin-staining (upper pictures) clearly show preserved joint cavities in the memantine-treated mouse compared to the massive cell infiltration and loss of joint space in the control group. Safranin O-fast green staining, a method for detecting depletion of proteoglycans, (lower pictures) shows a considerable loss of joint cavity and eroded cartilage in the control group, while the memantine-treated animal presents a healthy-looking joint.

### *INHIBITION OF METABOTROPIC GLUTAMATE RECEPTOR OR BROAD SPECTRUM EAA DOES NOT AFFECT CIA*

In addition to blocking the NMDA receptor, we exposed CIA animals to the metabotropic glutamate receptor1a antagonist AIDA and the broad spectrum excitatory amino acid (EAA) antagonist kynurenic acid (KA). None of these substances had any effect on the development of arthritis (Fig 11A,B).

**A**

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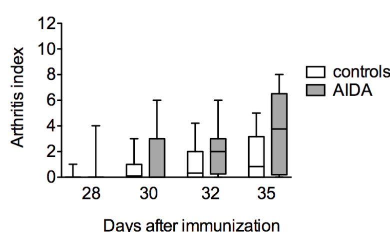
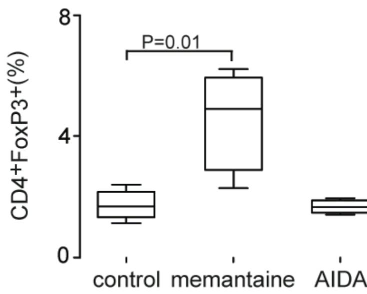



**Figure 11.** Arthritic severity and frequency of KA-exposed animals compared to controls shows no difference (A). Neither do AIDA-exposed animals compared to controls (B).

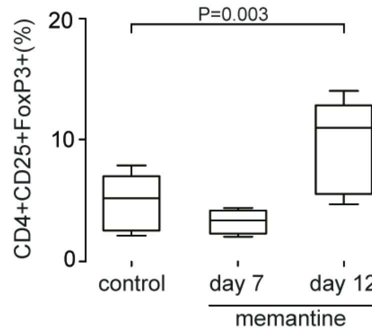
### *MEMANTINE INCREASES PRODUCTION OF REGULATORY T CELLS*

The effect of memantine on T cell populations was evaluated by flow cytometry. Naive NMRI mice were treated with memantine or AIDA for a duration of 7 or 12 days, splenocytes were isolated and analysed with FACS. Treatment with memantine resulted in an increase in Foxp3$^+$ in CD4$^+$ cells compared to controls (Fig 12A, $P$=0.01). For natural regulatory T cells, CD4$^+$CD25$^+$Foxp3$^+$, a significant increase in expression was seen after 12 days of treatment with memantine (Fig 12 B, $P$=0.003). The regulatory function of the CD4$^+$CD25$^+$Foxp3$^+$ cells were assessed in a mixed lymphocyte reaction (MLR) with CD4$^+$CD25-. The aCD3-induced proliferation of CD4$^+$CD25- effector T cells was decreased by 53% (data shown in Paper III). In consistency with this, a significant decrease in IL-2 was seen (Fig 12C) in the supernatants from CD4$^+$CD25$^+$ cells compared to levels in supernatants from CD4$^+$CD25$^-$ cells.

**A**




**B**




**C**

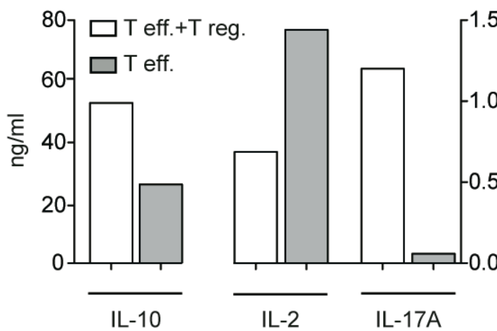



**Figure 12 Memantine-treatment increases production of regulatory T cells.** After 12 days of memantine-treatment, $CD4^+Foxp3^+$ cells were significanly increased ($P$=0.01) compared to controls (A). This was also the case for $CD4^+CD25^+Foxp3^+$, so called natural regulatory T cells. After 12 days of memantine-treatment the frequency was significantly increased ($P$=0.003)(B). When measuring cytokines in supernatans from the MLR reaction, IL-2 was shown to be significantly decreased in the $CD4^+CD25^+$ population compared to $CD4^+CD25^-$ cells. Cytokines IL-10 and IL-17A were increased in the $CD4^+CD25^+$ population compared to $CD4^+CD25^-$ cells (C). Data for the cytokine assay are based on cells isolated from 6 spleens.

### *MEMANTINE ACTIVATES B CELLS AND INCREASES ANTIGEN RESPONSE*

Memantine-treated mice express higher serum levels of antibodies towards CII (Fig 13A). This finding led us to further evaluate the effect of memantine on B cells. Flow cytometry was conducted with spleen cells from healthy NMRI mice treated in vivo with memantine or AIDA. The results showed that both antagonists caused enrichment of $CD19^+$ B cells in the spleen (Fig 13B), AIDA having the more pronounced effect. Memantine-exposed mice increased the production of CD25 on the surface of $CD19^+$ B cells (Fig 13C), indicating a more activated and mature B cell phenotype.

**A**

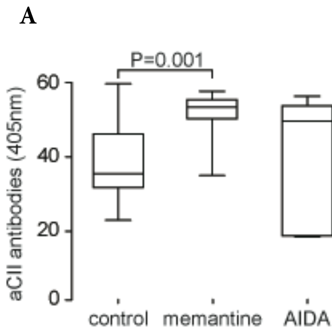
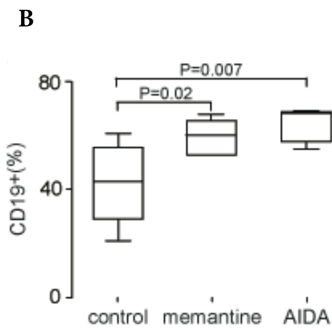
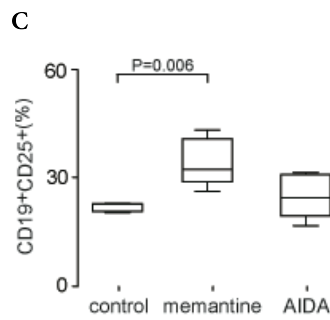



**Figure 13**. Serum analysis of aCII antibodies (A) showed that memantine-exposed animals produced significantly higher ($P$=0.001) levels of antibodies towards CII. This was also the case for animals treated with the metabotropic glutamate receptor antagonist AIDA. Results from flow cytometry on in vivo-treated healthy NMRI mice (B) show that both memantine ($P$=0.02) and AIDA ($P$=0.007) significantly increased the production of $CD19^+$ B cells in the spleen. Expression of $CD19^+CD25^+$ B cells was significantly increased in the spleens of memantine-treated animals compared to controls ($P$=0.006)(C).

**B**




**C**



# DISCUSSION

All studies included in this thesis are based on the same experimental animal model, the collagen-induced arthritis model. The genetic background, gender and age of mice used in the *in vivo* experiments are the same; male, inbred DBA/1 mice, haplotype $H2^q$, age 6-8 weeks when initiating experiments. For *in vivo* cell-mediated inflammatory response, *in vitro* and *ex vivo* studies, male outbred NMRI mice, age 6-8 weeks were used.
In all three studies presented in this thesis, a decrease in inflammatory joint disease and accompanying amelioration in systemic immune response was observed. The mechanisms behind these anti-arthritic effects are however different.

In **Paper I** we present data showing that chronic intake of ethanol and acetaldehyde in moderate doses, decreases the development and progression of CIA in mice. Through what mechanisms is this accomplished? When summarizing the effects of ethanol, an obvious effect on modified immune response is seen as the ethanol-exposed mice show **altered hormone levels**, **expression of nuclear transcription factors**, **cytokines** and **chemokines** and consequent reduction in inflammation and chemotactic activity. Where in the inflammatory cascade ethanol intervenes, can be speculated upon. A summary of affected read-outs from the ethanol-exposure is seen below.

**ETHANOL**

↓ NF-κB
↓ AP-1
↓ IL-6
↓ TNF-α
↓ MIP-1α
↓ leukocyte migration

↑ IL-10
↑ testosterone
↑ femural BMD

Measurements of hormones in the ethanol-exposed mice by week 5 from start of experiment showed a significant **increase in levels of testosterone** compared to controls. To investigate this further, mice were castrated prior to induction of CIA and ethanol-exposure. The absence of testosterone totally abolished the anti-inflammatory effect of ethanol; castrated mice developed arthritis in similar way irrespective of treatment provided. This is strong evidence for a **hormone-mediated impact of ethanol on CIA**. In addition, this increase in testosterone can directly be linked to a decrease in NF-κB and AP-1 as treatment of splenocytes from castrated mice with α-hydroxytestosterone down-regulates both spontaneous and Con A-induced activation of NF-κB and AP-1.

In ethanol-exposed mice, a decrease in levels of transcription factors **NF-κB** and **AP-1** were observed. Both factors are vital for pro-inflammatory cytokine/chemokine production. NF-κB is activated by intra- and extra-cellular stimuli, such as cytokines, bacterial or viral products. The pro-inflammatory cytokines TNF-α and IL-1, are both seen in increased levels in RA, and are known to activate NF-κB. Activation of AP-1 and NF-κB, in turn regulate the transcription of pro-inflammatory cytokines. Constitutive activation of NF-κB is associated with a number of inflammatory diseases, such as RA, inflammatory bowel disease and multiple sclerosis (Q. Li, et al., 2002). Increasead activity of both AP-1 and NF-κB is seen in both RA-patients and CIA mice (Han, et al., 1998).

The cytokine **IL-6**, mostly recognized as pro-inflammatory in RA, was significantly decreased in the ethanol-exposed mice. IL-6 is produced by monocytes, synovial fibroblasts, B and T cells, and is known to stimulate B cell proliferation and antibody production as well as T cell proliferation and differentiation. IL-6 also increasies production of hepatic acute phase proteins such as CRP and complement factors. Analysis of IL-6 levels are often used as a measure of inflammation in the CIA model of arthritis and a decrease in production is a clear sign of reduced inflammatory activity. The reduced IL-6 seen in ethanol-exposed CIA mice correlates well with the reduced clinical arthritis observed *in vivo*. The decrease in IL-6 and other inflammatory cytokine/chemokines mediated through ethanol, is explained by the previously mentioned decrease in levels of NF-κB and AP-1.

The ethanol administered to the CIA mice is shown to significantly **reduce the migratory capacity** of leukocytes. The leukocytes in the assay are mostly macrophages and granulocytes, major actors in the destructructive process seen in CIA mice. Peripheral macrophages recruited to the joint express pro-inflammatory cytokines, chemokines and MMPs, all involved in the pathogenic process of CIA (Howard, et al., 2009). Monocytes from RA synovial tissues will also, in the presence of M-CSF, differentiate into osteoclasts. In combination with pro-inflammatory cytokines (IL-6, IL-1, TNF-α) released by the cells this will promote osteoclastic bone resorption (Nakano, et al., 2007). The reduction in migration of leukocytes after ethanol exposure strongly implies that recruitment of inflammatory cells to the joints is reduced, leading to alleviatation of inflammation and destructive damage. The reduced migration of leukocytes is a somewhat surprising finding, since no effect of ethanol was seen in the DTH-reaction.

The pro-inflammatory cytokine TNF-α and chemokine MIP-1α was affected by ethanol-exposure *in vitro* and *ex vivo*. A significant **decrease in MIP-1α** and **TNF-α** levels were seen in spleen cells of NMRI mice after drinking ethanol for two months as well as in naive cells after incubation with 0.5% ethanol. This down-regulation in production of MIP-1α can explain the anti-migratory properties of ethanol observed in leukocytes from ethanol-drinking mice and *in vitro* ethanol-exposed naive splenocytes. The reduction in TNF-α, mainly produced by macrophages, is yet another sign of the reduced inflammatory activity in the spleen cells.

The ethanol-exposed mice, in addition to a less erosive disease, presented **higher mineral density in trabecular bone**. No differences were however seen in the cortical BMD. To evaluate any potential effect of ethanol on BMD in healthy mice, NMRI mice were subjected to 10% ethanol or water for two months. No differences were seen in either BMD or pQCT in between the two groups. This indicates that effects of ethanol on bone are related to reduced inflammation.
The significant decrease in levels of cortisol and IGF-1 has not been further studied.

The ethanol exposure to CIA mice showed no direct effect on the number of cells in the immune system; frequencies of T and B cells from spleen and bone marrow of ethanol-drinking mice were similar with the controls. This, along with the comparable levels of anti CII antibodies, similar response to DTH-reaction (T cell and macrophage-mediated) and olive oil-induced inflammation

(granulocyte-mediated) in the two groups, strongly indicate that ethanol does not directly affect B or T cells, macrophages or granulocytes. The lack of impact on the DTH-reaction and olive oil-induced inflammation, both being acute and subacute inflammatory reactions, indicates that ethanol mediates its anti-inflammatory effects in **chronic inflammatory conditions**.
Ethanol is unlikely to affect antigen presentation as the serum levels of aCII antibodies from ethanol-drinking mice were comparable to those in the control animals. This lack of effect from ethanol on production of antibodies, is further supported from CAIA-experiments. No difference in development of disease was seen between the ethanol drinking and the water-drinking groups when exposed to preformed antibodies towards collagen II. In conclusion, no direct effect on immune cells is shown to be mediated by ethanol. Rather, an indirect mediation via testosterone and reduction in transcription factors is more probable.

In **Paper II** we examined the effect of cigarette smoke (CS) and nicotine exposure on CIA mice. We exposed mice to CS from 16 weeks prior to collagen immunization and after induction of CIA continuing for another 6 weeks until end of experiment, giving a total of 22 weeks of smoke-exposure. This equals one fifth of their life-span, corresponding to 16,9 years of smoking in humans with a median life span of 80 years. The exposure of CIA mice to cigarette smoke did not aggravate the development of arthritis, contrary; it **delayed the onset and progression of disease**. A summary of the read-outs after smoking/nicotine-exposure is seen below.

**SMOKING / NICOTINE**

↓ aCII antibodies (CS)
↓ aCCP antibodies (CS)
↓ weight reduction in acute phase (CS)

↓ IL-6 by stimulated splenocytes *in vitro* (nic)

↑ weight gain in recovery phase (CS)

Smoking mice developed disease significantly later and to a less severe extent than did non-smoking controls. More so, the smoking mice had statistically significant **lower levels** of antibodies towards **collagen II** and **citrullinated peptides**. This decrease might be due to a **loss of ability to respond to antigen** (collagen and citrulline) and/or to **produce antibodies** towards the antigen. Likewise, smokers show lower frequency of response to vaccines than do non-smokers (Struve, 1992) and this reduction in antibodies is not related to a decrease in number of B cells but rather with **suppression** of their function and **ability to produce antibodies**. Such a **dampening effect on B cells** exercised by cigarette smoke or nicotine would be a plausible mechanism behind the anti-inflammatory effect observed in our CIA experiments. The decreased expression of aCII antibodies could also explain the **decreased joint destruction** observed in smoking animals. By discontinuation of experiment, clinical arthritis was comparable in between the smoking and non-smoking group and accordingly, so was the erosive scores from histological evaluation. However, a difference in numbers of mice with erosive engagement was observed; only 37% of smoking mice had destructive changes in their joints while 60% in the non-smoking control-group had. The decrease in antibodies towards CII could possibly reduce expression of **cross-reactive antibodies** towards endogenous collagen II in the joints and hence decrease the joint damage. Whether antibodies to CII are contributors to disease or merely products of a reaction to cartilage degradation, is still debated. Studies on arthritogenic mAbs to CII have been shown to directly contribute to cartilage destruction *in vitro* (Crombie, et al., 2005) and *in vivo* studies have shown that multiple epitopes of CII, while present in cartilage, are accessible for binding of antibodies (Holmdahl, et al., 1991).

One of many potent substances in cigarette smoke is **nicotine**, known to possess anti-inflammatory properties used therapeutically in for example ulcerative colitis (Sykes, et al., 2000). We therefore subjected another group of mice to nicotine in drinking water. The mice received nicotine dissolved in water (100 μg/ml) as only source of fluid. When measuring the amount of fluid consumed by the mice, an average of 3 ml/mouse/day was appreciated. This equals about an eight of the amount of nicotine that the cigarette smoking mice received (600 μg/cigarette x 4 cigarettes per day). When evaluating the clinical arthritis, the nicotine-exposed group showed a clear tendency to reduced arthritic score. Furthermore, in vitro experiments with LPS-stimulated naïve splenocytes, showed **that nicotine significantly decreased the production of IL-6.** For in vivo IL-6, no differences were seen in between the smoking and non-smoking group or nicotine-

exposed and control group. The absence of difference in IL-6 levels, is probably due to the fact that blood samples were taken when arthritis was similar in smoking/nicotine-exposed group and the control groups. Nicotine has been reported to decrease IL-6-production from TNF-α-stimulated fibroblast-like synoviocytes from RA-patients by suppressing activation of the NF-κB pathway (Zhou, et al., 2010). A very recent study of nicotine's effect on the CIA model, similar to ours but with intra peritoneal injections instead of per os administration, showed that nicotine decreased expression of TNF-α and IL-6 (T. Li, et al., 2010).

The importance of nicotinic ACh-receptors in RA is evident as RA patients have increased expression in synoviocytes and in synovia (van Maanen, et al., 2009; Waldburger, et al., 2008; Westman, et al., 2009). Additionally, exposing CIA mice to nicotine has shown to ameliorate arthritis, inhibit bone degradation and reduce TNF-α expression (van MaanenLebre, et al., 2009). nACh receptors have been found to be able to modulate release of other neurotransmitters in CNS, as the receptors often are expressed on axons other than ACh-nerves (Marchi, et al., 2002). The α7nAChRs have recently gained much interest, as it for example is localized on glutamatergic presynaptic cells (Lin, et al., 2010) where they facilitate Glu transmission (Hilmas, et al., 2001). This connection between glutamate and nicotinic receptors is of considerable interest and needs to be addressed further.

**Paper III** presents data on glutamate receptor modulation in CIA mice. In our studies, we exposed CIA mice to three different modulators of the Glu receptor; group 1 received the NMDA receptor antagonist memantine from day 0 and in a separate experiment from day 21; group 2 received the metabotropic Glu receptor antagonist, AIDA; and group 3 received the excitatory amino acid antagonist kynurenic acid (KA).

Interaction with the NMDA receptor with the antagonist memantine, proved to have a potent anti-inflammatory effect, as **clinical arthritis**, **synovitis**, **erosivity** and markers for **bone resorption** and **metabolism** were significantly decreased in the memantine-exposed group compared to control. The mechanisms behind the anti-inflammatory effects of the blockade of

the NMDA-receptor, are most probably mediated via multiple pathways. A summary of the read-outs after GluR modulation is seen below.

**GluR modulation**

↓ CTX-I bone resorption marker (M)
↓ OPN bone metabolism marker (M)

↑ aCII antibodies (M)
↑ CD19+ spleen B cells (M, AIDA)
↑ $CD19^+CD25^+$ (M)
↑ $CD4^+CD25^+Foxp3^+$ (M)
↑ IL-2, IL-4, IFN-γ, TNF-α

Memantine-exposed mice showed an **accumulation of $CD19^+$ B** cells in spleen and **up-regulation** of **CD25** on the surface of the $CD19^+$ cells. The $CD19^+CD25^+$ B cell population is thought to perform immunoregulatory functions in human and rodents (Amu, et al., 2006) and these cells may have played a protective role in this study, as mentioned below.

Memantine-exposure *in vivo* showed to **increase the production of natural regulatory T** cells in spleen; after 12 days of treatment the $CD4^+CD25^+Foxp3^+$ population was significantly increased.

The $CD4^+CD25^+$ population of cells from memantine-exposed mice were analysed for their regulatory function in a mixed lymphocyte reaction with $CD4^+CD25^-$ cells. aCD3-induced proliferation of the $CD4^+CD25^-$ effector T cells was decreased by 53% in the presence of $CD4^+CD25^+$ cells compared to $CD4^+CD25^-$ cell cultures alone. A significant **decrease in IL-2**, IL-4, IFN-γ and TNF-α levels in the supernatants owas observed in the $CD4^+CD25^-$ effector cells when mixed with the $CD4^+CD25^+$ cells. Additionally, the supernatants had significantly **increased levels of IL-17A and IL-10** compared to only $CD4^+CD25^-$ cells. This is complementing proof of the regulatory and anti-inflammatory effect exercised by Tregs from memantine-treated mice.

The increase in functional Tregs after memantine-exposure is indicative of a more **suppressive** activity towards **self-reactive T cells**. Dysfunctional Tregs are suggested to be a cause of

autoimmune disease (Sakaguchi, et al., 2010) and Treg cell-function has been found to be abnormal in patients with RA (Notley, et al., 2010) as well as in CIA mice (Kelchtermans, et al., 2005). Same study showed that a certain population of potent suppressive Tregs is induced after treatment with TNF-inhibitors.

Development of CIA is often said to be dependent on production of aCII antibodies. In spite of the significantly decreased arthritis, an **increase in frequency of B cells** and **aCII antibodies** in memantine-exposed mice was observed. This points to a more efficient antigen-presentation and antibody-production towards the collagen used for immunization. The anti-inflammatory mechanism exercised by memantine might then rather be due to an interaction in the process of cross-reactivity to endogenous collagen in the cartilage. The arthritogenicity of the collagen-antibodies found in memantine-exposed animals might also be different and of a less joint-destructive nature.

Levels of RF (IgG) and aCCP in sera from CIA mice were unaffected after treatment with any of the GluR modulators. Even though the levels of IgG in sera from the CIA mice were unchanged after memantine-exposure, the possibility of activation of other Ig-producing B cell-clones might explain the the increased antibodies.

The discrepancy between the production of antigen-specific antibodies (aCII abs) and autoantibodies (aCCP abs) may be viewed as a **relative supression of self-reactive lymphocytes**. This suppression could be performed by regulatory cells such as the $CD4^{+}CD25^{+}$ B cells and $CD4^{+}CD25^{+}Foxp3^{+}$ T cells, both found to be increased after memantine-exposure.

Memantine has been shown to block the $\alpha$7nACh receptor, actually more potently than it blocks the NMDA receptor in the brain (Aracava, et al., 2005). With this in mind, it is possible to speculate that our results are accounted for by interaction with the NMDA receptor and/or the $\alpha$7nACh receptor. Further experiments need to be performed to fully comprehend the complexity of the anti-inflammatory properties possessed by these two receptors.

The decrease in aCII antibodies observed after cigarette smoking in CIA mice and the increase in same antibodies seen after memantine-exposure to CIA mice might through different mechanistical pathways both lead to alleviation of arthritis. Development of CIA is often said to

be dependent on production of aCII antibodies. A decrease in CIA would then be accompanied by a decrease in levels of aCII antibodies. In this thesis we present three studies, all showing significant alleviation of CIA. The impact on aCII antibodies are, however, very different; in Paper I the levels are unchanged between ethanol-exposed mice and controls, in Paper II the aCCP in smoking mice are decreased compared to controls, and in Paper III, the aCCP-levels are higher in the memantine-exposed group compared to controls. This shows that the presence of anti-collagen antibodies in CIA are important but not crucial to development of the disease.

Studies of the anti-inflammatory properties exercised by ethanol, nicotine and NMDA-receptor blockade need further validation but proves from these studies to be potential therapeutical targets for rheumatic diseases.

The table below summarizes **in what step** and **through what mechanisms** the substances studied in this thesis intervene in the collagen-induced arthritis process.

| | ETHANOL | SMOKING/NICOTINE | GluR MODULATION |
|---|---|---|---|
| **1° immunisation** | | | |
| **Antigen presentation** | Reduced MIP-1α l → less migration of MΦ.<br>Reduced TNF-α → less recruitment of of neutrophils and Mϕ.<br>→ Reduced recruitment of APCs<br>Increased IL-10 suppres antigen-presentation of APCs | Dampening effect on B cells leading to reduced antigenresponse and/or antibody-production.<br>Reduced IL-6 → less activation of B and T cells | Activation of B cells and increase in antigen response<br>Increased antibody-production |
| **Activation of T and B cells** | Reduced IL-6 → less activation of B and T cells<br>Reduced TNF-α → less activation of T cells | Dampening effect on B cells leading to reduced T cell-activation. | Increase in freq of regulatory T and B cells → impairment of B and T cell functions |
| **Devopment of immuno-logic memory** | Reduced IL-6 → decreased proliferation and differentiation of T cells | Reduced IL-6 → decreased proliferation and differentiation of T cells | Increase in freq of regulatory T and B cells → impairment of B and T cell differentiation |
| **2° immunisation** | | | |
| **Effector phase (development of arthritis)** | All of the above → less migration and activity of inflammatory cells in the joint | Decreased CII-antibodies reduce cross-reactivity to endogenous CII. | Increase in regulatory T and B cells → suppression of self-reactive T and B cells |

# MAIN CONCLUSIONS FROM THE THESIS

## THE MAJOR RESULTS FROM THIS THESIS ARE:

### PAPER I

- Ethanol and the major metabolite acetaldehyde ameliorates and delay development of CIA in mice
- Ethanol down-regulates *ex vivo* production of MIP1-α and TNF-α by spleen cells through the NF-κB and AP-1 dependent cascade
- Ethanol down-regulates migration of peritoneal leukocytes
- Ethanol increases testosterone levels
- Ethanol prevents arthritis-induced loss of bone-mineral density

### PAPER II

- Smoking and nicotine exposure delay development of CIA in mice
- Smoking decreases production of aCCP and aCII antibodies in CIA mice
- Nicotine suppresses production of IL-6 by stimulated splenocytes

### PAPER III

- The NMDA receptor antagonist memantine ameliorates and delay development of CIA, while inhibition of metabotropic glutamate receptor or broad spectrum EAA does not
- Memantine activates B cells and increases specific antibody production
- Memantine increases production of regulatory T cells suppressive towards self-reactive T cells

# POPULÄRVETENSKAPLIG SAMMANFATTNING

Reumatoid artit (RA), ledgångsreumatism, är en autoimmun sjukdom där kroppens immunförsvar av okänd anledning attackerar den egna vävnaden som om den vore främmande. Ungefär 1% av Sveriges befolkning är drabbade av RA och det är en av de största folksjukdomarna. Den bakomliggande orsaken till varför vissa människor drabbas av sjukdomen är ännu inte klarlagd. Dock har ett flertal olika bidragande faktorer identifierats som alla påverkar risken för att drabbas av sjukdomen. Många av dessa har påvisats i epidemiologiska studier, en typ av studie där man studerar om det finns samband mellan en viss exponering och någon sjukdom. Sådana studier är ofta baserade på intevjuer med patienter. Epidemiologiska studier på RA har visat att ett flertal olika miljötoxiner (bl.a. alkohol och rökning) påverkar risken för att drabbas av sjukdom. En epidemiologisk studie kan alltså visa på ett samband mellan en sjukdom och en specifik riskfaktor, dock kan den inte säga något om de bakomliggande mekanismerna. För att kunna klarlägga detta krävs experimentella studier. I de arbeten som presenteras i denna avhandling har vi valt att undersöka miljötoxinerna etanol och nikotin, de två mest missbrukade substanserna bland befolkningen. Då dessa visat sig ha anti-inflammatoriska egenskaper, valde vi att studera hur de påverkar ledinflammation hos möss.

I samtliga studier redovisade i denna avhandling har vi använt oss av en väletablerad djurmodell av RA; den så kallade kollagen-inducerad artrit modellen (CIA). Genom att injicera möss med kollagen, en beståndsdel i brosk, sätter man igång en sjukdomsprocess i djuret som på många vis liknar RA hos människor. Mössen utvecklar kraftig inflammation i sina leder då de invaderas av inflammatoriska celler som ger svullnad samt efterföljande nedbrytning av ledens brosk och ben. Samtidigt som vi sätter igång inflammation i försöksdjuren, startar vi exponering med olika substanser med förhoppningen om att kunna lindra ledbesvären.

I **Artikel I** visar vi att hanmöss som dricker 10% etanol under hela försökets gång får mindre inflammation och nedbrytning i sina leder. Möss som är kastrerade får inte samma minskning i

inflammation, vilket påvisar att testosteron har en central roll i den anti-inflammatoriska effekt som etanol utövar. Flera andra viktiga delar i den inflammatoriska processen påverkas också till det bättre av etanol.

I **Artikel II** utsätter vi mössen för dels cigarettrök (aktiv rökning) och dels nikotin. Möss som rökt får mindre inflammation i sina leder än de som inte rökt. Möss som fick dricka nikotinsyra i vatten visade också på en minskning i ledinflammation, den var dock inte lika tydlig som hos de rökande mössen.

I **Artikel III** visar vi att en substans som blockerar NMDA-receptorn (en receptor som även blockeras av etanol) resulterar i minskning av inflammation och nedbrytning av mössens leder. Vi förklarar detta med att mössen får fler s.k regulatoriska T-celler (Tregs). Dessa celler är viktiga för att kontrollera immunförsvarets funktioner och är centrala vad gäller att förhindra autoimmuna sjukdomar, det vill säga undvika attack på den egna vävnaden, som sker vid RA. Patienter med RA har visats ha defekta sådana Tregs.

Sammanfattningsvis har vi studerat effekten av vanligt förekommande miljötoxiner, och de av människor mest brukade drogerna etanol och nikotin, på utvecklingen av experimentell artrit hos möss. Vi visar att etanol mycket effektivt fördröjer utveckling av och lindrar inflammation samt förstörelse av leder genom att påtagligt höja nivåerna av testosteron hos musen. Viktiga inflammatoriska signalmolekyler dämpas och celler betydelsefulla vid förstörelse av lederna blir funktionellt nedsatta. Rökning minskar ledinflammation hos möss genom att effektivt minska produktionen av antikroppar och signalmolekyler som bidrar till sjukdomsprocessen i musen. Blockering av NMDA-receptorn verkar dämpande på inflammation och ledförstörelse genom att öka frekvensen av regulatoriska T-celler. Dessa celler verkar dämpande på auto-reaktiva celler som bidrar till sjukdomsprocessen vid både kollagen-inducerad artrit och RA.

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Söta tjejer, tack för att ni stöttat mig med uppmuntrade ord, KAKOR, GODIS & NAGELLACK under arbetet med ramen. Annelie: tack för att du handlat mat åt mig och uppmuntrat så de senaste månaderna - du är en liten pärla!

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Tack för upplysning om Medical art! Du ser bilderna va ;)?

**Karin C**
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