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Reduced ROS production triggers arthritis / The role of T cells in arthritis pathogenesis

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The role of T cells in arthritis pathogenesis

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The Faculty of Medicine, Lund University, has appointed Professor Cornelis L. Verweij as an opponent at the public defence of Jens Holmberg´s PhD thesis. The defence will take place at the Biomedical Center, Rune Grubb lecture hall, on Friday, the 10th of December 13.00, and will be held in English.

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TO MY PARENTS

Mona Konnberg

Kent-Olof Holmberg

Dissertation for the degree of Doctor of Philosophy,
Faculty of Medicine at Lund University, 2004

Jens Holmberg

Reduced ROS production triggers arthritis

The role of T cells in arthritis pathogenesis

Abstract

The aim of this thesis is to defend a novel mechanism that triggers arthritis susceptibility in both rats and mice through reduced production of reactive oxygen species (ROS), but also to introduce a novel model for chronic relapsing arthritis. Five papers are included, all of which employ animal models for rheumatoid arthritis and one that also uses animal models for multiple sclerosis. *Paper I* aimed at identifying strong dominant loci operating early in PIA by linkage analysis of a large F2 backcross. We identified eight dominant QTLs regulating arthritis traits, of which *Pia4* was the strongest in suppressing arthritis. *Paper II* aimed at cloning the gene responsible for the strong arthritis-suppressive effect of *Pia4*. *Ncf1* was positionally identified as the only gene within a minimal *Pia4*-fragment with polymorphisms inside the coding region that differed between parental strains. *Paper III* aimed at showing that the arthritis-regulatory effect of *Ncf1* is not species-specific. We showed that *Ncf1*-mutant mice were more susceptible to arthritis as well as to MOG protein-induced EAE than heterozygous littermate controls. *Paper IV* aimed at characterizing the immune response in PIA. Through several cell transfer experiments, we could conclude that T cell antigens must be involved in PIA, since arthritis transferred by T cells was restricted by MHC class II DQ and DR molecules. *Paper V* aimed at characterizing the chronic relapsing arthritis induced by adoptive transfer of PIA. High levels of COMP, AGP and IgG2b in blood at the chronic phase suggested cartilage destruction, systemic inflammation and Th1-mediated antibody production, respectively. Also, Methotrexate, anti-IFN-γ or anti-TNF-α treatment with Etanercept ameliorated arthritis severity at the chronic stage.

This thesis is based on the following papers that will be referred to by their roman numerals:

I. Identification and isolation of dominant susceptibility loci for pristane-induced arthritis.
Peter Olofsson*****, Jens Holmberg*****, Ulf Pettersson, Rikard Holmdahl.
The Journal of Immunology. 2003 Jul 1;171(1):407-16.
***** Equal contribution to this work

II. Positional identification of *Ncf1* as a gene that regulates arthritis severity in rats.
Peter Olofsson, Jens Holmberg, Jesper Tordsson, Shemin Lu, Bo Åkerström, Rikard Holmdahl.
Nature Genetics. 2003 Jan;33(1):25-32.

III. Enhanced autoimmunity, arthritis, and encephalomyelitis in mice with a reduced oxidative burst due to a mutation in the *Ncf1* gene.
Malin Hultqvist, Peter Olofsson, Jens Holmberg, Thomas Bäckström, Jesper Tordsson, Rikard Holmdahl.
Proc Natl Acad Sci U S A. 2004 Aug 24;101(34):12646-51.

IV. Pristane, a non-antigenic adjuvant induces MHC class II-restricted, arthritogenic T cells.
Jens Holmberg, Hisakata Yamada, Shemin Lu, Peter Olofsson, Rikard Holmdahl.
Submitted for publication.

V. Chronic relapsing arthritis, induced by pristane-sensitized Th1 cells.
Jens Holmberg, Jonatan Tuncel, Malin Hultqvist, Lena Wester, Peter Olofsson, Rikard Holmdahl.
Manuscript.

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Abbreviations

AGP	α1-acid glycoprotein
AIA	adjuvant-induced arthritis
APC	antigen-presenting cell
B10.Q	disease-susceptible C57B1/10 mouse with H-2^q
BAC	bacterial artificial chromosome
BN	arthritis-resistant inbred rat strain
CGD	chronic granulomatous disease
CIA	collagen-induced arthritis
CII	collagen type II
cM	centi Morgan (genetic distance)
CNS	central nervous system
COMP	cartilage oligomeric matrix protein
Con A	concanavalin A
Cyb	cytochrome b
DA	arthritis-susceptible inbred rat strain
DBA/1	arthritis-susceptible inbred mouse strain
DMARD	disease-modifying antirheumatic drug
DTH	delayed-type hypersensitivity
E3	arthritis-resistant inbred rat strain
EAE	experimental allergic encephalomyelitis
H-2	histocompatibility 2, the mouse MHC
HC gp39	human cartilage glycoprotein-39
HLA	histocompatibility locus A, the human MHC
IFN	interferon
Ig	immunoglobulin
IL	interleukin
LAT	linker for activation of T cells
MAG	myelin associated glycoprotein
MBP	myelin basic protein
MHC	major histocompatibility complex
MMP	metalloproteinase
MOG	myelin oligodendrocyte glycoprotein
MOSP	myelin oligodendrocyte specific protein
MS	multiple sclerosis
MTX	methotrexate
NADPH	nicotinamide adenine dinucleotide phosphate
Ncf	neutrophil cytosolic factor
NSAID	non-steroidal anti-inflammatory drug
OIA	oil-induced arthritis
PAC	P1-derived artificial chromosome
PLP	proteolipid protein
phox	phagocyte oxidase
PIA	pristane-induced arthritis
PK	protein kinase
PMA	phorbol myristate acetate
PX	phox homology domain
PxxP	proline-rich domain
QTL	quantitative trait locus
RA	rheumatoid arthritis
rad	radiation absorbed dose
RF	rheumatoid factor
ROS	reactive oxygen species
RT	rat transplantation antigen, the rat MHC
TCR	T cell antigen receptor
Th	T helper
TNF	tumor necrosis factor

Introduction

Autoimmune diseases arise when the immune system attacks self-antigens and causes tissue injuries. These diseases affect a few percent of the population, and could be crippling or fatal, and often occur in the prime of life, generally afflicting more women than men. We do not know what initiates autoimmune responses, or whether organ-specific and systemic autoimmune diseases share similar etiologic mechanisms, but we believe that both genetic and environmental factors contribute to the disease expression. The role of genetic factors are supported by several studies demonstrating an association between expression of particular major histocompatibility complex (MHC) alleles and susceptibility to autoimmunity, which also argues for the involvement of T cells mediating autoimmune diseases. In fact, the unifying characteristic in all autoimmune diseases is damage of self-tissues mediated by humoral and/or cellular immune pathways, in both of which T cells appear to be an absolute requirement. To unravel pathogenic mechanisms and the complex inheritance of autoimmune diseases it is important to limit the genetic heterogeneity as well as the influence of the environment. The use of adequate animal models is essential for such a study. The aim of this thesis is to defend a novel mechanism that triggers arthritis susceptibility in both rats and mice through reduced production of reactive oxygen species (ROS), but also to introduce a novel model for chronic relapsing arthritis. Five papers are included, all of which employ animal models for rheumatoid arthritis and one that also uses animal models for multiple sclerosis. To follow these papers, an introduction is given for rheumatoid arthritis, multiple sclerosis, reverse genetics and for the ROS producing NADPH oxidase.

Rheumatoid arthritis

Rheumatoid arthritis (RA) is one of the most common autoimmune diseases affecting approximately 1 % of the world population (Feldmann et al. 1996; Wiles et al. 1999). The disease has a peak of onset at the age of 50-60 years, and women are affected three times more often than men (Silman 1994; Symmons et al. 1994). The chronic inflammation of RA primarily affects diarthrodial joints, where synovial inflammation branches into cartilage destruction, bone erosion and ultimately joint deformity and loss of joint function. A comparative study of twin cohorts in Finland and the United Kingdom have further estimated a concordance rate in monozygotic twins of 60 % (MacGregor et al. 2000), leaving room for non-genetic environmental factors, such as infectious agents, oral contraceptives and smoking, contributing to the disease (Feldmann et al. 1996; Lee et al. 2001).

Symptoms

Patients suffering from RA develop symmetrical swelling and pain in the joints, often accompanied with stiffness and fatigue. However, the disease varies from mild symptoms such as tenderness in few joints to severe pain accompanied with life-threatening systemic manifestations, and RA is therefore diagnosed according to certain criteria. These criteria are based on clinical symptoms and signs, biochemical markers and radiographic changes. To be diagnosed for RA, patients must fulfill four out of seven criteria, as defined by the American Collage of Rheumatology (ACR) (Arnett et al. 1988): **1**) morning stiffness, lasting for at least one hour; **2**) swelling in at least three joints; **3**) swelling in hand joints; **4**) symmetrical joint swelling; **5**) rheumatoid nodules; **6**) abnormal serum rheumatoid factor; and **7**) erosion or decalcification on X-ray of hand. Criteria **1** to **4** must be present for at least six weeks.

Pathogenesis

Pannus formation

The pathology of RA is complex and involves oedema, fibrin deposition, hyperplasia of synoviocytes and synovial infiltration of mononuclear inflammatory cells in affected joints at an early stage. New blood vessles are dispersed and vessel endothelial cells transform into high endothelia venules in the early disease facilitating the transit of blood leukocytes into the synovium. At a later stage of RA, a cellular pannus is formed, composed of mononuclear cells and fibroblasts. Eventually, this cellular pannus is replaced by fibrous pannus, composed of a less vascularized layer of pannus cells and collagen overlying cartilage. Bone and cartilage erosions exist during the whole disease course, and ultimately ankylosis is formed and articular function is lost (Scutellari et al. 1998).

HLA class II

We do not know what triggers the self-destructive process in RA, or why it persists. However, genetic linkage analyses of patients with RA have revealed that a common motif in the peptide-binding pocket of the histocompatibility locus A (HLA) class II molecule was shared between different disease linked alleles, an observation that lead to the shared epitope hypothesis. The hypothesis claims that HLA-DR molecules sharing a similar third hypervariable region are thought to bind arthritogenic peptides, mediating susceptibility to RA (Gregersen et al. 1987). This strongly suggests a role for $CD4^+$ T cells in RA, since the only known function of HLA-DR molecules is to present antigens to $CD4^+$ T cells as the first step of activation.

T cells

The vast majority of lymphocytes infiltrating RA synovia are αβT cells, most of which are $CD4^+$ αβT cells (Bankhurst et al. 1976; Janossy et al. 1981; Zvaifler et al. 1994). Several reports show that many of these synovial T cells, compared with peripheral blood T cells, are highly differentiated, expressing surface molecules associated with prior exposure to antigen (*e.g.* CD45RO, CD27) (Tak et al. 1996), activation (*e.g.* ICAM-1, CD40L, CD69, CD71, HLA class II) (Takahashi et al. 1992; Afeltra et al. 1993; Kohem et al. 1996; Liu et al. 2001), and adhesion (*e.g.* VLA, CD29, CD44) (Hemler et al. 1986; Takahashi et al. 1992). Although synovial T cells display such a distinct repertoire of surface molecules, no predominant T cell antigen receptor (TCR) subset required for RA development has yet been identified. However, among the small population of $CD25^+$ T cells in synovial fluid, a frequent skewing of the TCRVβ repertoire (*e.g.* Vβ3, Vβ14 and Vβ17) has been observed, suggesting a local activation of oligoclonal T cells by a common antigen stimuli (Howell et al. 1991; Rittner et al. 1997).

The search for T cell antigens required for RA development has so far been disappointing. However, cartilage-responsive T cells associated with cartilage destruction in RA patients have been documented (Alsalameh et al. 1990). Also, evidence for clonally restricted synovial T cells towards specific type II collagen (CII) epitopes have been observed in RA patients (Londei et al. 1989; Snowden et al. 1997), but unfortunately in healthy controls as well (Snowden et al. 1997). Nevertheless, the human cartilage glycoprotein-39 (HC gp39), specifically produced by chondrocytes in rheumatoid lesions, but not in non-arthritic joints, seems like a plausible cartilage antigen (Verheijden et al. 1997). In fact, injection of this protein in mice triggered the development of chronic relapsing arthritis (Verheijden et al. 1997), suggesting that the HC gp39 protein is a target of the T cell-driven immune response in RA.

It has been difficult to assess the role for infiltrating T cells in RA synovium due to several reasons. Firstly, although synovial T cells mainly are of the Th1 type (Dolhain et al. 1996; van der Graaff et al. 1999), Th1 cytokines that should drive the rheumatoid inflammation are scarce in synovial tissue (Firestein et al. 1988; Smeets et al. 1998). Instead, most soluble inflammatory mediators in the synovium appear to be produced by macrophages, synovial lining cells, fibroblasts and endothelial cells (Firestein et al. 1988). Secondly, synovial T cells in RA display a phenotype arguing for apoptosis (*e.g.* Bcl-2 low, Fas high) and are susceptible for spontaneous apoptosis on removal from the joint (Salmon et al. 1997). It seems that the local environment protects synovial T cells from apoptosis, since these cells can be rescued by interaction with synovial fibroblasts or by IL-2R gamma chain signalling cytokines (Salmon et al. 1997). Thirdly, synovial T cells are hyporesponsive against mitogenic and antigenic stimuli. It has been suggested that this hyporesponsiveness is due to a defective TCR-signaling. In fact, synovial T cells show an impaired phosphorylation of proteins involved in TCR-signaling, such as LAT (linker for activation of T cells) and the TCR zeta-chain (Maurice et al. 1997; Gringhuis et al. 2000). In addition, local exposure of high levels of ROS in the RA synovium has been suggested to affect TCR-signaling by altering the intracellular redox balance (Maurice et al. 1997; Gringhuis et al. 2000).

B cells and autoantibodies

B cells are a minority of the infiltrating lymphocytes in the RA synovium (Natvig et al. 1989), consisting predominantly of memory B cells and plasma cells (Reparon-Schuijt et al. 2000). In some RA patients, large follicle-like structures resembling germinal centers are detected in the synovia (Schroder et al. 1996). These structures comprise $CD4^+$ T cells, follicular dendritic cells as well as clonally expanded B cells, indicating that activated B cells can differentiate into plasma cells locally (Kim et al. 1999). The plasma cells in the RA synovium have been suggested to take part in the pathogenesis as producers of autoantibodies. These antibodies are distinguished between RA-associated antibodies present in RA and in other autoimmune or infectious diseases, and RA-specific autoantibodies exclusively present in RA (Smolen et al. 2001; van Boekel et al. 2002).

To diagnose RA, the most helpful RA-associated autoantibodies are the rheumatoid factors (RFs), specific against self-IgG Fc fragments (Williams 1994), and studies have shown that the early presence of RF in blood sera is a predisposing factor for RA development (Masi et al. 1976; Paimela et al. 1995). Other RA-associated autoantibodies include antibodies recognizing cartilage oligomeric matrix protein (Souto-Carneiro et al. 2001), human cartilage glycoprotein-39 (Vos et al. 2000), and various collagens (*e.g.* collagen type I, II, IX and XI) (Charriere et al. 1988) etc. An example of RA-specific autoantibodies include antibodies recognizing cyclic citrullinated peptide (CCP) (Schellekens et al. 1998). The question is whether local production of autoantibodies has a direct role in the pathogenesis of RA, or is a secondary effect of inflammation. Another question is whether synovial B cells could play a pathogenic role as antigen-presenting cells (APCs) as well.

Macrophages and cytokines

Other cells in the RA synovia that could contribute to the chronic inflammation and joint destruction are macrophages and fibroblast-like synoviocytes (Kinne et al. 2000; Firestein et al. 2002; Firestein 2003), as well as granulocytes, dendritic cells and mast cells (Thomas et al. 1994; Woolley et al. 2000). During the last decade, macrophages have been the most promising target for therapy. This is because macrophages are the main producers of tumor necrosis factor-alpha (TNF-α) and IL-1, both of which are readily detected in RA synovium (Firestein et al. 1990; Feldmann et al. 1996). These cytokines are strong proinflammatory mediators in the signal transduction between inflammatory cells in RA synovitis. IL-1 acts on fibroblasts and chondrocytes, which are stimulated to produce metalloproteinases, such as stromelysins and collagenases (Duff 1994). However, the effect of IL-1 can be strongly inhibited by antibodies neutralizing TNF-α, and TNF-α has therefore been suggested to be a major inducer of IL-1 in RA (Brennan et al. 1989). In fact, TNF-α also induces production of other proinflammatory cytokines, such as IL-6 (inducer of the hepatic acute phase response) and IL-8 (chemotactic factor for neutrophils) (Feldmann et al. 1996), which puts TNF-α at the top of the proinflammatory cytokine cascade.

Therapy

There are several drugs used for RA patients. Drugs often used are non-steroidal anti-inflammatory drugs (NSAIDs), disease-modifying antirheumatic drugs (DMARDs) and glucocorticoids. NSAIDs reduce the symptoms of RA, but do not retard the disease progress. Methotrexate (MTX), a folic acid antagonist, has generally been accepted as the leading DMARD (Pincus et al. 1992; Bannwarth et al. 1994). MTX shows a rapid onset, inhibition of neutrophil chemotaxis (Sperling et al. 1990), reduced IL-6 in blood sera (Barrera et al. 1993), reduced acute phase reactants (Bannwarth et al. 1994) and fewer adverse effects than other DMARDs (Bondeson 1997). In addition, a few DMARDs such as the anti-rheumatic gold-compound, auranofin, as well as the antimalarial, quinacrine, have been shown to be potent inhibitors of the induction of IL-1 and TNF-α (Danis et al. 1991; Bondeson et al. 1998). Glucocorticoids restrain clonal T cell proliferation by inhibiting certain transcription factors, but these drugs are mainly used in severe cases due to adverse effects. However, new promising, but expensive, cytokine-targeted therapies have been developed that can be used for patients that do not respond to DMARDs. These cytokine-targeted therapies include TNF-α trapping drugs, such as Etanercept (a fusion protein combining the ligand binding portion of human TNF receptor 2 (p75) with the Fc-portion of human IgG) (Franklin 1999) and Imfliximab (a recombinant chimeric antibody against human TNF-α, consisting of human IgG1 constant and mouse variable regions) (Antoni et al. 1999), as well as IL-1Ra (a recombinant human interleukin-1 receptor antagonist) (Watt et al. 2001). In addition, treatment with cytokine-trapping drugs can be improved by combination with DMARDs, as has been reported for Etanercept combined with MTX (Klareskog et al. 2004).

Animal models

Pristane-induced arthritis

Pristane-induced arthritis (PIA) in rats fulfills the criteria necessary for diagnosis of RA in humans. The disease is induced by an intradermal injection of non-antigenic pristane oil (2,6,10,14-tetramethylpentadecane). The susceptible DA rat, but not the resistant E3 rat, develops a sudden onset 10-12 days after injection with an incidence of 100 %, followed by a chronic relapsing disease course with an erosive and symmetric destruction of peripheral joints (Vingsbo et al. 1996). PIA also induces elevated levels of α1-acid glycoprotein (AGP) as well as cartilage oligomeric matrix protein (COMP) in peripheral blood, suggesting an acute systemic inflammation and cartilage degradation, respectively (Olofsson et al. 2002). Other reactants indicating systemic reactions in PIA involve elevated levels of fibrinogen and IL-6 in peripheral blood (Vingsbo et al. 1996; Olofsson et al. 2002).

Within a week after pristane injection, lymphocytes expand 10-fold in draining lymph nodes, and the $CD4^+$ T cells continuously expand in numbers until the disease onset (paper IV). A few days after onset, there is a pronounced pannus formation in ankle joints with cartilage and bone erosions (Vingsbo et al. 1996) and a selective accumulation of highly differentiated $CD4^+$ T cells in the synovia (paper IV). Like other adjuvant-induced arthritis models in rats, such as oil-induced arthritis (OIA) (Kleinau et al. 1993; Svelander et al. 1997) and adjuvant-induced arthritis (AIA) (Taurog et al. 1983; Taurog et al. 1983), PIA is a T-cell driven disease (Vingsbo et al. 1996) and pristane-sensitized $CD4^+$ T cells alone can adoptively transfer PIA to naive syngenic recipients upon re-activation *in vitro* (paper IV). The transferred $CD4^+$ T cells have also been shown to target the recipient synovia upon intravenous injection (paper IV; paper V).

Most interestingly, the transfer of PIA can be ameliorated by prophylactic treatment of recipients with either anti-RT1B (against MHC class II DQ in the rat) or anti-RT1D (against MHC class II DR in the rat) monoclonal antibodies, suggesting the involvement of a set of antigens in this model, although pristane oil itself is non-antigenic (paper IV). The MHC restriction was also confirmed in rats congenic for the MHC region, showing that at least one syngenic MHC allele is required for irradiated recipients to become susceptible for transferred PIA (paper IV). Additionally, T cells that transfer PIA produce high levels of IFN-γ and TNF-α, but not IL-4, suggesting a Th1 response for PIA. In fact, prophylactic treatment as well as treatment at the chronic stage of transferred PIA with MTX, anti-IFN-γ antibodies or Etanercept significantly ameliorate arthritis development (paper IV; paper V).

Like in RA, the PIA model is genetically associated with the MHC region. One of the strongest quantitative trait loci (QTL) for PIA harbours the entire MHC region, denoted *Pia1*, and was identified on rat chromosome 20 (Vingsbo et al. 1996; Nordquist et al. 2000; Olofsson et al. 2003). However, the superior locus regulating PIA is the *Pia4* QTL on rat chromosome 12. *Pia4* was first discovered through linkage analysis of a pristane injected (E3xDA)F2 cross as an DA additive promoting region (Vingsbo-Lundberg et al. 1998), and later confirmed in a high-powered DA(E3xDA) cross (paper I). Through positional cloning of the *Pia4* QTL in rats, we have identified a naturally occurring polymorphism of *Ncf1* (encoding neutrophil cytosolic factor 1, a component of the NADPH oxidase complex) regulating arthritis severity (paper II). This polymorphism was further shown to significantly regulate other rat arthritis models than PIA, such as oil-induced arthritis, collagen type II-induced arthritis and hexadecane-induced arthritis (paper II). The *Ncf1* polymorphism was also shown to act by regulating the production of ROS in neutrophils, and prophylactic treatment with phytol oil (3,7,11,15-tetramethyl-2-hexadecen-1-ol) that induces ROS production, abrogated the development of PIA (paper II). To confirm *Ncf1* as an arthritis-regulating gene in another animal species, we proceeded to investigate the development of collagen type II-induced arthritis in mice mutated in the *Ncf1* gene.

Collagen-induced arthritis

Collagen type II-induced arthritis (CIA) is perhaps the most extensively studied animal model for RA. This is not surprising, since collagen type II (CII) is a major component of joint cartilage, and some RA patients produce autoantibodies against this protein (Charriere et al. 1988). Unlike PIA, CIA can be induced in mice with a high incidence, as well as in rats (Trentham et al. 1977; Courtenay et al. 1980; Holmdahl et al. 1990). The susceptibility is strongly associated with the MHC region for both mice (Wooley et al. 1981; Wooley et al. 1985) and rats (Griffiths et al. 1981; Griffiths 1988). Susceptible mouse strains carry either the H-2^q or H-2^r haplotypes (Wooley et al. 1985), while rats are highly susceptible if they carry the $RT1^a$ haplotype for the MHC region (Griffiths et al. 1981). In mice, CIA is normally induced with heterologous (*i.e.* rat, chicken or bovine) CII emulsified in complete Freund´s adjuvant (Wooley et al. 1981), while the DA rat strain, carrying the $RT1^a$ haplotype, is susceptible also to homologous CII (Goldschmidt et al. 1991).

The disease course of CIA resembles RA in many aspects, including the destruction of cartilage in peripheral joints, fibrin deposition and pannus formation. However, CIA is not as chronic or symmetric as PIA. Several reports suggest that CIA could be a B cell-driven disease, since CIA can be transferred with serum (Stuart et al. 1982; Stuart et al. 1983). Also, mice lacking B cells due to deletion of the IgM heavy chain are resistant to CIA (Svensson et al. 1998). However, immunization of CII induces a strong T cell-dependent anti-CII autoantibody response (Goldschmidt et al. 1991), suggesting that CIA is also very much dependent on T cells.

The T cell dependence in CIA has been widely investigated both through administrations of antibodies against the αβTCR, CD4 or CD8 molecules, but also in mice that are deficient for these molecules. Administration of antibodies against αβTCR (Moder et al. 1992; Maeda et al. 1994) or CD4 (Ranges et al. 1985) in mice were shown to reduce the susceptibility to CIA. Also, treatment with anti-CD8 neutralizing antibodies ameliorate the development of CIA in mice (Arai et al. 1996). TCRβ knock-out mice, deficient in αβT cells, are resistant to CIA (Corthay et al. 1999), while CD4-deficient B10.Q mice (Ehinger et al. 2001) or CD8-deficient DBA/1 mice (Tada et al. 1996) are less susceptible to CIA.

We have recently shown that *Ncf1*-mutated mice, with aberrant Ncf1 function, will enhance a T cell-dependent response in CIA compared with wildtype and heterozygous mice (paper III). After immunization with CII in the *Ncf1*-mutated mice, both a CII-specific delayed-type hypersensitivity (DTH) reaction and T cell-dependent IgG responses against CII are enhanced, suggesting that Ncf1 is involved in the selection of T cell autoreactivity (paper III). The *Ncf1*-mutated mice were also shown to produce increased levels of antibodies against specific C1-, J1- and U1-epitopes of CII (paper III), some of which are recognized in human RA as well (Burkhardt et al. 2002).

Multiple sclerosis

Multiple sclerosis (MS) is a chronic autoimmune disease of the central nervous system (CNS) affecting 0.1-0.2 % of the Caucasian population (Sadovnick et al. 1993; MacDonald et al. 2000). The disease onset usually occurs between ages of 20 and 40, and women are affected twice as often as men (Noseworthy et al. 2000). In MS, the immune system attacks the myelin sheets surrounding nerve cells in the CNS, where infiltration of mononuclear cells are observed (Steinman 1996). The demyelination is visualized as plaques primarily located in the optic nerve, periventricular white matter, brain stem, cerebellum and the spinal cord (Steinman 1996; Lucchinetti et al. 2000; Lucchinetti et al. 2001; Bruck et al. 2002). A concordance rate in monozygotic twins of 20-30 % (Ebers et al. 1994) argues for genetic factors (Ebers et al. 1995; Compston 2000) as well as environmental factors (Granieri et al. 2001; Steiner et al. 2001) contributing to the disease. As in RA, the only identified genetic association to MS is conferred by the HLA region (Ligers et al. 2001; Marrosu et al. 2001; Lang et al. 2002).

Symptoms

Patients suffering from MS develop visual loss, limb weakness and motor disturbances. Severe cases can lead to paralysis and ultimately death. The disease is diagnosed according to several criteria (Poser et al. 1983), but is often complemented with magnetic resonance imaging (MRI) of the CNS (Miller et al. 1998) to provide specificity and sensitivity of the diagnosis. The disease course of MS is often relapsing but can progress without pauses (Lublin et al. 1996).

Pathogenesis

A crucial step in MS pathogenesis is the brain tissue infiltration by peripheral blood mononuclear cells (PBMC). Endothelial cells, surrounding glial cells and immigrated PBMC regulate the process of cellular extravasation by expression of adhesion molecules and chemokines (Trebst et al. 2001). One hypothesis of MS pathogenesis is that myelin-reactive T cells migrate from the periphery into the CNS where they secrete proinflammatory cytokines inducing expression of chemokines and metalloproteinases (MMPs) (Bar-Or et al. 1999), later causing demyelination. MS and RA thus share some autoimmune characteristics, and several reports suggest the involvement of autoreactive $CD4^+$ T cells (Ben-Nun et al. 1981) of Th1 phenotype (Renno et al. 1994; Pette et al. 1997) in MS.

Several myelin proteins have been suggested as potential autoantigens in MS, and there are reports demonstrating T cell responses against the CNS, recognizing immunodominant epitopes of myelin basic protein (MBP) (Ota et al. 1990) and proteolipid protein (PLP) (Markovic-Plese et al. 1995) in patients. In addition, antibodies against MBP and other myelin proteins found in plaques suggest a role for B cells as well in MS (Gerritse et al. 1994; Genain et al. 1999; Cross et al. 2001). In addition, there are clear associations between common viral infections and the development of MS (Sibley et al. 1985). However, no infectious pathogen has yet been identified as relevant for the induction of MS.

Therapy

Myelin-specific T cells treated with recombinant interferon-beta (IFN-β) have been reported to express immunosuppressive cytokines (Rudick et al. 1998), and over the past decade, there has been much interest in the role of IFN-β to reduce disease activity in adults with MS. Trials have shown clinical and MRI improvement, reduction in relapse frequency and severity, as well as improvement in disability scores (Paolillo et al. 2002; Fernandez et al. 2003). Other immunotherapies include *glatiramer acetate*, a random mixture of sythesized peptides that reduce MS relapses (Arnon et al. 2004), cytostatic drugs such as mitoxantrone and cyclophosphamide (Lynch et al. 1996; Jain 2000), as well as immunosuppressive corticosteroids (Cook et al. 1997).

Animal models

Experimental allergic encephalomyelitis

Experimental allergic encephalomyelitis (EAE) is an animal model for MS. The disease can be induced in rodents by intradermal immunization of various myelin proteins or whole spinal cord homogenate emulsified in Freund´s adjuvant. This will trigger a T cell-driven response against the CNS within a few weeks (Whitehouse et al. 1969; Bergsteinsdottir et al. 2000; Shao et al. 2004). Pathogenic myelin proteins that can induce EAE include myelin basic protein (MBP) (Pettinelli et al. 1982), myelin oligodendrocyte glycoprotein (MOG) (Amor et al. 1994), myelin associated glycoprotein (MAG) (Weerth et al. 1999), myelin oligodendrocyte specific protein (MOSP) (Stevens et al. 1999), and proteolipid protein (PLP) (Yamamura et al. 1986). In addition, EAE can be transferred with myelin-reactive $CD4^+$ T cells (Ben-Nun et al. 1981; Sedgwick et al. 1989), demonstrating that EAE is a T cell-driven autoimmune disease.

The disease course of EAE could be either acute, chronic progressive or chronic relapsing, as in human MS (Lublin et al. 1996). Symptoms and signs that are similar to MS include motor disturbances and limb paralysis, multifocal perivascular CNS infiltrates composed of T cells and monocytes, and association with the MHC region (Gasser et al. 1973; Williams et al. 1973; Abdul-Majid et al. 2000). However, it has been reported that the major genetic control of susceptibility to EAE is located outside the H-2 complex (Montgomery et al. 1982; Tuohy et al. 1988), suggesting a pathogenic role for non-MHC genes as well. As in PIA, EAE is a T cell-driven disease, but the T cell receptor genes have not generated strong evidence of linkage (Livingstone et al. 1995). However, one of the non-MHC loci showing strong linkage to severity in EAE is the *Pia4* locus (containing *Ncf1*) in rats (Bergsteinsdottir et al. 2000). The importance of *Ncf1* in EAE was confirmed in *Ncf1*-mutated mice with aberrant Ncf1 function. In fact, *Ncf1*-mutated mice were more susceptible to MOG protein-induced EAE, but less susceptible to MOG peptide-induced EAE compared with heterozygous controls, which manifests a regulatory role for Ncf1 in EAE (paper III).

Reverse genetics for complex diseases

Diseases that are not inherited in a Mendelian mode are often called complex diseases. Autoimmune diseases, like RA and MS, are examples of complex diseases, where both genes and environment operate in consert contributing to the disease, a phenomenon referred to as *multifactorial inheritance*. Estimations of both the genetic and the environmental impact have been extensively studied by measuring the risk for siblings divided by the risk for the population to develop disease (Risch 1987). For example, in RA, the relative risk value varies between 2 and 17 (Seldin et al. 1999), depending upon discrepancies for disease diagnosis as well as the incidence for RA that varies among different populations (Wiles et al. 1999). Another way to estimate the genetic heritability and the environmental impact of complex diseases is by comparing monozygotic twins (genetically identical) with dizygotic twins (approximately 50 % genetically identical). The heritability in RA has then been estimated to 60 % (MacGregor et al. 2000). However, a tendancy of overestimation has been suggested for twin studies, because of the shared environment. To avoid a variable environment in order to focus on the genetic contribution for complex diseases, it is important to use animal models to study these diseases. By using genetically segregating crosses of animals for disease induction, one could link disease traits to specific chromosomal regions. As this is done with an undetermined mind, there are no prerequisites concerning the function and identities of the genes that you might find. The progress of letting the disease symptoms guide you to what genes are regulating it is called *reverse genetics*.

Genetically segregating crosses

There are several advantages in using animal models to study the genetic contribution in complex diseases. Firstly, many animals can be used in a single study where the environment is controlled. Secondly, inbred strains can be crossed using defined crossing procedures to provide controlled genetic segregation in the offspring in order to identify chromosomal regions, or even genes, that regulate the disease. Thirdly, the function of identified chromosomal regions or isolated genes can be extensively studied by several techniques, for example, transfer of bone marrow or inflammatory cells, as well as various treatments and vaccinations. Inbred rat and mouse strains have normally been inbred for at least 20 generations, which means that all loci are homozygous within each species, and there are several inbred strains that are either susceptible or resistant to induced complex diseases. Depending upon what genetic effects one wishes to investigate for a complex disease, there are different ways to cross inbred strains.

In principle, there are two genetically segregating crosses, the F2 intercross and the F2 backcross. The first generation of outcrossing (F1) is produced by crossing two different inbred strains, for example, a disease-susceptible inbred strain with a disease-resistant inbred strain. The produced F1 offspring is heterozygous for all loci. An F2 intercross is then established by crossing F1 animals with each other producing genotypes that segregate in a Mendelian 1:2:1 manner. The F2 intercross provides information concerning both recessive and dominant genes and is the most common setup for studying complex diseases in animals. However, to establish a strong analysis of only dominant genes controlling a disease, an F2 backcross should be considered (Darvasi 1998). An F2 backcross is established by crossing F1 animals with either of the parental strains, producing genotypes that segregate in a Mendelian 1:1 manner. Once agreed on the setup, linkage analysis is performed.

Linkage analysis

Linkage analysis of a genetic disease links disease traits to chromosomal fragments. The purpose is to identify genes that regulate the studied disease. In fact, a few genes associated with autoimmune diseases in humans have already been suggested through linkage analysis of human families, followed by classic case-control studies, for example programmed cell death 1 (*PDCD1*) in systemic lupus erythematosis (Prokunina et al. 2002) and a caspase recruitment domain (*CARD15/NOD2*) in inflammatory bowel disease (Lesage et al. 2002). However, no single genes have so far been identified for RA or MS, which could be due to several reasons, some of which are mentioned below:

Incomplete penetrance, when a phenotype is vague, but the contributing gene is present
Phenocopies, when non-genetic factors bridge the genetic contribution to a phenotype
Locus heterogeneity, when different genes provide the same phenotype
Allelic heterogeneity, when different alleles within a locus express the same phenotype
Epistasis, when a gene interferes with or prevents the expression of another gene
Modifier genes, genes that interact with other genes to alter a phenotype
Anticipation, when the effect of a gene is enhanced for each generation
Polygenecity, when many genes contribute to a phenotype

To reduce the impact of non-genetic factors (*e.g.* the environment), as well as the genetic heterogeneity for linkage analyses of polygenic diseases, animal models are often used. To perform a linkage analysis, each individual of a genetically segregated cross, often rodents, must be genotyped and phenotyped. Genotyping locates recombinations in the genome, and is performed by amplification of DNA markers, such as microsatellite markers, spanning the entire genome. Phenotyping provides numerical (quantitative) values for disease traits, such as disease scores and other responses. Linkage analysis is then performed by statistically analyze the likelihood for a given disease phenotype to be correlated with a genotype at a chromosomal locus, and the term for such a linkage is quantitative trait locus (QTL). The statistical significance of such a QTL should then be evaluated by a so-called *permutation test*, providing an empirical significance limit for both non-parametric and non-continuous phenotypes in relation to a studied genotype (Doerge et al. 1996).

When a linkage analysis has been completed, it is important to confirm QTLs. This could be performed in an animal strain, congenic for the QTL of interest. A congenic strain for a QTL, could be a disease-susceptible inbred strain into which the QTL from the disease-resistant strain has been bred, or vice versa (reciprocal congenics). By continual backcrossing and genotyping, it is possible to pick a founder from an offspring that has reduced heterozygocity within the QTL due to recombination, thus minimizing the disease-regulating fragment. In fact, recombinations within a large number of offspring display normal distribution. Therefore, to speed up this procedure, one could genotype the entire genome of a large offspring for each generation, and pick a founder that displays less heterozygocity than expected from random segregation, thus allowing less backcrossing to establish a genetically pure background (Wakeland et al. 1997; Wong 2002). If the trait used for QTL identification can be confirmed in an animal, congenic for the QTL, the inheritance of the trait can often be treated as a Mendelian trait. Once a functional congenic strain comprising a fragment less than 1 cM has been established, one should consider starting analysing the genes harboured by the congenic region to identify genes for the disease trait.

Positional cloning

Positional cloning is the final result of reverse genetics leading to the identification of a gene regulating a quantitative trait. Once a minimal disease-regulating congenic fragment has been established through extensive backcross or intercross breeding, there are several techniques that should be used to identify and explain the disease-regulating gene/s. *cDNA sequencing*, *differential expression* and *functional studies* (if possible) of all genes within the congenic fragment should be tested for both the disease-susceptible and the disease-resistant parental strains. The cDNA sequencing is performed to identify strain-specific polymorphisms that could result in amino acid substitutions or truncations of the protein. For example, Olofsson et al. (2003) (paper II) succeeded to positionally clone the *Ncf1* gene in rats, regulating the severity of pristane-induced arthritis. *Ncf1* was identified as the only gene within a minimal *Pia4*-fragment on DA background with polymorphisms inside the coding region that differed between parental strains. These polymorphisms resulted in amino acid substitutions in the Ncf1 protein, but no differential expression of Ncf1 was observed between DA and DA.pia4 congenic littermates, suggesting that the major disease-ameliorating effect of *Pia4* must arise from the structural polymorphism of Ncf1 (paper II). However, differential expression techniques might be useful to identify polymorphisms outside the coding region regulating the expression of certain genes that could influence the studied disease.

It is also important to verify a positionally identified gene for an induced disease in another species in order to demonstrate that the genetic effect is not species-specific. For example, *Ncf1*-mutated mice were shown to be more susceptible to arthritis than heterozygous littermates (paper III). It is therefore possible that *Ncf1* polymorphisms could regulate arthritis in humans as well. In addition, functional studies should be performed to explain a possible mechanism for the positionally identified gene. For *Ncf1*, it was shown that the arthritis-promoting allele contributed to a reduced production of ROS from neutrophils, suggesting that reduced ROS production triggers arthritis. This hypothesis was further strengthened by ameliorating arthritis development by prophylactic treatments of arthritis-susceptible DA rats with phytol oil, which triggers ROS production (paper II). Other functional studies, such as transfer of bone marrow or inflammatory cells, as well as thymus transplantation experiments between strains that display structural polymorphisms in the disease-regulatory gene should be performed to study where and when the gene is operating.

The NADPH oxidase

When professional phagocytes, like neutrophils and macrophages are ingesting microorganisms, the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase is activated to reduce oxygen molecules to superoxide ions (O_2^-) that is subsequently converted to microbicidal oxidants (Roos et al. 2003). The oxidants produced are collectively called *reactive oxygen species* (ROS), including the superoxide ion, hydrogen peroxide, hydroxyl radicals and hypoclorous acid (Babior 1999), and the process by which ROS is produced is called *the respiratory burst*. A functional NADPH oxidase is necessary for the innate immune response to produce ROS in order to kill invading microorganisms. This is clearly exemplified in chronic granulomatous disease (CGD) in humans, caused by inherited mutations of phagocyte oxidases, such as gp91phox (alias Cybb or Nox2) or p47phox (alias Ncf1), protein components of the NADPH oxidase complex (Noack et al. 2001; Jirapongsananuruk et al. 2002). Patients suffering from CGD have a non-functional NADPH oxidase due to such mutations leading to the inability for macrophages and neutrophils to produce ROS and kill invading microorganisms, resulting in recurrent infections (Segal et al. 2000). In this thesis, we show that reduced ROS production also affects the adaptive immune response. In fact, polymorphisms as well as truncation of Ncf1, a crucial member of the NADPH oxidase, reduce ROS production and triggers susceptibility to T cell-mediated arthritis in animal models (paper II; paper III). The mechanism by which ROS is produced and the importance for Ncf1 in regulating this production is mentioned below.

ROS production

The NADPH oxidase is a multicomponent enzyme located in phagosomal membranes, as well as in the plasma membrane of various cells. In phagocytic cells, it is composed of the oxidase-specific proteins p22phox (Cyba), p47phox (Ncf1), p67phox (Ncf2) and gp91phox (Cybb or Nox2), as well as the GTPase (Rac) (Quinn et al. 2004). Another oxidase-specific protein (p40phox (Ncf4)) and a second GTPase (Rap1A) have also been suggested to regulate the NADPH oxidase activity (Quinn et al. 2004). During oxidase activation, a cytosolic multimeric complex consisting of equimolar (1:1:1) amounts of Ncf1, Ncf2, and Ncf4 is formed, phosphorylated and translocated to a membrane-bound heterodimer (flavocytochrome b) composed of Cyba and Cybb oxidase components (Park et al. 1992; Iyer et al. 1994; Lapouge et al. 2002). This process is highly regulated and involves several phosphorylation steps, translocation and conformational changes (Quinn et al. 2004). Briefly, phosphorylation of Ncf1 in the cytosolic multimeric complex unmasks essential binding domains (*e.g.* the tandem Src homology 3 (SH3) domains, the polybasic region (+++) and the N-terminal phox homology (PX) domain) as shown in Figure 1 below. The cytosolic complex is then translocated to the membrane-bound heterodimer, and the tandem SH3 domains of Ncf1 interact with a proline-rich target in Cyba, and the PX domains of both Ncf1 and Ncf4 stabilize a functional NADPH oxidase by binding to membrane phosphoinositides with apparent preference for phosphatidylinositol-3,4-bisphosphate (Kanai et al. 2001). Once assembled, the NADPH oxidase catalyzes the reduction of extracellular or intraphagosomal oxygen molecules (O_2) to superoxide ions (O_2^-) through Cybb and subsequently NADPH is oxidized to $NADP^+$ (Quinn et al. 2004) as shown in Figure 1 below.

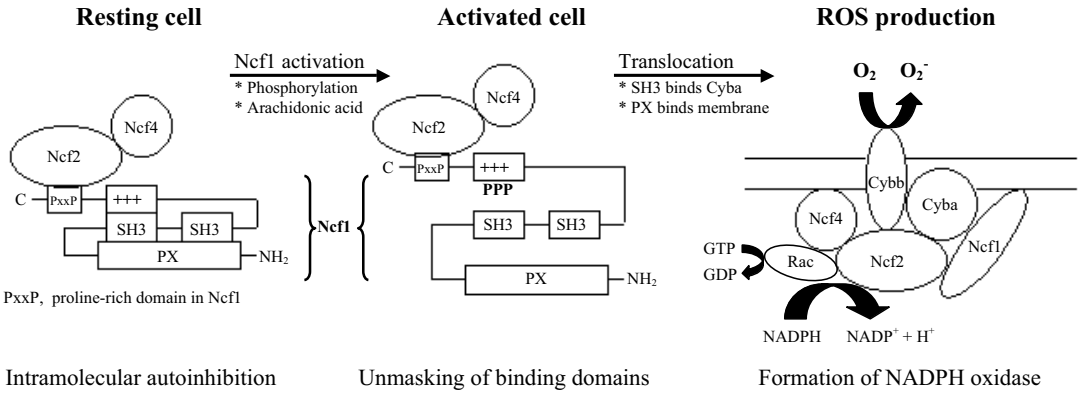

Figure 1. Activation of Ncf1 triggers ROS production

Regulation of the NADPH oxidase

Although all oxidase components are necessary for activation of the NADPH oxidase, Ncf1 is regarded as a key component for both activation and deactivation of this enzyme. In resting cells, the cytosolic multimeric complex consisting of Ncf1, Ncf2 and Ncf4 is stochiometrically prevented to interact with Cyba, since the tandem SH3 domains of Ncf1 interact with the polybasic domain (+++) as well as with the N-terminal phox homology (PX) domain within Ncf1 (Figure 1, left). Upon cell activation, Ncf1 is phosphorylated, primarily in the polybasic domain and the C terminus, which induces conformational changes that unfold the intramolecular autoinhibitory interaction of Ncf1 (Ago et al. 1999; Groemping et al. 2003) (Figure 1, middle). This phosphorylation of Ncf1 is one of the key intracellular events associated with NADPH oxidase activation. A number of kinases have been proposed to participate in the phosphorylation of Ncf1, such as protein kinase C (PKC) (el Benna et al. 1994), extracellular signal-regulated kinase 1/2 (ERK1/2) (Dewas et al. 2000), p21-activated kinase (PAK) (Knaus et al. 1995), protein kinase B (PKB) (Didichenko et al. 1996), casein kinase 2 (Park et al. 2001), and a phosphatidic acid-activated kinase (Waite et al. 1997), of which PKC plays a dominant role in this process (Fontayne et al. 2002).

However, activation of Ncf1 *in vivo* requires a synergistic action of phosphorylation in presence of arachidonic acid (Dana et al. 1998; Shiose et al. 2000). In fact, high concentrations of long-chain polyunsaturated membrane-soluble fatty acids such as arachidonic acid (Shiose et al. 2000) or omega-3 fatty acids such as eicosapentaenoic or docosahexanoic acid (Schneider et al. 2001) can directly activate and unmask binding domains of Ncf1. Once Ncf1 has been activated, the cytosolic multimeric complex can interact with the membrane-bound heterodimer, as described above. Conclusively, the activated conformation of Ncf1 involves both phosphorylation and direct activation through common lipid structures, and is crucial for successful formation of the NADPH oxidase and the subsequent ROS production. Apart from Ncf1, other cytosolic oxidases, like Ncf2 and Ncf4, seem to play a regulatory role for the activation of the phagocyte NADPH oxidase. Ncf2 is expressed in limiting levels in the cytosol (el Benna et al. 1994), but is essential for the activation of the NADPH oxidase through interaction with RAC (Diekmann et al. 1994). Although little is known about Ncf4, it has been suggested to act as a stabilizer of the cytosolic multimeric complex (Wientjes et al. 1993). In addition, members of the Ras family of GTPases, Ras and Rap1, have also been reported to regulate ROS production (Remans et al. 2004).

Present investigation

This thesis is based on five papers, four of which mainly concern the pathogenesis of pristane-induced arthritis (PIA) in rats, and one that concerns collagen-induced arthritis (CIA) as well as experimental allergic encephalomyelitis (EAE) in mice. However, there is a linkage between all of these papers. *Paper I* aimed at identifying strong dominant loci operating early in PIA by linkage analysis of a large F2 backcross. We identified eight dominant QTLs regulating arthritis traits, of which *Pia4* was the strongest in suppressing arthritis. *Paper II* aimed at cloning the gene responsible for the strong arthritis-suppressive effect of *Pia4*. *Ncf1* was positionally identified as the only gene within a minimal *Pia4*-fragment with polymorphisms inside the coding region that differed between parental strains. *Paper III* aimed at showing that the arthritis-regulatory effect of *Ncf1* is not species-specific. We showed that *Ncf1*-mutant mice were more susceptible to arthritis as well as to MOG protein-induced EAE than heterozygous littermate controls. *Paper IV* aimed at characterizing the immune response in PIA. Through several cell transfer experiments, we could conclude that T cell antigens must be involved in PIA, since arthritis transferred by T cells was restricted by MHC class II DQ and DR molecules. *Paper V* aimed at characterizing the chronic relapsing arthritis induced by adoptive transfer of PIA. High levels of COMP, AGP and IgG2b in blood at the chronic phase suggested cartilage destruction, systemic inflammation and Th1-mediated antibody production, respectively. Also, MTX, anti-IFN-γ or anti-TNF-α treatment with Etanercept ameliorated arthritis severity at the chronic stage.

Paper I. **Identification and isolation of dominant susceptibility loci for pristane-induced arthritis**

This article is discussed in The Journal of Immunology, 2003, 171: 1-2.

This paper demonstrates how the genetic complexity of arthritis can be broken up into simple Mendelian traits, leading to the identification of chromosomal regions harbouring genes conferring susceptibility and resistance. This is important in order to understand the inheritance and pathogenesis of RA in humans, and to facilitate the development of target-specific drugs.

We used the PIA model in rats. The inheritance of PIA was determined by genomes from two inbred strains, *i.e.*, the arthritis-resistant E3 and the arthritis-susceptible DA rat strains. We used a high-powered backcross strategy using as many as 650 progeny from DA(E3xDA) rats to identify E3 dominant loci operating early in PIA. Clinical score, disease onset, paw swelling, AGP (indicating systemic inflammation), COMP (indicating cartilage destruction) and CD4/CD8 T cell ratio in spleen (indicating expansion of arthritogenic $CD4^+$ T cells) were used as disease traits. Five new dominant PIA QTLs were identified, three of which were DA-promoting; *Pia10* (chr. 10), *Pia13* (chr. 7), *Pia14* (chr. 8), and two that were E3-promoting; *Pia12* (chr. 6) and *Pia15* (chr. 18). Three previously identified DA-promoting QTLs regulating PIA were reproduced with high significance, but also verified in congenic strains; *Pia1* (MHC region, chr. 20), *Pia4* (chr. 12) and *Pia7* (chr. 4). These three dominant loci were also found to operate in concert. In addition, both COMP and AGP were linked to *Pia4*, and the CD4/CD8 phenotype revealed linkage to the MHC region *Pia1*. *Pia4* was by far the strongest dominant locus regulating PIA, and by stratifying the material to exclude animals affected by this locus, we were able to observe an increase in significance for other QTLs, especially *Pia1* and *Pia7*. As *Pia1* was originally identified as a regulatory locus of chronic arthritis in MHC congenics with LEW background, one could suggest the possibility for more than one gene in the *Pia1/MHC* region regulating PIA. However, we could not reproduce *Pia2* or *Pia3* loci that were previously shown to regulate onset in an F2 intercross, which could be explained by the fact that there are more possible genetic interactions in an F2 intercross than in an F2 backcross.

Paper II. **Positional identification of *Ncf1* as a gene that regulates arthritis severity in rats**

This article is discussed in Nature Reviews Drug Discovery, 2003, 2: 93-94.

This paper demonstrates that it is possible to positionally clone a gene that is responsible for the effect of an identified QTL regulating disease in animal models. A naturally occurring polymorphism in the protein neutrophil cytosolic factor 1 (Ncf1) was identified and shown to regulate the severity of arthritis through mechanisms involving production of reactive oxygen species and arthritogenic T cells.

The positional cloning of *Ncf1* was performed on the DA.pia4 congenic strain, containing a dominant protective genomic fragment (*Pia4*) from the arthritis-resistant E3 strain on the arthritis-susceptible DA background. DA alleles within the *PIA4*-fragment were shown to promote arthritis development in an additive fashion. Elevated plasma levels of COMP and AGP were also observed for DA rats compared with DA.pia4 congenic rats. An extensive backcross breeding of the DA.pia4 congenic strain was performed to obtain recombinations within the *Pia4*-fragment. A physical map of a consensus *Pia4* congenic fragment of 1 cM was constructed, using microsatellite markers covering a functional 1 cM *Pia4*-fragment to obtain relevant P1-derived artificial chromosome (PAC) clones. By using end sequences from these PAC clones, partially sequenced bacterial artificial chromosome (BAC) clones and the sequence of the corresponding gene region in mice, a draft physical map of *Pia4* was constructed. The 1 cM *Pia4*-fragment was further dissected and a minimal arthritis-protective interval of 300 kb containing only two genes (*e.g. Ncf1* and *Gtf2i*) was identified. The cDNA of these two genes were sequenced for both DA and E3 strains. Three polymorphisms were identified for the *Ncf1* gene, two of which resulted in amino acid substitutions between DA and E3. Only one polymorphism, outside the translated region, was identified for the *Gtf2i* gene. Neither *Ncf1* nor *Gtf2i* were differentially expressed between DA.pia4 and DA littermates, suggesting that *Gtf2i* was not a candidate gene for *Pia4* and that the major disease-protective effect of this QTL must arise from the structural polymorphism of *Ncf1*. To explain a possible mechanism for the structural polymorphism of Ncf1, we proceeded to perform functional studies. We observed that phorbol myristate acetate (PMA)-activated neutrophils from arthritis-susceptible DA rats produced significantly less ROS than DA.pia4 congenic littermates and arthritis-resistant E3 and BN rat strains, suggesting that reduced ROS production triggers arthritis. This hypothesis was further strengthened by ameliorating arthritis development by treatment of arthritis-susceptible DA rats with phytol oil, which triggers ROS production. To investigate whether the disease-promoting allele of Ncf1 is involved in the generation of arthritogenic T cells or operates locally in the joint, we performed reciprocal adoptive transfer of activated spleen cells between DA and DA.pia4 congenic littermates. We observed that donor cells from DA, but not from any of the DA.pia4 congenics, could adoptively transfer arthritis to both DA and DA.pia4 congenics, suggesting a regulatory role for Ncf1 in the priming phase of arthritis. We also sequenced the three identified single-nucleotide polymorphisms (SNPs) in *Ncf1* (SNP codon 106, SNP codon 153 and SNP codon 383) in other inbred rat strains and in wild rats, and could observe a high degree of polymorphisms in both populations, suggesting that these polymorphisms were naturally occurring. In fact, the arthritis-resistant BN rat strain shared the same sequences of SNP codon 106 and SNP codon 383 with the arthritis-susceptible DA rat, and the same sequence of SNP codon 153 with the arthritis-resistant E3 rat, suggesting that the threonine at position 153 mediates the disease protection.

Paper III. **Enhanced autoimmunity, arthritis, and encephalomyelitis in mice with a reduced oxidative burst due to a mutation in the *Ncf1* gene**

This paper demonstrates that *Ncf1*-mutant mice with aberrant Ncf1 function are highly susceptible to arthritis. This is important, because it confirms the reduced ROS production and the enhanced arthritis susceptibility observed in rats expressing the disease-promoting *Ncf1* allele. Since different *Ncf1* mutations affect both mice and rats similarly, it is possible that *Ncf1* polymorphisms resulting in altered ROS production could regulate arthritis development in humans as well.

We observed that mice with aberrant Ncf1 function developed a significantly more severe disease course of CIA than heterozygous and wild-type littermate controls. The *Ncf1*-mutated mice also showed a more pronounced cartilage destruction as well as active inflammation at the chronic stage of CIA than littermate controls, as measured by elevated levels of COMP in serum and histologic sections, respectively. To evaluate the enhanced autoimmune response for *Ncf1*-mutated mice, we proceeded to investigate antibody responses against collagen type II (CII). We observed elevated levels of IgG1, IgG2a, IgG2b and IgG3, but not IgM antibodies against CII for *Ncf1*-mutated mice compared with littermate controls, suggesting a T cell-mediated antibody production. In fact, elevated responses against C1-, J1- and U1-epitopes of CII were also observed for *Ncf1*-mutated mice. We next investigated T cell-specific DTH responses against CII or OVA in mice immunized with these antigens, respectively. The DTH response against CII, but not against OVA, was elevated for the *Ncf1*-mutant mice, suggesting an enhanced activity of CII-reactive T cells mediating a more severe chronic arthritis in these mice compared with littermate controls. The *Ncf1*-mutated mice were also used to study the development of EAE, since it has been reported that mice deleted for the *Ncf1*-gene are protected against EAE, induced with the MOG_{35-55} peptide. The *Ncf1*-mutated mice and littermate controls were immunized either with MOG_{79-96} peptide or MOG protein. We could confirm earlier data by showing that the *Ncf1*-mutated mice were less susceptible to MOG_{79-96} peptide-induced EAE than littermate controls. However, the *Ncf1*-mutated mice developed significantly more severe MOG protein-induced EAE, which could mean that Ncf1 affects the processing and presentation of antigens for immune recognition. In addition, we have observed spontaneous chronic arthritis in *Ncf1*-mutant female mice, starting a few days after delivery (postpartum). Histologic sections of these mice showed a severe destruction of bone and cartilage. We also found elevated levels of IgG antibodies against C1-, J1- and U1-epitopes of CII in these mice compared with healthy littermate controls, resembling the response observed in CIA.

Paper IV. **Pristane, a non-antigenic adjuvant induces MHC class II-restricted, arthritogenic T cells**

This paper demonstrates that a single intradermal injection of pristane oil (2,6,10,14-tetramethylpentadecane) induces arthritis that can be transferred by MHC class II restricted oligoclonal Th1 cells. This is important, because it argues for the involvement of a set of antigens in an arthritis model induced by a non-antigenic adjuvant. It raises the question whether non-antigenic factors could trigger an autoimmune T cell response against self-antigens in humans as well.

We observed a selective accumulation of highly differentiated $CD4^+$ αβT cells, but few $CD8^+$ αβT cells and B cells in arthritic synovia by flow cytometry. We proceeded to investigate lymphocyte expansions in draining lymph nodes, and observed that $CD4^+$ αβT continuously expanded in numbers until disease onset. Isolated cells from draining lymph nodes or spleen around onset could adoptively transfer arthritis after re-activation with Con A *in vitro*. Cell selection experiments revealed that $CD4^+$ αβT cells alone, but not $CD4^-$ cells could transfer arthritis. In addition, isolated cells stimulated with plate-bound mitogenic anti-Vα4 TCR, anti-Vβ10 TCR, or anti-Vβ16 TCR, but not with anti-Vβ8.5 TCR monoclonal antibodies transferred arthritis, suggesting that oligoclonal T cells mediate PIA. We proceeded to investigate whether pristane-sensitized T cells were restricted to MHC class II molecules. We observed that prophylactic treatment of recipients with antibodies against MHC class II DQ or DR molecules, both ameliorated transferred PIA, suggesting the involvement of antigens in this model, although pristane oil itself is non-antigenic. In addition, adoptive transfer to irradiated recipients that were semi-allogenic to the MHC region developed arthritis similar to syngenic animals, but MHC allogenic recipients showed no signs of arthritis, which confirmed MHC restriction. We next investigated whether the arthritogenic T cells target the recipient synovium by transferring donor cells from (DAxDA.1I)F1 rats to irradiated DA.1I recipient rats. We could detect donor cells in recipient synovium by immunohistochemistry, using a DA haplotype-specific anti-MHC class I antibody. Synovial tissue was also excised from recipient joints and flow cytometry of isolated synovial cells revealed that although total spleen cells were transferred, more than 98 % of these cells were $CD4^+$ αβT cells. Finally, we showed that the arthritogenic T cells produced high levels of IFN-γ and TNF-α, but not IL-4, suggesting a Th1 response. Prophylactic treatment of recipients with anti-IFN-γ or Etanercept significantly ameliorated the development of transferred arthritis.

Paper V. **Chronic relapsing arthritis, induced by pristane-sensitized Th1 cells**

This paper demonstrates a novel model for chronic relapsing arthritis, induced by injection of pristane-sensitized Th1 cells in irradiated syngenic recipients. The key to this observation is that recipients need to be irradiated to become susceptible for chronic relapsing arthritis. We have also observed that thymectomy enhances the irradiation-induced susceptibility to chronic relapsing arthritis, leading to increased synovial infiltration of inflammatory cells and to an elevated acute systemic inflammatory response. In addition, chronic relapses can be ameliorated with classic drug therapy used for RA patients. These observations are important, and provide an arthritis model for basic and therapeutic analysis of T cell effector pathways leading to chronic relapsing arthritis.

We examined if transferred PIA could become chronic by adjusting the transfer procedure. We observed that pristane-sensitized T cells from draining lymph nodes, but not from spleen, could induce chronic relapsing arthritis in irradiated, but not in non-irradiated, syngenic recipient rats. The chronic relapsing disease course was still ongoing at the end of experiment at 124 days after transfer, and histologic sections of affected joints revealed a pronounced destruction of cartilage and bone, as well as a massive pannus formation. High levels of COMP, AGP and IgG2b antibodies in blood plasma suggested cartilage destruction, acute systemic inflammatory response and selective Th1-mediated antibody production at this stage. We proceeded to investigate if irradiation-induced susceptibility to chronic relapsing arthritis could be explained by depletion of T cells in recipients. We were unable to show that irradiation-induced susceptibility for chronic arthritis transfer is due to depletion of $CD4^+$ or $CD8^+$ recipient T cells, since successful antibody-depletions of $CD4^+$ or $CD8^+$ T cells in non-irradiated thymectomized recipients could not induce any significant susceptibility for chronic arthritis transfer compared with groups of irradiated recipients. Interestingly, thymectomy significantly enhanced arthritis susceptibility for both the acute and the chronic phases of arthritis in irradiated recipients compared with non-thymectomized irradiated controls. We could observe that thymectomy enhanced the acute inflammatory response, as measured by plasma levels of AGP, in irradiated recipients and triggered proliferation of $CD4^+25^+$ T cells, macrophages, NK cells and granulocytes in spleen, and contributed to an increased synovial infiltration of granulocytes and donor $CD4^+$ lymphocytes in affected joint synovia at chronic stage, compared with non-thymectomized irradiated controls. We proceeded to investigate whether $CD4^+$ $\alpha\beta$T cells alone could adoptively transfer chronic relapsing arthritis and if the arthritogenic LN T cells produce Th1 cytokines, as shown for arthritogenic spleen T cells. We observed that 20 million $CD4^+$ $\alpha\beta$T cells (>95% pure), isolated from inguinal lymph nodes 14 days post-injection of pristane and re-activated *in vitro* with Con A was enough to provoke chronic relapsing arthritis in 3 out of 3 irradiated syngenic recipients. Pristane-sensitized LN cells were also cultured with or without Con A to examine the cytokine profile upon re-activation *in vitro*. In absence of Con A-stimulation, supernatants showed no detection for IFN-γ, low secretion of TNF-α and IL-4, while in the presence of Con A, a highly significant response for IFN-γ and TNF-α was observed, but not for IL-4, suggesting a Th1 response for arthritogenic T cells. To further examine the role of IFN-γ and TNF-α in this chronic arthritis transfer model, and to test available drugs commonly used for RA patients, we treated chronic relapses of arthritis with anti-IFN-γ, MTX or with Etanercept (recombinant TNF-α receptor), all of which significantly ameliorated arthritis severity by a single injection of doses equivalent for humans.

Concluding remarks

It is impossible to directly judge which gene is responsible for the disease-regulatory effect of a congenic fragment identified through genetic linkage analysis. For example, the minimal *Pia4* congenic fragment, in which *Ncf1* was positionally identified, comprised only two genes, *Gtf2i*, involved in the B cell receptor pathway, and *Ncf1*, a regulatory subunit of the NADPH oxidase that produces oxygen radicals as a result of infections. I would have guessed that a gene controlling the adaptive immune response should be more important for autoimmunity than a gene controlling the innate immune response, but I was wrong. Peter Olofsson managed to sequence these genes from both the arthritis-resistant E3 and the arthritis-susceptible DA strains, and demonstrated that only *Ncf1* comprised polymorphisms in the coding region that differed between parental strains. In fact, through functional studies of the NADPH oxidase as well as SNP marker sequencing of various wild rats and inbred rat strains, we could suggest that the threonine at the position 153 in *Ncf1* mediated the disease protection. This disease-protective mutation is situated close to the N-terminal SH3-domain of *Ncf1*, and might allow an additional post-translational modification upon Ncf1 activation, like phosphorylation. Perhaps this could facilitate the unmasking of essential SH3-binding domains, which is necessary for interaction with Cyba, or even help stabilizing the interaction of the PX domain with membrane phosphoinositides, thus leading to a greater activation and formation of the NADPH oxidase, resulting in enhanced production of ROS and arthritis protection (Figure 2, below).

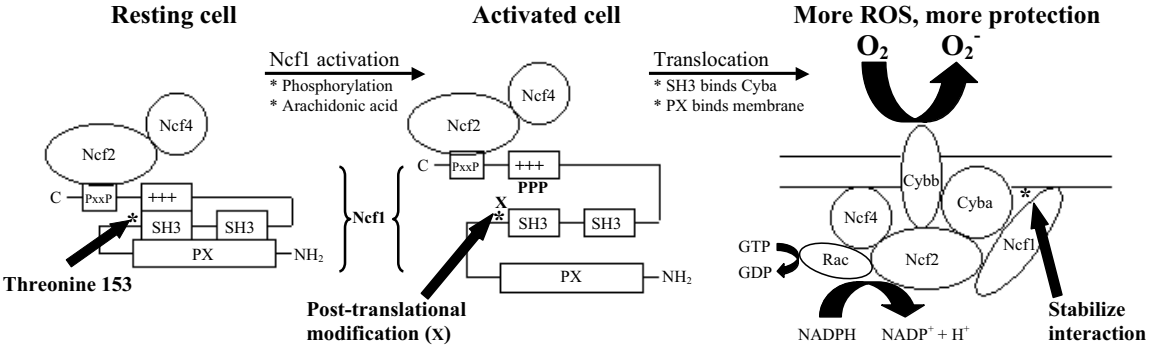

Figure 2. Possible mechanisms for arthritis-protective threonine at position 153

It is important to study where and when production of ROS regulates arthritis-susceptibility. As shown in paper II, we can ameliorate arthritis development by early treatments with phytol oil, which triggers activation of the NADPH oxidase, and it could therefore be interesting to study drugs that specifically induce ROS production in RA patients as well. Also, we showed that only rats with the disease-promoting allele of *Ncf1* could be used as donors for adoptive transfer of PIA, indicating a role for ROS production in regulating the priming phase of arthritis. We plan to start reciprocal thymus transplantation experiments between DA and DA.pia4 rats to clarify whether the disease-promoting allele of *Ncf1* in phagocytic cells in thymus results in an aberrant negative selection, leading to the development of autoreactive T cells. We believe that irradiated thymectomized DA rats transplanted with DA.pia4 thymus in the kidney capsule should be protected from arthritis compared with positive controls (*i.e.* irradiated thymectomized DA rats transplanted with DA thymus), if injected with pristane after T cell reconstitution in draining lymph nodes.

As shown in paper III, the arthritis-promoting effect as well as the reduced ROS production was also observed in *Ncf1*-mutated mice with an aberrant Ncf1 function, and therefore not species-specific, suggesting that mutations in *Ncf1* might trigger an autoimmune response in humans as well. Future experiments that should be considered include reciprocal transfer experiments of isolated cell subsets from different lymphoid tissues between *Ncf1*-mutant mice and wildtype mice to identify which cells that regulate the autoreactive T cells and where and when this regulation occurs. However, now it is time to study the effect of *Ncf1* in humans as well. One should investigate whether PMA-activated neutrophils from blood of RA patients produce less ROS than healthy controls. This method could then be used for early diagnosis. Other future prospects for *Ncf1* in humans involve the identification of arthritis-promoting alleles of *Ncf1* to support early diagnosis for RA as well as for other autoimmune diseases. This study could be extended to include other regulatory subunits of the NADPH oxidase.

In paper IV, we showed that pristane-sensitized Th1 cells transfer arthritis to syngenic recipients and are restricted to MHC class II DR and DQ molecules. It argues for the involvement of a set of antigens in this model, although pristane oil is non-antigenic. This could mean that non-antigenic factors could trigger an autoimmune T cell response against self-antigens in humans as well. We have already started to analyze fractions of joint-proteins from both naive and arthritic rats in order to identify T cell antigens responsible for arthritis onset as well as for perpetuation of chronic relapsing arthritis. The identification of a T cell antigen will be very useful, not only for RA diagnosis, but also for vaccination with this antigen or with antigen-specific T cell receptors. In paper V, we started to optimize the T cell transfer procedure by injecting pristane-sensitized spleen or lymph node cells to irradiated or non-irradiated recipients. We were surprised when irradiated recipients injected with lymph node cells, but not with spleen cells, failed to recover as normally occurs 30-40 days after adoptive T cell transfer of PIA. In fact, these rats showed severe chronic relapses starting around day 50 until the end of experiment at day 124 after transfer. Histologic sections of affected joints and analysis of blood plasma samples at this stage revealed a severe destruction of bone and cartilage as well as an acute systemic inflammatory response and Th1-mediated IgG2b antibody production. The reason for why spleen cells cannot transfer chronic relapsing arthritis should be further investigated. As shown in paper V, spleen cells induce a significantly more rapid arthritis with a maximum score already at day 8 post-transfer compared with lymph node cells with a maximum score at day 14 post-transfer, regardless of irradiation. This could mean that arthritogenic T cells in spleen, compared with lymph nodes, are more differentiated, but also more susceptible for apoptosis upon T cell transfer to recipients. There could also be a higher frequency of arthritis-suppressive cells in spleen, compared with lymph nodes, that proliferate after transfer to reduce arthritis severity.

We do not know why irradiation to recipients, before T cell transfer of PIA, induces susceptibility to chronic relapsing arthritis. Our first hypothesis was simple but not complete, suggesting that irradiation depletes suppressive T cells in the recipient, also leading to extra space for donor T cells to proliferate and exert their pathogenecity. However, although a few T cell-depleted thymectomized or only thymectomized recipients developed a mild relapsing arthritis it was not comparable to what was observed for irradiated groups of recipients, all of which developed severe chronic relapses. Perhaps irradiation could activate macrophages to trigger presentation of autoantigens or create neoepitopes in cartilage. In addition, we observed that thymectomy in combination with irradiation enhanced the severity of both the acute phase and the chronic relapsing phase of arthritis, and also enhanced the acute systemic inflammatory response, compared with non-thymectomized irradiated recipients. It was further shown that thymectomy triggered expansion of donor $CD4^+$ T cells as well as other inflammatory cells, in both spleen and affected joints of irradiated recipients, which brings us back to the initial idea supporting a role for suppressive T cells in the recipient, which will be further studied.

Populärvetenskaplig sammanfattning

Brist på fria radikaler kan ge artrit

Fagocytiska celler i vårt immunförsvar producerar fria syre-radikaler för att eliminera fagocyterade (uppätna) mikroorganismer. Produktionen av dessa syre-radikaler sker med hjälp av ett enzymkomplex (NADPH oxidas) i cellmembranet samt i membranet på de fagosomer, innehållande mikroorganismer, som bildas i och med fagocytos. Människor med nedsatt funktion av detta NADPH oxidas p.g.a. specifika mutationer i detta komplex kan inte bilda tillräckligt med syre-radikaler och har därför nedsatt immunförsvar mot mikroorganismer. Sjukdomen kallas CGD (kronisk granulomatös sjukdom) och är mycket sällsynt. Vi har upptäckt att olika funktionsnedsättande mutationer i Ncf1 (neutrofil cytosolisk faktor 1), som är en proteinkomponent i NADPH oxidas-komplexet, leder till försämrad produktion av fria syre-radikaler samt till ökad mottaglighet för artrit hos både råttor och möss. Ur ett genetiskt perspektiv skiljer vi oss inte mycket från råttor och möss. Det är därför troligt att funktionsnedsättande mutationer i Ncf1 hos människa kan leda till ökad mottaglighet för reumatoid artrit (RA)*, även kallad ledgångsreumatism.

Denna upptäckt är ny och revolutionerande. Aldrig tidigare har man kunnat följa ett sjukdomsförlopp för att kunna identifiera en enskild gen. Tekniken bakom upptäckten kallas *Linkage analysis* (kopplingsanalys), och används ofta i djurmodeller för komplexa sjukdomar. I djurmodeller korsas i regel sjukdoms-resistenta djur med sjukdoms-mottagliga djur på ett sådant sätt att avkomman får slumpmässiga kombinationer av sjukdoms-resistenta och sjukdoms-mottagliga kromosomfragment. Genom att inducera sjukdom på samma sätt i ett stort antal djur av denna genetiskt segregerade avkomma, kan man koppla sjukdoms-symptom till olika kromosomfragment. Vi injicerade pristanolja i 650 st. råttor med slumpmässiga artrit-resistenta och artrit-mottagliga kromosomfragment. Vi kunde identifiera åtta st. dominanta kromosomfragment som reglerade sjukdomsförloppet pristan-inducerad artrit (PIA). Det överlägset starkast reglerande kromosom-fragmentet, *Pia4*, innehöll flera s.k. kandidatgener som skulle kunna orsaka reglering av artrit. Genom att korsa råttor på sådant sätt att ett minimalt *Pia4*-fragment från en artrit-resistent råtta fortfarande dämpade artrit väl inne i en artrit-mottaglig råtta kunde samtliga kandidatgener analyseras.

I detta minimala *Pia4*-fragment fanns dock endast två gener, varav *Ncf1* var den enda som genetiskt skiljde sig mellan den artrit-resistenta råttan och den artrit-mottagliga råttan. Vi identifierade mutationer (genetiska förändringar) i *Ncf1* hos den artrit-mottagliga råttan som gav upphov till nedsatt produktion av fria syre-radikaler. Omvänt kunde vi visa att stimulerad produktion av fria syre-radikaler genom injektion med fytololja i dessa råttor hämmade utvecklingen av PIA. För att förvissa oss om att denna upptäckt inte var specifik för inducerad artrit hos råttor studerade vi en annan mutation av *Ncf1* hos möss. Möss med muterad *Ncf1* producerade inga syre-radikaler alls och var förutom kollagen-inducerad artrit även mottaglig för spontan utveckling av artrit. Faktum är att *Ncf1*-muterade möss även uppvisade ökad mottaglighet för MOG protein-inducerad experimentell allergisk encefalomyelit (EAE) som är en djurmodell för multipel skleros (MS)[#]. Kanske har mutationer i *Ncf1* betydelse för uppkomst av ytterligare autoimmuna sjukdomar där kroppens immunförsvar angriper kroppsegen vävnad.

* RA drabbar ca: 1 % av befolkningen och är en autoimmun sjukdom där kroppens eget immunförsvar angriper och förstör leder. Vad som orsakar denna rubbning i immunförsvaret är ännu oklar men vi vet att både genetiska (arv) och icke-genetiska (miljö) faktorer bidrar till sjukdomsförloppet.

[#] MS drabbar ca: 0,1-0,2 % av befolkningen i nordiska länder och i Nordamerika, och är en autoimmun sjukdom där kroppens eget immunförsvar angriper och förstör det proteinskal (myelin) runt nervtrådar som påskyndar nervimpulser. Både genetiska och icke-genetiska faktorer bidrar till sjukdomsförloppet.

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