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**IDENTIFICATION OF GENES THAT REGULATE
ARTHRITIS AND IgE PRODUCTION
IN RAT AND HUMAN**

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ABSTRACT

Multiple genome-wide scans have identified quantitative trait loci (QTLs) that influence inflammatory diseases on chromosome 4q42 in DA rats. Several of the QTLs overlap with the QTL designated *Oia2* (Oil-induced arthritis-2), which was originally identified in a genome-wide linkage analysis in oil-induced arthritis (OIA), a model that shows similarities to rheumatoid arthritis (RA) in human.

When initiating this thesis, the major candidate disease gene(s) within *Oia2* was the natural killer cell gene complex (*NKC*), because the DA *NKC* encodes aberrant NK cell-mediated natural toxicity that could be suspected to influence arthritis and other immunopathologies.

The main aim with this thesis was to identify genes within *Oia2* that regulate immunopathologies, as this could provide new knowledge of disease pathways leading to RA and possibly other inflammatory conditions in human.

Congenic and subcongenic strains were established by transfer of chromosomal intervals covering the *Oia2*-region, between DA rats and arthritis-resistant MHC-identical PVG.1AV1 and LEW.1AV1 rats. A novel congenic strain DA.C4R3(PVG), harboring PVG alleles in a 16.8 Mb *Oia2*

interval on DA background, deviated from DA in both arthritis susceptibility and total serum IgE levels. It was resistant to OIA and showed protection against arthritis induced by both collagen type II (CIA) and squalene (SIA). Serum levels of IgE in naïve DA.C4R3(PVG) were increased compared to DA. Genetic fine-mapping in 16 congenic and subcongenic strains in oil-induced arthritis demonstrated that the *NKC* is situated outside the arthritis-regulating region, thus excluding *NKC* as being *Oia2*. Rats made congenic for *Oia2* in a 1.44 Mb region, and designated DA.C4R11(PVG), were resistant to OIA and had lower levels of IgE compared to DA. This suggests that the larger 16.8 Mb interval in DA.C4R3(PVG) contains more than one gene that regulates serum IgE levels, which is a phenotype that may correlate with asthma. Subsequently, an *Oia2* congenic strain was produced, DA.C4R17(PVG), which was arthritis resistant, and which defines a C-type lectin-like receptor gene complex, designated Antigen Presenting Lectin-like receptor gene Complex (APLEC). Sequencing of the APLEC cDNAs from DA and PVG.1AV1 revealed a nonsense mutation in the gene for Dendritic Cell Activating Receptor 1 (*Dcar1*) in the DA rat, and RT-PCR displayed significantly reduced mRNA levels of *Dcar1* in lymph nodes of DA.

In humans, the homologous APLEC genes located on 12p13 were tested in case-control materials. Frequency comparisons of single nucleotide polymorphisms (SNPs) of the Dendritic Cell Immunoreceptor (*DCIR*), which is closely related to rat *Dcar1*, revealed association with RA as well as with total IgE-levels in atopic asthma. In addition, the *DCIR* was also associated with

forced expiratory vital capacity (FVC), which demonstrates influence on asthma severity.

In conclusion, genes encoded from *APLEC* influence both arthritis and total IgE-levels in both humans and rats.

Keywords: RA, experimental animal model, QTL, congenic strain, *APLEC*, asthma, total IgE

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LIST OF ABBREVIATIONS

AGP	α -1 acid glycoprotein
AIA	Adjuvant-induced arthritis
APC	Antigen-presenting cell
APLEC	APC lectin-like receptor gene complex
APLR	APC lectin-like receptor
AvIA	Avridine-induced arthritis
BAC	Bacterial artificial chromosome
BcR	B cell receptor
BDCA-2	Blood DC antigen-2
BN	Brown Norway (an inbred rat strain)
CCP	Cyclic citrulline peptide
cDNA	Complementary DNA
CFA	Complete Freund's adjuvant
CIA	Collagen-induced arthritis
CII	Collagen type II
CLECSF	C-type lectin super family
cM	centiMorgan
COMP	Cartilage oligomeric matrix protein
CpG	Cytosine-Phosphate-Guanosine
CRD	Carbohydrate recognition domain
DA	Dark Agouti (an inbred rat strain)
DASH	Dynamic allele-specific hybridization
DC	Dendritic cell
DCIR	DC immunoinhibitory receptor
DCAR	DC immunoactivating receptor
Dectin-2	DC-associated C-type lectin-2
DLEC	DC lectin
DNA	Deoxyribonucleic acid
EAE	Experimental Autoimmune Encephalomyelitis
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FEV1	Forced expiratory volume per liter
FVC	Forced expiratory vital capacity
GPI	Glycose-6-phosphatase isomerase
d.p.i	Days post-injection
HEV	High endothelial venule
HLA	Human leukocyte antigen
IFA	Incomplete Freund's adjuvant
Ig	Immunoglobulin
IL	Interleukin
ITAM	Immunoreceptor tyrosine-based activation motif

ITIM	Immunoreceptor tyrosine-based inhibitory motif
kb	Kilobasepair(s)
LD	Linkage disequilibrium
LEW	Lewis (an inbred rat strain)
LINES	Long interspersed nuclear elements
LOD	Logarithm of odds ratio
LPS	Lipopolysaccharide
mAb	Monoclonal antibody
Mb	Megabasepair(s)
MCL	Macrophage C-type lectin
MDP	Muramyl dipeptide
MIA	Mycobacteria-induced arthritis
MHC	Major histocompatibility complex
Mincle	Macrophage-inducible C-type lectin
MOG	Myelin Oligodendrocyte Glycoprotein
mRNA	Messenger ribonucleic acid
MØ	Macrophage
MS	Multiple Sclerosis
MMU6	<i>Mus musculus</i> chromosome 6
NADPH	Nicotinamide adenine dinucleotide phosphate
Ncf1	Neutrophil cytosolic factor 1
NKC	Natural Killer (gene/cell) complex
NO	Nitride oxide
OIA	Oil-induced arthritis
PAC	Plasmid artificial chromosome
PCR	Polymerase chain reaction
PIA	Pristane-induced arthritis
PVG	Piebald Virol Glaxo (an inbred rat strain)
RNO4	<i>Rattus norvegicus</i> chromosome 4
QTL	Quantitative trait locus / loci
RA	Rheumatoid arthritis
RF	Rheumatoid factor
RT-PCR	Real time PCR
RT1	<i>Rattus norvegicus</i> MHC
SF	Synovial fluid
SIA	Squalene-induced arthritis
SINES	Short interspersed nuclear elements
SLE	Systemic Lupus Erythematosus
SNP	Single nucleotide polymorphism
SSLP	Simple sequence length polymorphism
TcR	T cell receptor
Th	Helper T cell
TLR	Toll-like receptor
TNF- α	Tumor necrosis factor- α

PREFACE

The twentieth century has experienced a tremendous amount of technical innovations and great progress within medicine development. However, after the millennium, the struggle is ongoing in the aim to find cures for multifactorial inflammatory diseases, such as multiple sclerosis, systemic lupus erythematosus, asthma and rheumatoid arthritis. Improvements have been made to alleviate the diseases, but we still have limited knowledge of their causative factors. The genetic and environmental factors need to be delineated in order to understand and possibly interfere with the biochemical pathways involved in rheumatoid arthritis.

In this thesis, I present the results of the search for genes involved in arthritis development, and which could also influence other immunopathologies.

The thesis frame contains an introduction to the immune system, focusing on the autoimmune response and rheumatoid arthritis. This is followed by an account of experimental animal models for RA and the gene mapping strategy, which led to the identification of a gene complex in which *DCIR* was shown to be associated with both rheumatoid arthritis and atopic asthma in humans.

My wish is that my research one day in the future can contribute, if only to a small extent, to solutions of how to abrogate, and most of all, prevent the onset of rheumatoid arthritis as well as other inflammatory diseases.

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INTRODUCTION

Activation of the immune system - for good and bad

All vertebrates have in common a delicate defense system to evade harmful attacks from infectious agents, the immune system. The mechanisms of the immune system are highly regulated for optimal, effective elimination of foreign invaders or pathogens (i.e. bacteria, virus, fungi and parasites), with minimal harm of the individual.

The immune system is generally divided into two kinds of responses; the innate, non-specific, immune response, and the adapted, antigen-specific immune response. However, both responses are dependent on each other and act in concert. The innate immune mechanisms act immediately, by help of the complement system and so-called phagocytic and cytotoxic cells, which recognize the pathogens as nonself, and either digest them or secrete fatal toxins. The pathogen-recognizing receptors on these cells have been strongly conserved during evolution, and bind to specific structures that are common to many pathogens. Thus these receptors are also known as pattern-recognition molecules.

The adaptive immune response involves antigen-specific effector cells and memory cells, which have been activated by antigen-presenting cells (i.e. macrophages, dendritic cells and B cells), and chemoattractant-secreting cells (mainly macrophages and neutrophils). The innate response acts in parallel and participates in the removal of pathogens which have been targeted by the adaptive immune response.

Autoimmunity

The initiation of autoimmunity occurs when tolerance to a self-antigen is broken. In a healthy individual there are always some cells that have receptors which bind to self-antigen. As long as these interactions are weak and transient, these cells cause no harm. But when the binding is irreversible and triggers long-lasting damage to tissues, an autoimmune reaction has occurred. Autoimmune diseases embrace a large group of disorders which are described by having detectable autoantibodies or autoreactive T lymphocytes.

The reasons for development of autoimmunity are unclear, but most probably several factors need to coincide to initiate an autoimmune response. The genetic predisposition as well as the environment both influence the

homoeostasis of the immune system. An ongoing infection and / or a non-autoimmune disease can act as a trigger for autoimmunity. Molecular mimicry has also been suggested as a trigger for autoimmunity, and where peptides shared by pathogen and host activate autoantibodies or T cells to crossreact with self antigens¹⁻⁴.

Autoimmune diseases are often due to variations in genes coding for molecules involved in the regulation in the immune system. A conformational change of a protein structure may also cause induction of an immune response, and has been reported for citrullinated proteins⁵⁻⁷. The genes coding for the major histocompatibility complex (MHC) are of great importance, and some MHC haplotypes (i.e. genotypes), also known as the human leukocyte antigen (HLA) genotypes, are more common than others in autoimmune diseases. Other genetic polymorphisms that can contribute to susceptibility have been reported for the genes of the T cell immunoglobulin and mucin-domain-containing (TIM) proteins^{8,9} and the autoimmune regulator (AIRE)¹⁰. Additional immunoregulatory molecules that may influence disease susceptibility are co-receptors of the T cell receptor (TCR), the inhibitory co-receptors CTLA-4^{11,12} and PD-1¹³⁻¹⁵, and positive regulatory co-receptor CD28¹⁶, and the inducible co-stimulator ICOS^{17,18}.

The autoimmune response most often causes severe and sometimes even fatal damage to the individual and acts locally, as in multiple sclerosis (MS) and primary Sjögren's syndrome or systemically, as in systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA).

Rheumatoid arthritis

Rheumatoid arthritis is a chronic, systemic autoimmune disease characterized by symmetrical inflammation of the peripheral joints, cartilage and bone erosion. The disease causes severe pain, stiffness and functional disability. Patients with RA have premature mortality (10-15 years) in comparison with the general population^{19,20}, and about 50% of the deaths are due to cardiovascular causes such as myocardial infarction^{21,22}.

The prevalence of RA is about 0.5-1% in western populations²³⁻²⁵ and is two-to-three times more frequent in women compared to men. The disease is multifactorial and more like a syndrome comprising several diseases. The etiology is for the most part unknown, but is certainly caused by both genetic and environmental factors. RA is known to be associated with the MHC and is suggested to be caused by TH₁ cells specific for an as yet unidentified antigen.

There is still no cure for RA, but there are several treatments which help to improve life quality and prevent functional disability. Among them are NSAIDs (Non-Steroidal Anti-inflammatory Drugs, e.g. Cox-2-inhibitors), DMARDs (Disease Modifying Anti-Rheumatic Drugs, e.g. enzyme inhibitors) and cytokine inhibitors (e.g. anti-TNF and anti-CD20 agents). Glucocorticosteroids are used as a general anti-inflammatory treatment.

At least four of seven criteria must be satisfied for an RA diagnosis (Table I), according to the 1987 revised criteria of the American Rheumatism Association (ARA), and where criteria 1-4 must be present for at least 6 weeks²⁶. However, these criteria cannot be used to identify patients with early RA.

Table I. 1987 revised criteria for rheumatoid arthritis classification, set by the American College of Rheumatology (ACR). Adapted and modified from Arnett et al. *Arthritis Rheum*, 1988²⁶

1. Morning stiffness
 2. Arthritis of three or more joint areas
 3. Arthritis of hand joints
 4. Symmetric arthritis
 5. Rheumatoid nodules
 6. Serum rheumatoid factor
 7. Radiographic changes
-

Pathology of RA

Local manifestations

The target organ in RA is the synovium (or synovial membrane) (Fig. 1), which is a soft connective tissue lining the closest spaces of diarthrodial (synovial) joints, tendon sheaths and bursae. The synovium is built up by two-layers of so-called synoviocytes; the intima, the lining layer containing fibroblast-like cells and macrophage-like cells, and the adventitia, the sublining layer, containing blood vessels, fat and monocytes.

Early RA is characterized by synovitis (inflammation of the synovium), where the synovium is thickened because of expansion of inflammatory synovial lining cells

in the presence of edema, deposition of fibrin and vascular proliferation of so-called high endothelial venules (HEVs)^{27,28}. This is followed by an infiltration of mostly CD4⁺ T cells, but also CD8⁺ T cells, B cells, dendritic cells and some macrophages²⁹, and which is facilitated by the HEVs²⁸. These cells produce proinflammatory cytokines (TNF α , IL-1, IL-6), which attract macrophages and polymorphonuclear cells, primarily neutrophils, to accumulate in the synovial fluid and on the cartilage surfaces. Recently, macrophages in synovial fluid of RA patients, as well as in rats in experimental arthritis, were found to secrete high amounts of a proinflammatory molecule designated HMGB-1^{30,31}. In addition, polykaryons, so-called multinucleated giant cells, of macrophage and /or synoviocyte origin, have been reported in the synovium³².

The expansion of synovial lining cells that extends to the cartilage and bone, and the infiltration of inflammatory cells and immune complexes leads to a pannus formation (Fig. 1). An increase of the synovial fluid volume within the joint is also evident, and hyaluronan, an important component for cartilage function, is abundant in both the synovium and in the synovial fluid.

The inflammation leads to articular pain and swelling and the inflammatory cells cause degradation of the cartilage and bone tissue.

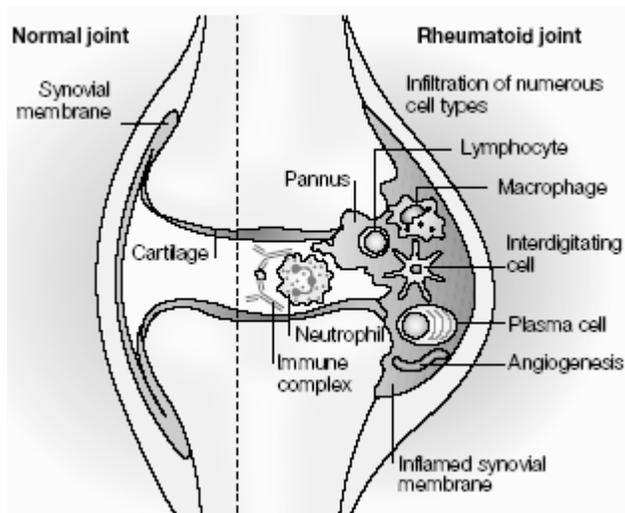


Fig. 1. Comparison of normal (left) and rheumatoid joint (right). Adapted from Feldmann, *Nature Reviews Immunology* 2002³³.

Extra-articular manifestations

Besides local inflammation, systemic abnormalities are common in patients with severe RA. Some extra-articular manifestations are rheumatoid nodules, caused by fibrinoid necrosis as a result of small vessel vasculitis, secondary Sjögren's Syndrome, vasculitis, lymphadenopathy and neuropathy. High titers of different

autoantibodies are common in RA patients. About 75% of all RA patients are seropositive for Rheumatoid Factor (RF), where the most common RF is IgM against the Fc-portion of IgG. However, RF is highly non-specific, since it also occurs in other autoimmune diseases such as Sjögren's syndrome, infectious diseases, as well as in the healthy population (3-5%) in which titers increase with age. Other autoantibodies are ANA (antinuclear antibody) and the more specific anti-cyclic citrullinated peptide antibody (anti-CCP). Antibodies against citrullinated peptides are for example APF (anti-perinuclear factor), AKA (anti-keratin antibodies), AFA (anti-filaggrin antibodies) and anti-citrullinated collagen type I antibodies⁵⁻⁷.

Moreover, there is evidence for association of a dysfunctional hypothalamo-pituitary-adrenal (HPA) axis, an essential component of the neuroendocrine response to stress and which is important for the maintenance of homeostasis, with RA patients suffering from chronic pain. This feature has been confirmed in rat models of arthritis³⁴⁻³⁶.

Etiology of RA

The disease etiology is to the most part unknown. Both genetic and environmental factors influence disease development, and most probably they act in concert. A classic approach for illustrating the influence of genetic and environmental risk factors in RA are twin studies, for which a higher concordance rate in monozygotic twins in comparison to dizygotic twins corresponds with inherited determinants. The classification criteria of RA may limit the estimation of environmental risk factors since it for example does not take into account whether the patients are positive or negative for RF, which seem to have different profiles of risk factors.

Environmental risk factors

There are several rather than one single determinant causing RA. Most of the suggested risk factors have not been verified or are not convincing. To date, smoking is the only firmly verified environmental risk factor for RA³⁷⁻⁴¹. A recent study demonstrates that smoking over a long period of time (>20 years) was needed to significantly increase the disease risk⁴¹. Cigarette smoking is also associated with rheumatoid factor, both in RA patients and healthy individuals. Furthermore, hydraulic oils have been suggested as risk factors for RA⁴², and

there is strong evidence that exposure to silica^{43,44} increases the relative risk for RA.

Non-genetic host factors, in the form of hormones, may influence RA susceptibility. However, it is not clear whether the hormonal changes are a result of RA development or if the hormonal changes contribute to the development of RA. Oestrogen has an alleviating and sometimes a preventing effect on arthritis, both in laboratory animals and in humans⁴⁵⁻⁴⁸. Male sex hormones, particularly testosterone, are generally lower in men who have RA⁴⁹, whereas levels of female sex hormones do not differ between RA patients and controls⁵⁰. The hormone prolactin, important for the production of breast milk, increases during pregnancy and is suggested to act as a proinflammatory mediator as reports indicate that exposure to breastfeeding and women who breastfed after their first pregnancy are at the greatest risk^{51,52}. However, it should be emphasized that breastfeeding can be a co-variate with very many immunological changes during this period.

Exposure to infection (i.e. Epstein-Barr virus, parvovirus and some bacteria such as *Proteus* and *Mycoplasma*) may also act as disease trigger⁴⁹. Finally, socio-economic factors or occupation may also be of importance for disease susceptibility.

Genetic factors

The genetic influence in RA has been demonstrated in twin studies, in which the concordance rate in monozygotic twins is higher than that in dizygotic twins, 12–15% and 3–4%, respectively^{53,54}. Nevertheless, the concordance between twins is dependent on the prevalence of disease. In other words, the concordance increases as the population prevalence increases. A suitable way to assess heritability is to make a series of assumptions of genetic and environmental factor sharing between twin types. An attempt to take this strategy into account has been applied in a study using data from both English and Finnish mono- and dizygotic twins, and which suggests that approximately 50–60% of the prevalence of RA in twins is explained by shared genetic effects⁵⁵.

Various reports demonstrate that the occurrence of RA differs geographically. The native American-Indian populations have the highest recorded presence of RA, with a prevalence of 6.8% among the Chippewa Indians⁵⁶ and of 5.3% among the Pima Indians⁵⁷. On the contrary, populations in south east Asia have

a very low incidence, with the lowest reported occurrence in China and Japan (0.2-0.3%)^{58,59}.

The fact that the prevalence of RA varies between different populations supports the existence of genetic risk factors, that they are important and explain differences in disease risk. It also reflects the fact that these populations have different life habits, diet, smoking frequency etc., and that these environmental conditions may influence susceptibility to RA.

The genetic background for RA is complex, involving genes both within and outside the MHC. About 30% of the genetic factors are contributed by alleles of the MHC on chromosome 6^{60,61}. The HLA (Human leukocyte antigen) DRB1 alleles have been known as a risk factor of RA for almost 30 years, where the HLA DRB1*0401 and HLA DRB1*0404 alleles are much stronger susceptibility factors than those of HLA DRB1*0101⁶². In addition, individuals who are heterozygous for the genotype HLA DRB*0401/*0404 have a considerably greater risk than individuals who carry single HLA DRB*0101 alleles. However, there are indications that the relationship between HLA and RA is related to severity as well as to susceptibility for RA⁶² or it may be related to interactions between HLADRB1 and environmental factors⁶³.

All the DRB1 alleles that are associated with RA have in common a conserved amino-acid sequence (QKRAA, QRRAA), designated "the shared epitope" (SE), which is located in the third hypervariable region⁶⁴. A suggested hypothesis for the onset of RA has been the molecular mimicry, since sequence homology between the SE and prokaryotic sequences from Epstein-Barr virus and *Escherichia coli* exists⁶⁵.

Those individuals who are homozygous for SE genes have a greater risk for developing RA than those who carry only one copy. There are indications that SE in combination with smoking⁶³, rheumatoid factor and TNF α (Tumor Necrosis Factor)⁶⁶ increases disease risk. A recent study reported that presence of anti-citrulline antibodies together with SE gene carriage is strongly associated with future onset of RA⁶⁷.

The non-MHC genes are not clearly identified. Several candidate genes have been investigated, but the results are controversial. However, confirmation has recently been made for the association between RA and a functional single-nucleotide polymorphism (a substitution in Arg620Trp) located in the coding region of *PTPN22* (intracellular protein tyrosine phosphatase nonreceptor 22)⁶⁸⁻⁷⁰. *PTPN22* encodes for lymphoid-specific phosphatase (Lyp), which is a suppressor of T cell activation⁷¹. The polymorphism leads to disruption of Lyp in the binding

to C-terminal Src kinase (Csk), which exerts an inhibitory function on the TCR. *PTPN22* has also been reported to be associated with SLE⁷² and type I diabetes^{73,74} and is thus constitutes an example of a gene with regulatory effects in several autoimmune diseases. One more example of such a gene may be *MHC2TA* (MHC class II Transactivator), which has been shown to be associated with RA, MS and myocardial infarction⁷⁵. In addition, corticotrophin releasing hormone (CRH)⁷⁶, SEC8L1⁷⁷ (a component of the exocyst complex), CYP19 (also known as aromatase or oestrogen transferase)⁷⁸, IFN- γ ^{79,80} and other cytokines (such as IL-10, IL-2 and IFN γ ⁸¹⁻⁸³) have been reported to be associated with RA.

In order to identify disease-linked chromosomal region harboring candidate genes, a common method is the genome scan using microsatellite markers for linkage to RA, and which will be further described in following sections. Four such genome scans have been performed in RA⁸⁴⁻⁸⁷. In addition, three replication scans have been performed⁸⁸⁻⁹⁰.

Another strategy is to use results and genome screens of animal models of arthritis to see whether syntenic regions are also linked to RA in humans. In such a study linkage was suggested to 17q22 ($P < 0.001$)⁹¹, which corresponds to the arthritis-linked QTLs *Oia3*⁹² and *Cia5*⁹³ in the rat. The *Oia3* was identified in a genome-wide scan in the rat using oil-induced arthritis (OIA), an experimental model that shows similarities to RA.

Experimental animal models

The complexity and polygenicity of the RA has hampered understanding of the underlying disease mechanisms and the identification of disease-linked genes. The use of experimental animal model enables reproducible studies under controlled experimental conditions, and are valuable for identifying susceptibility genes and biochemical pathways leading to disease. However, it should be emphasized that an animal model may only represent one of several possible ways of developing arthritis. Nevertheless, it enables mapping of quantitative trait loci (QTLs) that regulates traits such as incidence, severity, day of onset of the disease, gender differences, changes in serum (i.e. acute phase response, antibody production). Furthermore, animal models facilitates profound studies of biochemical processes such as inflammation, erosion of bone and cartilage.

Animal models for RA

There are several animal models for RA, most being rodent models. The arthritis is either spontaneous or requires induction with external stimuli. Animals that develop spontaneous models are for example the MRL-lpr/lpr mice⁹⁴ and the K/BxN mice⁹⁵, originally a cross between a T cell receptor transgenic line and the NOD (Non-Obese Diabetic) strain, that produce antibodies directed against the ubiquitous antigen glucose-6-phosphate isomerase (G6PI). Other spontaneous rodent models occur in two rat strains that are transgenic for genomic clones of HLA-B27 and its associated light chain human β_2 -microglobulin, these having been established on inbred LEW and on F344 rat strain backgrounds, respectively⁹⁶.

Interestingly, many loci identified in RA models are also found in other models of autoimmune diseases. Moreover, susceptibility loci for arthritis in the rat have also been shown to link with arthritis in mice as well as in humans, and which will be demonstrated in the papers of this thesis.

Rats have several advantages in arthritis models in comparison with mice. They are more sensitive to induction of arthritis similar to that of RA, and the inflammatory response is better documented and the larger size of the rat facilitates clinical examinations. Also, the fact that the rat, in contrast to the mouse, can develop arthritis not only after injection with antigen but also by injection with non-antigenic so-called adjuvant alone (lat. Adjuvare – to help), enables parallel studies of different disease pathways using the same strain.

The following sections describe arthritis models in the rat and are focused on models used in this thesis.

Arthritis induced by cartilage-derived proteins (CIA, C^{XI}IA, COMPIA)

Three different cartilage-derived proteins in combination with adjuvant have been demonstrated to induce arthritis in rats: Collagen type II (CII), collagen type XI (CXI) and cartilage oligomeric matrix protein (COMP).

Collagen-induced arthritis (CIA) was established as a model nearly 30 years ago⁹⁷, through injection with CII mixed in CFA. Today, CIA is generally induced by CII mixed in IFA. The rats develop a severe, erosive polyarthritis 2-3 weeks after injection with autologous CII, followed by a chronic relapsing disease course⁹⁸⁻¹⁰⁰. The disease has a female preponderance¹⁰¹, with marked bone and cartilage erosions and presence of enthesopathy and new bone formation⁹⁷. T cell infiltration into the joints is barely significant. High levels of circulating

COMP¹⁰² and a significant presence of anti-CII antibodies^{99,100} are also associated with CIA.

CXI-induced arthritis (C^{XI}IA) resembles CIA, but has a more aggressive chronic disease course and has a male preponderance. In addition, presence of B cell clusters are seen in the joints.

Cartilage oligomeric induced arthritis (COMPIA) is an acute, non-chronic disease with presence of anti-COMP antibodies¹⁰³. No gender preponderance has been recorded and bone and cartilage are left intact.

Recently, increased immunogenicity of CII by chlorination with hypochlorous acid (HOCl) in IFA has been reported to increase arthritogenicity in the LEW.1AV1 rat strain, whereas non-chlorinated CII in IFA alone did not induce arthritis in this strain¹⁰⁴. The same research group has also demonstrated that citrullinated CII increase immunogenicity and arthriticity, and where LEW.1AV1 rats, injected with citrullinated CII in IFA, developed a disease with higher incidence and earlier onset than LEW.1AV1 rats injected with unmodified CII in IFA¹⁰⁵.

Mycobacteria-induced arthritis (MIA)

The mycobacteria-induced arthritis (MIA) model is also known as classical adjuvant-induced arthritis (AIA). In 1954, Storck demonstrated that rats develop joint erosions after injection with Freund's complete adjuvant (CFA)¹⁰⁶, which contains heat-killed mycobacteria in the emulsifying oil incomplete Freund's adjuvant (IFA). Two years later MIA was established as the first model for arthritis¹⁰⁷. MIA is not joint-specific¹⁰⁸. The disease is severe with a female preponderance, causing swelling of joints, bone and cartilage erosions, enthesopathy (inflammation and tenderness where the tendons meet the bones) and new bone formation¹⁰⁹. Pronounced inflammatory T cell infiltrates¹¹⁰ and granulomas (formation of a core of infected macrophages surrounded by activated lymphocytes) in several organs leading to necrosis (tissue degradation) are also evident. However, MIA is non-chronic and the rats recover within a few months.

Oil-induced arthritis (OIA)

Oil-induced arthritis (OIA) shows phenotypical similarities with RA, but has a milder disease course than that of the auto-antigen-induced arthritis models. OIA is induced by injection of the mineral oil IFA. Mineral oil is commonly used together with antigen in order to enhance the immune response (i.e. antibody production) after a primary injection, or to boost the immune response in repeated injections. However, injection of IFA was found to cause arthritis in the extremely disease-prone DA rat¹¹¹. The mineral oil contains no antigenic peptides and is made up of 85% non-metabolizable mineral oil (liquid paraffin oil Bayol F) and 15% mannide monooleate Arlacel[®] A, which functions as a surfactant. These components are separately sufficient to induce arthritis in the DA rat¹¹¹. Interestingly, repeated injection of IFA after recovery from a first injection can not induce a second inflammation¹¹¹. The cause of this phenomenon is unknown.

OIA is an acute, monophasic disease with symmetric inflammation of peripheral joints. Disease onset is about 12-14 days post-injection (d.p.i.). There is no significant gender preponderance, although the males have a slightly more severe disease course than females¹¹². Bone and cartilage erosions are rare and mild^{111,113}. T cell infiltrations occur at a low level in the joints¹¹³. Passive transfer of T cells from arthritic lymph nodes induces a CD4⁺ TH₁-cell dependent arthritis in naive, irradiated DA rats^{114,115}.

Squalene-induced arthritis (SIA)

The squalene-induced arthritis (SIA) model is induced by injection of the shark-liver-oil-derived squalene (C₃₀H₅₀). Squalene is an endogenous cholesterol precursor present in all mammals. The adjuvant also occurs as a component in human vaccines. Anti-squalene antibodies have, in some cases, been reported in individuals who have received anthrax vaccine^{116,117} and in the blood of Gulf War era veterans^{118,119}, of which some developed GWS (Gulf War Syndrome), characterized by muscle and joint pain, fatigue, skin rash and gastrointestinal and respiratory symptoms¹²⁰.

The disease course of SIA is similar, but slightly more aggressive, than that of OIA. Bone and cartilage erosions are clearly present, as well as T cell infiltration in the joints¹²¹. Furthermore, squalene mostly accumulates in hyperplastic lymph nodes draining the injection site, and not in the joints, and most of the adjuvant is stored within cells rather than in the ECM¹²².

In addition to the DA rat, the MHC-identical rat strain LEW.1AV1 also develops arthritis, although a very mild form. As in OIA, passive transfer of arthritic lymph node cells induces a CD4⁺ T cell-dependent arthritis in naive, irradiated DA rats¹²³. The recipients also showed an increase of the pro-inflammatory cytokines IL-1 β and IFN γ just before onset of disease. A female preponderance has been reported for LEW.1AV1, but not for DA rats¹²¹.

Pristane-induced arthritis (PIA)

Pristane-induced arthritis (PIA) is the most aggressive model of adjuvant-induced arthritis and fulfills almost all the criteria for RA. The arthritis model is induced by the shark-liver-oil-derived pristane (*pristis* lat. shark) (C₁₉H₄₀), a lipid component of chlorophyll which is present in normal tissue (including thymus and lymphoid peripheral tissue) in vertebrate animals^{124,125}. PIA has a chronic relapsing disease course with a female preponderance, and is characterized by pronounced bone and cartilage erosions¹²⁶. Similar to OIA and SIA, PIA involves symmetric inflammation of the peripheral joints¹²⁷. T cell infiltration in the joints¹²⁶ and high levels of circulating COMP¹²⁸ are also present. In common with OIA and SIA, no antibodies for cartilage antigens have been reported for PIA. Enthesopathy and new bone formation are clearly present. In contrast to OIA and SIA, PIA also includes presence of RF. The disease onset is slightly later in PIA than in SIA or OIA.

In addition, there are several other adjuvants that can cause joint inflammation, such as the synthetic lipoidal amine avidine (CP-20,961)^{129,130} and different linear hydrocarbon compounds¹³¹ such as the lipids hexadecane (C₁₆H₃₄) and heptadecane (C₁₇H₃₆).

Pathology of adjuvant-induced arthritis in rat

The induction of joint inflammation in experimental arthritis is normally induced by one single intradermal injection of an antigen and/or adjuvant. Depending on agent and strain of rat, the model shows at least some of the following characteristics¹³²:

- Tissue-specific disease, affecting primarily diarthrodial, cartilaginous peripheral joints. Systemic manifestations may occur.

- The inflammation can not be explained by any infectious agents
- Bone and cartilage erosion
- Chronicity
- Association with certain MHC haplotypes
- The disease is non-spontaneous, and induced by agents in rats that harbor susceptibility alleles (Box 1). Rats lacking these alleles are partly or completely resistant.

Mapping of genes in experimental arthritis

Genetic diseases

A genetic disease can either be monogenic, caused by a single gene, or polygenic, involving more than one gene. Single gene defects are referred to as Mendelian disorders (single locus disorders, which either act in an autosomal dominant, autosomal recessive or X-linked way). The gene defect (i.e. mutation) may be inherited from parents to children or may occur spontaneously. The mutation will then exist in every cell of the child. The mutated gene can either code for a dysfunctional or nonfunctional protein, or it can become a pseudogene, not able to produce any protein at all. Examples of monogenic diseases are cystic fibrosis, phenylketonurea and the X-linked disorder Hemophilia.

In contrast, polygenic diseases are more complex in that they involve several genes and often epistatic interactions, as well as interactions between genetic and environmental factors. In polygenic diseases each of these genes are assumed to work independently, but cause the same phenotype. In the population there may be a sum of these different monogenic diseases as a complex disease with subsequent problems with identification of a single gene in this pool of diseases. These circumstances hamper the identification of disease-causing genes. In addition, penetrance is incomplete (<<100%) in complex diseases in comparison to that of monogenic diseases (~100%), reflecting different mechanisms underlying genetic influence. The genes involved in complex diseases are referred to as susceptibility genes rather than as causative genes, since it is most unlikely that one single gene can directly be responsible for the induction of a complex disease.

The following sections will describe genetic approaches to dissecting complex diseases.

Approaches in gene mapping in human complex diseases

The recent completions of the sequence of the human^{133,134}, mouse¹³⁵ and rat¹³⁶ genomes offer new opportunities for gene candidate approaches and positional cloning, for example *in silico* analysis of DNA by use of web-based tools. This progress in sequencing has and will greatly reduce the work with candidate gene approaches and other approaches, since you know better where the genetic markers (Box 1) are. However, traditional approaches in gene mapping are still necessary in the initial steps.

For an unbiased approach, the mapping of genes that affect disease susceptibility in humans is initiated by a genome-wide scan in order to find quantitative trait loci or QTLs (Box 1), that are linked with the disease phenotype. The following steps in the mapping process include narrowing the QTL, which harbors the susceptibility gene/genes of interest to a small enough size (usually less than 2 Mb) to initiate positional cloning (Box 2) and sequencing of the candidate gene of interest.

The classical genome-wide analysis has two different approaches. The first method is *linkage analysis*, which is either genetical model-dependent, such as the lod score analysis (Box 2), or genetical model-independent, such as the sib pair method (Box 2). The linkage analysis in humans often lacks statistical power, since it is difficult to obtain enough large pedigrees of closely related individuals. This circumstance results in low statistical power for detecting weak effects¹³⁷ and low mapping resolution, which hampers gene identification even after a region has been detected¹³⁸. A more modern and increasingly used approach in genome scans is the SNP (Box 1) genome-wide scan, which makes it possible to perform genome scans at very high resolutions.

The second method is the *allelic association study*, one of the most common ways of identifying disease-linked genes of complex diseases. The association study is either based on case-control studies, with comparisons of allele (i.e. single nucleotide polymorphism) frequencies in a set of unrelated affected individuals to a set of controls, or is family-based, comparing the frequencies of alleles of A with frequencies for alleles of B transmitted to the affected child to the alleles not transmitted. The allele frequencies are most often based on frequencies of SNPs, where the SNPs could either be close to or within the gene of interest.

A variant of the association study is the admixture mapping¹³⁹ (Box 2) of genetically distinct populations has recently experienced a renaissance, where hypertension loci in the African American population were identified¹⁴⁰. This mapping approach uses samples from recently admixed populations to identify susceptibility loci in order to detect "ancestry association" where the risk alleles have different frequencies in the original contributing populations. However, this method has the disadvantage of the risk of generating false-positive associations when cases and controls are not properly matched¹⁴¹.

Generally, it should be emphasized that the genome scan methods in humans are limited in that they are difficult to replicate, because of weak statistical power and polygenicity¹⁴². Although several loci have been identified through genome-wide scans^{84,85,88,90,143,144}, and of which several are overlapping with loci in other inflammatory diseases, such as SLE, MS and type I diabetes^{145,146}, only one gene has been verified¹⁴⁷. This gene is the earlier described *PTPN22*, the gene for the protein tyrosine phosphatase Lyp, which is associated with both RA⁶⁸ and SLE⁷², and is essential for down-regulation of T cell responses. The traditional microsatellite marker-assisted genome scans for loci involved in RA show, however, few overlaps between the different studies, which weakens the reliability of the results. In order to circumvent these problems it is convenient to perform genetic studies in animal models, which offer controlled experimental conditions (environment, nutrition, night and day cycle etc.), as well as large numbers of age- and sex-matched study populations.

Identification of QTLs in experimental arthritis

Genome-wide linkage analysis

Rodent models are common in genome-wide scans for disease-linked QTLs. Rats offer some advantages in comparison with mice in that some rat strains are extremely disease-prone to several arthritis-models induced by either antigen or adjuvant. This enables parallel studies using the same rat strain in different arthritis models involving different disease pathways.

Today, more than 40 arthritis-linked QTLs have been identified in MIA¹⁴⁸, CIA^{93,148-153}, PIA^{127,152,154,155}, OIA⁹² and SIA¹⁵⁶ by use of genetic marker-assisted genome-wide linkage analysis in the rat (Fig. 2). Several of these loci also overlap with loci in other inflammatory diseases^{85,86,91,157-173} in the rat, as shown in Figure 2. This indicates a general regulatory function of several QTLs in inflammation,

either in different biochemical pathways or as a component in a pathway common for various inflammatory conditions.

The interval mapping¹⁸³ is the most common method for identification of QTLs and which comprises testing for presence of the QTL between two mapped loci. The significance of linkage between these tested loci and the analyzed phenotype is calculated by a probability value expressed as a logarithm of odds ratio (LOD) (Box 1).

Linkage analysis of an arthritis-regulating QTL in the rat is performed in crosses between differentially arthritis susceptible strains. The QTLs are localized by use of multiple genetic markers (i.e. microsatellites¹⁸⁴, SNPs¹⁸⁵ or RFLPs¹⁸⁶) (Box 1) distributed over the genome, approximately every tenth cM. In order to find susceptibility loci located outside the MHC gene complex, the rat strains can be selected to be MHC-identical. Thus there will be no interfering regulatory effects from genes within the MHC. One commonly shared MHC haplotype is RT1^{av1}, which derives from the DA(RT1^{av1}) inbred strain, from now on simply designated DA.

There are three main alternative crosses used for linkage analysis in rat models. The first is an F1 backcross, where the progeny (F1) of a cross between a resistant and a susceptible inbred strain are backcrossed onto one of the parental strains (P). The second is an F2 intercross, where the progeny in F1 are intercrossed to produce an F2 generation. Each animal of the progeny of F1 backcross or the F2 intercross will have a unique genotype which enables identification of arthritis regulating QTLs. The third is the advanced intercross line (AIL)¹⁸⁷, which derives from two inbred strains that have been randomly intercrossed for several generations, thus giving rise to a very high number of recombinations. Here it is important to maintain a large number of breeding pairs, thus avoiding brother and sister mating to prevent homozygosity. This approach yields a higher mapping resolution. The statistical significance is normally estimated using a permutation test¹⁸². A recent report describes linkage analysis in a partial advanced intercross line (PAI)¹⁸⁸, an AIL approach based on a cross between congenic (Box 1) mice strains in CIA and EAE.

The most common linkage analysis is based on an F2 intercross or F1 backcross, where the F2 intercross enables a higher mapping resolution than the F1 backcross. The F2 intercross is more informative with regard to recessive alleles and alleles with additive effects from either of the parental strains, and this makes the possibility of finding QTLs greater in an F2 intercross than in an F1 backcross¹⁸⁹.

To sum up, the F1 backcross requires more animals in comparison with the F2 intercross in order to obtain similar results, but is more efficient (half amount of animals) when the goal is to detect only the QTLs. One of the most commonly used inbred rat strains in gene mapping studies of inflammatory diseases is the DA rat, which is extremely disease-prone to several experimental diseases and to

all experimental arthritis models. Figure 3 shows a schematic view of a genome-wide linkage analysis in an F2 intercross between DA and MHC-identical LEW.1AV1, and which resulted in the identification of the QTL *Oia2* on rat chromosome 4 (RNO4)⁹². The QTL *Oia2* was later reproduced in an F1 backcross between DA and PVG.1AV1¹⁹⁰ and became the central theme of this thesis, as described in Papers I-IV.

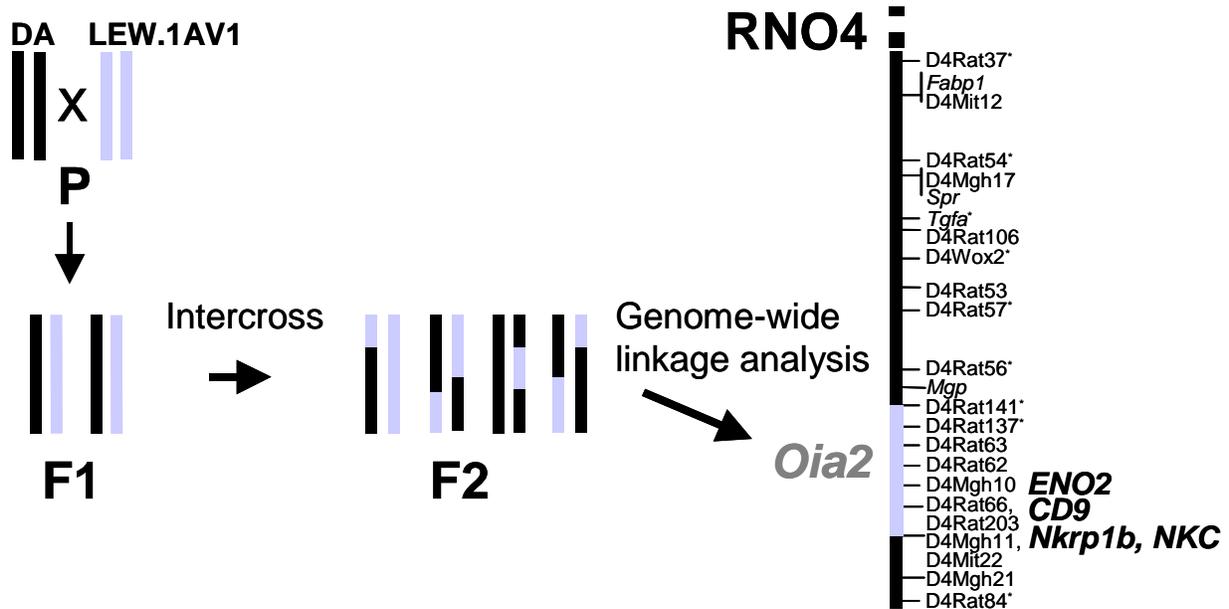


Fig. 3. Simplified procedure of genome-wide linkage analysis and which led to the identification of the non-MHC QTL *Oia2* on rat chromosome 4 (RNO4) in oil-induced arthritis (OIA). Aligned RNO4 are microsatellite markers that are polymorphic between the parental strains. *Oia2* was identified at the marker D4Mgh10 with a *P*-value of 4×10^{-13} . Candidate genes are given in bold italics. The original genome scan was performed in an F2 intercross between DA and LEW.1AV1⁹² and was replicated in a backcross between DA and PVG.1AV1¹⁹⁰.

High resolution mapping of a QTL in congenic strains

The QTL analysis comprises detection, map estimation and fine mapping. The strength of linkage signal, usually expressed as a “LOD score” (Box 1), of each QTL is calculated by statistical probability calculations. Computer-based software programs using hypothesis-testing algorithms such as MAPMAKER EXP/QTL^{183,191}, GENEHUNTER¹⁹² or R/qtl, enable the creation of a recombination linkage map of genetic markers (not in R/QTL), usually microsatellite markers. The R/qtl allows two-QTL linkage mapping.

Any identified QTL has to have a significant linkage¹⁸¹ between a disease trait and the chromosomal interval it has been linked to. In order to estimate

significance thresholds for each analyzed phenotype in a specific experiment it is often suitable to use a permutation test¹⁸², which is applied in R/qtl. After a statistical verification (threshold specific for the experiment) of a QTL, the next step is to reproduce the QTL in congenic strains, where the genomic fragment containing the QTL is transferred from a resistant strain onto the genome of the susceptible strain, or *vice versa*. Figure 4 describes the steps towards susceptibility gene identification.

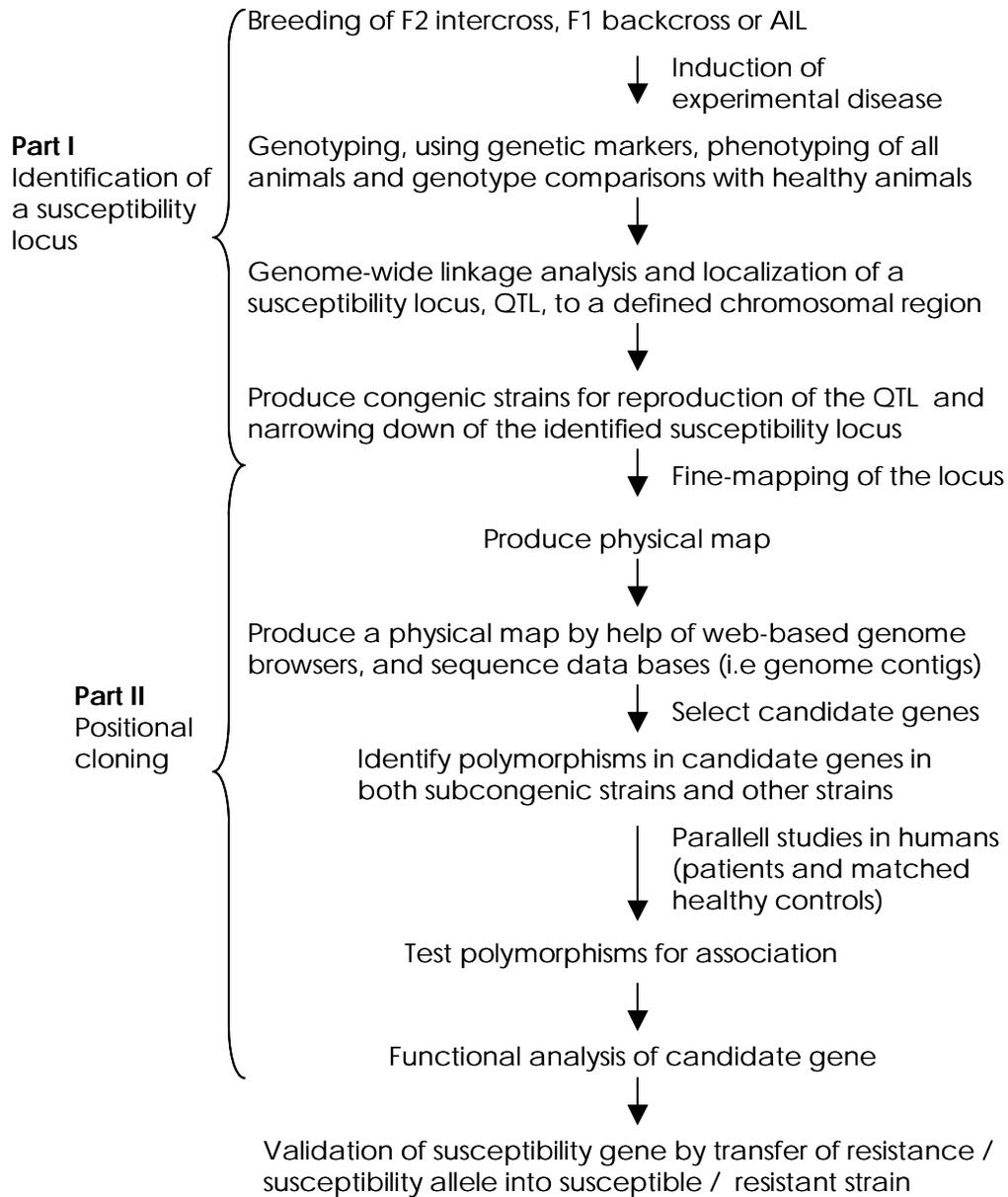


Fig. 4. Pathway for identification of a susceptibility gene in complex disease. Adapted and modified from Morahan and Morel, *Current Opinion in Immunology*, 2002¹⁹³

The establishment of a congenic strain is generally performed by selective backcrossing, starting with F1 animals, for more than 9 generations, onto either of the parental strains. This gives a theoretical cleanliness of the recipient background genome of 99.998% ($100 - 0.5^9 = 99.998$) or a donor background (outside the congenic region) of less than 0.002 %.

After at least the ninth backcross generation the so-called recombinant progeny is intercrossed in order to produce a strain which is homozygous for the QTL of interest. This is the congenic strain and can now be compared to the parental strains for the disease trait.

The procedure is exemplified with the reproduction of the QTL *Oia2* in Figure 5,

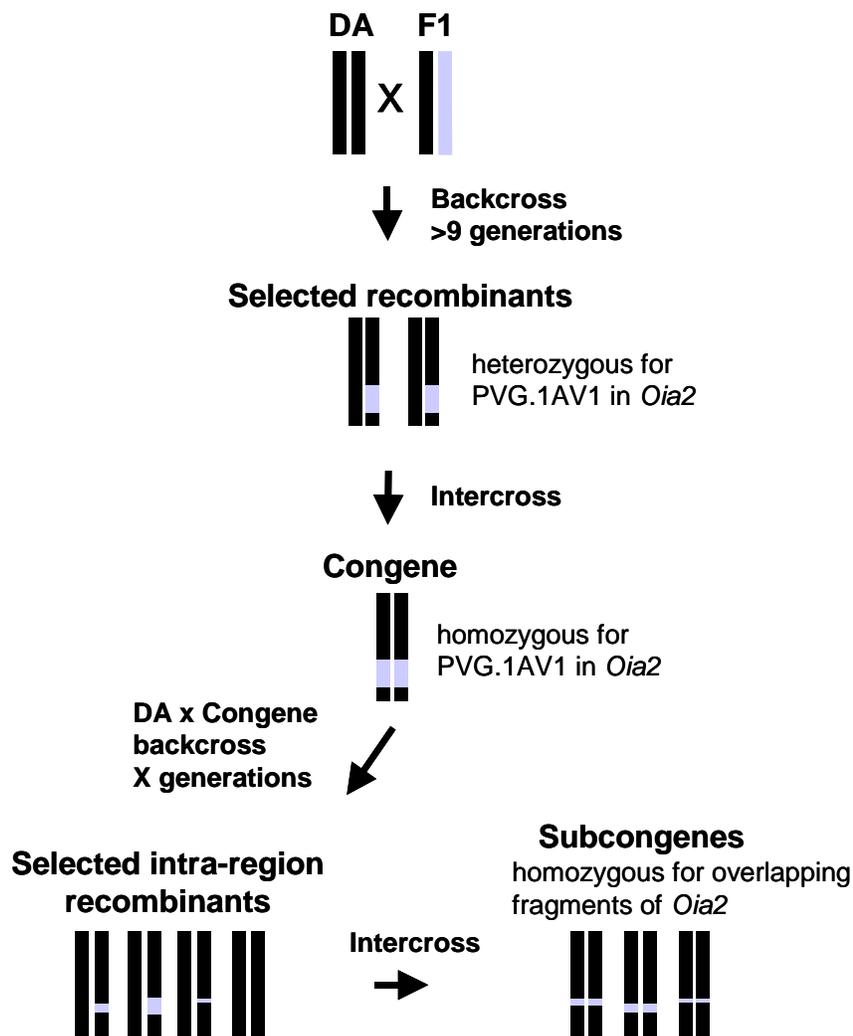


Fig. 5. Simplified procedure of reproduction and mapping of the arthritis-regulating QTL *Oia2* in congenic and subcongenic strains. The F1 generation is progeny of a cross between DA and PVG.1AV1. The progeny are then backcrossed onto the disease-prone DA rat for isolation of protective gene-*s* within *Oia2*. The selection of congenic and subcongenic strains are based on maintained protective effect of the studied arthritis phenotype in oil-induced arthritis (OIA).

where the QTL is reproduced by transfer of alleles covering *Oia2* from the arthritis-resistant PVG.1AV1 rat onto the arthritis-prone DA rat.

An alternative method to conventional backcross breeding is to use marker-assisted breeding, so-called speed congenics^{194,195}, which takes about half the time of backcrosses.

In order to narrow down the QTL to allow a candidate gene approach, it is necessary to produce subcongenic strains, thus delimiting the disease-regulating genomic interval to a size of approximately 1 Mb, which on average contains 10-30 genes. The subcongenic strains are produced by further backcrossing of the congenic strain onto the parental strain used in the production of the congenic strain. These subcongenes carry parts of the originally transferred chromosomal region, and allow fine-mapping and candidate gene approach. In complex disease models, such as experimental arthritis, subcongenic animals in some cases have revealed several sub-QTLs, as demonstrated for RNO4 and RNO10 in arthritis^{156,174,175,196} and for MMU17 in a murine model for SLE¹⁹⁷.

Positional cloning of candidate genes in experimental arthritis

Subcongenic strains carrying a QTL, containing approximately 10 genes, are suitable for the initiation of candidate gene analysis. The techniques for positional cloning of candidate genes are for example chromosome walking (Box 2), shot gun sequencing (Box 2) and exon trapping. An example of a successful shot gun sequencing is the completion of the human genome by Craig Venter's team¹³⁴ in 2001. In my project exon trapping was used in the aim of finding candidate genes close to the microsatellite marker D4Rat90, which was isolated in our smallest subcongenic strain. Exon trapping is based on identification of potential exons in unknown sequences close to a genetic marker¹⁹⁸. Here, D4Rat90 was chosen as a probe for hybridisation with superpools of a PAC-library¹⁹⁹ covering the whole genome of BN rat. The assay was initialized by screening the PAC-library, using PCR, for identification of superpools containing the sequence for the microsatellite marker. To select single positive PAC-clones, the microsatellite marker was labelled with ³²P and used for hybridization onto nylon filters, each containing all clones in a specific superpool. Two overlapping clones, positive for D4Rat90, were chosen for exon trapping and inserts in positive PAC-clones were subcloned from their pPAC4 vectors into splicing vectors, pSPL3b, which contains a multiple cloning site flanked by a functional splice donor and an acceptor site. Finally, subcloned genomic BN DNA inserts were amplified in *E. coli*, and isolated and transfected into human

293T cells. In the mammalian cells, cloned DNA fragments with exon were spliced between the vector and insert sequences. Total RNA from the 293T cells was then isolated and used for cDNA synthesis. Analysed sequences were compared with genome data bases and the positions of exons of potential candidate genes close to D4Rat90 could be estimated. The results are further discussed in Results and Discussion.

In conclusion, there are several methods of identifying polymorphisms in genes once they have been identified. In addition, new important genes may also be discovered. The limiting factors are time, money and manpower. Besides gene sequencing, there are complementary methods for studies at mRNA and protein levels. These methods are extremely valuable when performing functional analysis and validation of selected candidate gene(s).

Box 1. Glossary

Allele – a variant of a gene

centiMorgan – cM, a measure of recombination frequency between genes/genetic markers.

A non-physical distance between two linked gene pairs, where 1% of the products of meiosis are recombinant. 1 cM = 1% recombination between two loci. The unit in linkage maps before completion of the sequencing of human mouse and rat genomes.

Congenic – inbred strain that delineates a genomic region containing a trait locus

Epistasis – interaction of several genes and where the net phenotypic effect of carrying more than one gene is different than would be predicted by simply combining the effects of each individual gene.

Genetic marker – short DNA sequence (i.e. microsatellite, SNP etc.) that is polymorphic and useful for mapping by linkage or association

Genotype – The pair of alleles at a locus

Haplotype – Unique set of alleles or SNPs at different sites on the same chromosome

Haplotype block – Segment of the genome that is unbroken in an investigated population by reshuffling or recombination during meiosis

Linkage – tendency for alleles of different genes / loci to be passed together from one generation to the next without being separated by recombination

Linkage disequilibrium – LD, A measure of co-segregation of alleles in a pop. Relationship between 2 alleles, which arises more often than it can be accounted for by chance, because those alleles are physically close on a chromosome and rarely separated from one another by recombination

Locus – Any polymorphic stretch of DNA in the human genome, (e.g. gene)

Lod score – the base₁₀ logarithm of the likelihood of the odds ratio for linkage. The lod scores for genome-wide significant linkage are 3.3 for linkage analysis in human and in the range between 3.3 (backcross and intercross (1d.f. additive) and 4.3 (depending on study design) in mouse or rat¹⁸¹

$$\text{LOD} = \log_{10} \frac{\text{Likelihood of QTL at position}}{\text{Likelihood of no. QTLs at position}}$$

Meiosis – Division process of the DNA in the production of germ cells (gametes). Involves independent assortment of chromosomes and crossing-overs (recombinations). Results in separation of the chromosomal pairs; one copy of each chromosome to each of the daughter cells (gametes)

Microsatellite – Short tandem repeat of DNA near or within a gene, consisting of repetitive base-pair units (ex. CACACA, a dinucleotide microsatellite) and flanked by a unique sequence which acts as the recognition tag for this microsatellite

Odds ratio – a measure of relative risk that is usually estimated from case-control studies

Phenotype – The detectable outward feature (trait) of a specific genotype

Polymorphism – variation (insertion, deletion, SNP, microsatellite etc) ranging from a single bp to thousands of bp in size) in DNA sequence

QTL – Quantitative trait locus

Quantitative trait – biological trait that shows continuous variation, and is measured quantitatively (e.g. length) rather than falling into distinct qualitative categories (e.g. affected or non-affected)

Penetrance – proportion of individuals with a particular genotype that express that phenotype

RFLP – Restriction fragment length polymorphism, constitutes by a restriction enzyme cleavage site which is unique for different individuals of the same species.

SNP – Single nucleotide polymorphism, bi-allelic (typically) base-pair substitution. The most common form of genetic polymorphism.

Box 2. Genetic approaches for identification of disease-linked genes

Lod score analysis – Parametric method, which is dependent on an assumption of a genetic model and specification of certain parameters (i.e. mode of inheritance of a disease and the markers, disease and marker allele numbers and frequencies, mutation rate and penetrance). Provides an estimate of the recombination fraction, and thereby evidence of linkage between two loci. Lod score 3.3 or more provide evidence of linkage.

Sib pair method – Non-parametric method (does not require knowledge about mode of inheritance), where affected pairs of siblings are collected and the degree of sharing of marker alleles between the siblings are compared. Can be applied both to quantitative and qualitative traits.

Admixture mapping – Genetic mapping using individuals whose genomes are mosaics of fragments that are descended from genetically distinct populations. Utilizes differences in allele frequencies in the founders to determine ancestry at a locus in order to map traits.

Positional cloning – A three step mapping technique where an identified QTL, usually no larger than 2 Mb and containing the gene/genes of interest, is cloned (copied) in an experimental system (often bacterial) and sequenced for mutations (polymorphisms). The procedure also includes a function analysis of sequenced “candidate” genes. Often in a murine model.
Step 1) Localization of a disease gene to a chromosomal subregion, generally by using traditional linkage analysis
Step 2) Searching in public databases for a candidate gene within that subregion
Step 3) Testing the candidate gene for disease-causing mutations

Chromosome walking – sequential isolation of clones carrying overlapping sequences of DNA. Used in positional cloning in an experimental system, a so called genomic library, built up by for example bacterial artificial clones (BAC library) or P1 (bacteriophage) artificial clones (PAC library), and which starts from a known location, the genetic marker, closest to the candidate gene of interest. The known DNA sequence is used as a probe for the adjacent unknown DNA sequence, which in turn, after being sequenced, is used as probe for the next portion of the genome. When a sufficiently large series of overlapping sequenced clones are isolated it is possible to identify polymorphisms in the candidate gene.

Shotgun sequencing – sequencing method used in positional cloning and where the genome is mechanically split into lots of short fragments and cloned in BACs, which are sequenced and assembled by computer-based software programs on the basis of overlap.

Atopic asthma

Atopic asthma is a complex, multifactorial, chronic airway inflammation characterized by elevated levels of IgE antibodies and eosinophilia in response to an inhaled allergen. The disease prevalence has increased worldwide over the last decades, with a preponderance in the industrialized countries,.

Together with eczema and allergic rhinitis (hay fever), atopic asthma is embraced under type I hypersensitivity reactions, which are characterized by an IgE-dependent (atopic) TH₂ immune response. The hypersensitivity causing allergen is an antigen, which selectively induces TH₂ cells to produce the cytokines IL-4 and IL-13, leading to a switch of the antibody isotype production from IgM to IgE. The allergen is a relatively small and highly soluble protein carried on dried airborne particles, such as pollen grains or mite feces. It normally does not evoke an immune response, but can cause harmful immune reactions, even at very low doses, in individuals with a certain genetic predisposition for hypersensitivity reactions. In addition to allergen-mediated activation of asthma, superantigens of toxins, produced by the bacteria *Staphylococcus aureus*, are suggested to be involved in the activation of CD4⁺ T cells of TH₂ subclass in asthma²⁰⁰.

As in RA, the environmental factors and living habits are thought to have an influence the tendency of an individual to develop an allergic reaction. In the year 1989, the concept "Hygiene hypothesis" was proposed as a responsible factor for the increasing prevalence of atopic diseases²⁰¹. In short, the hypothesis attempts to explain the increase of asthma and other allergic diseases as being linked to reduced exposure to germs through declining family sizes, more limited exposure to animals, higher general standards of cleanness, and that repeated exposure to microbes at an early age actually helps the immune system to adapt and not over-react to routine environmental stimuli, such as potential allergens. In comparison with RA, twin studies of clinical asthma are difficult to interpret because of variable diagnostic criteria among different studies and lack of a definitive biochemical marker.

The central factor in atopic diseases is the IgE antibody, although more is known about the pathophysiology of IgE-mediated responses than about the normal physiological role of IgE itself. However, polymorphisms of the C-type lectin-like receptor, *DCIR*, involved in down-regulation of dendritic cells, macrophages, granulocytes (mostly neutrophils) and B cells, shows significant correlation with changes of total IgE levels humans suffering from atopic asthma. This is further described in Paper IV.

Pathology of atopic asthma

Asthma is classified as mild, moderate or severe, according to severity of asthma attacks of coughing, wheezing and dyspnea. The pathophysiology of asthma involves activation of mast cells in the lower airways. The mast cells releases histamine, leukotrienes and other mediators that lead to contraction of bronchial smooth muscle tissue and increased mucus production (edema) and airway inflammation. Furthermore, proinflammatory cytokines, enzymes and growth factors produced by the damaged bronchial epithelium lead to structural changes of the bronchial tissues and which is referred to as "Remodelling"²⁰².

The characteristic features of atopic asthma are listed in Table II. The bronchoconstriction results in breathing difficulties, whereby inhaled air is trapped in the lungs. The affected individual suffers from asthma attacks of wheezing and dyspnea, which can be life-threatening without treatment with anti-inflammatories (corticosteroids and leukotriene receptor antagonists) for preventive use and bronchodilators (β 2-agonists) for acute need. However, the treatment has to be life-long, unless the symptoms do not disappear by themselves, since there is no cure for asthma. Asthma vaccines have been developed, but their effects are disputed .

Table II. Pathology of atopic asthma. Adapted and modified from Fireman, P. *Allergy and Asthma Proc, 2003*

1. Hyperinflammation of the lungs
 2. Smooth muscle hyperplasia
 3. Basement membrane thickening
 4. Mucus gland hyperplasia
 5. Mucosal epithelium sloughing
 6. Tissue edema
 7. Increased level of IgE antibodies
-

On the cellular level, there are increased numbers of eosinophils, neutrophils, lymphocytes and plasma cells in the bronchial tissues, secretions and mucus.

The chronicity of the inflammation causes hyperreactive airways and other factors than the original allergen that can thereby cause future asthma attacks. The diagnosis of asthma is based on presence of reversible airway obstruction, which can be demonstrated by the use of bronchodilators. Using spirometry tests of quantitative parameters respiratory function can be analyzed. These are: FVC (Forced Expiratory Vital Capacity - total volume of air expired from the lungs during a forced maneuver after a maximum inspiration), FEV₁ (Forced Expiratory Volume) and PEF (Peak Expiratory Flow - maximum flow generated during expiration performed with maximal force and started after a full inspiration). The parameters are sometimes given as units per second (for ex. FEV₁).

The IgE antibody

The IgE antibody was discovered in the sixties^{203,204}. The normal biological function of IgE antibodies is in the immune defense against parasitic infections²⁰⁵. The IgE antibody is the least abundant antibody isotype, with a concentration of about 150 ng/ml in serum, in comparison with 10 mg/ml for IgG in normal non-atopic individuals. In atopic individuals, who have increased risk of developing allergies, the serum IgE concentration may exceed 10 times the normal level. IgE antibodies are produced by plasma cells in lymph nodes, which are situated in the vicinity of the antigen entry point, or in newly produced germ centers at the sites where the allergic reaction takes place. Inhalation of allergens activates myeloid dendritic cells in the respiratory mucosa to migrate to these lymph nodes where they activate TH₂ cells to produce IL-4 and IL-13. Together with the membrane-bound co-stimulatory signal CD40 ligand the TH₂ cells induce isotype-switch from IgM to IgE in plasma cells. The IgE response can be further amplified by mast cells, basophils and eosinophils, which all express IL-4 and CD40-ligand upon crosslinking of soluble IgE by IgE-specific high-affinity receptors (i.e. FcεRI). The less common γδ T cells has also been reported to induce isotype switching in B cells in direct response to allergen^{206,207}.

Genetic influence of IgE production

Asthma is phenotypically heterogenous and there are several parameters that can be analyzed in atopic asthma (i.e. IgE, respiratory function and hypersensitivity). In Paper IV, total serum IgE was analyzed for association with one of the genes identified in Paper III.

Several human linkage studies have been performed with regard to IgE levels, and where linkages has been observed between serum IgE and 12p13, the syntenic region to rat 4q42, harbouring *Oia*^{208,209}. At least 22 genome-wide linkage scans for asthma susceptibility genes have been performed during the last decade^{208,210-230}. Ten regions of linkage were reproducible between screens and three regions, 7p14-15²²³, 14q24²²⁶, 20p13²²⁷, were statistically significant but not replicated by other groups²³¹. Within these regions, four asthma genes or gene complexes related to the IgE regulation have been identified by positional cloning. These are the *PHF11* (Plant Homeodomain (PHD) finger protein 11) on human chromosome 13q14²³², the *DPP10* (homologue of dipeptidyl peptidase) on chromosome 2q14-2q32²³³, and *ADAM33* (a disintegrin and metalloproteinase domain 33) at 20p13²²⁷. Furthermore, *GPRA* (G-protein-coupled receptor for asthma susceptibility) showed association with serum IgE, and an unknown gene, designated *AAA1*, with asthma, at 7p13, as it was studied using family materials from Finland and Canada²³⁴. In a haplotype analysis in one study, the most common haplotype of the TNF gene, associated with total serum IgE²³⁵.

Furthermore, in an analysis of the gene *CARD15* (Caspase-Recruitment Domain containing protein 15, also named *NOD2*) one significant polymorphism was reported for association with total serum IgE. Finally, in a recent Swedish study of one of the haplotypes analyses the IL4R (IL-4 receptor) gene was reported to have significant ($P < 0.0001$) association with asthma²³⁶.

In a study of the gene *AICDA* (Activation-Induced Cytidine Deaminase) a suggested association was reported between a CT polymorphism at position 7888 (7888C/T) and atopic asthma, but also with regulation of total serum IgE levels²³⁷. Additional suggested candidates for IgE-dependent asthma are genes for cytokines (i.e. IL-4, IL-5, IL-13), adhesion molecules and transcription factors (STAT-6, Eotaxin1)^{235,238}. The Eotaxin gene and some HLADR β 1 alleles show strong association with total serum IgE levels²³⁹. All mentioned studies were based on either genome-wide linkage or candidate gene association approaches.

As for RA, several animal models have been established for investigation of the pathophysiology and genetics of asthma²⁴⁰⁻²⁴². In the mouse, several studies have linked IgE regulation with chromosome 6. The 5-lipoxygenase, located on the central region of mouse chromosome 6, has been linked to IgE-regulation²⁴³. A QTL analysis in a [(C3H/HeJ x A/J)F1 x A/J] backcross showed significant linkage for ACh-induced AHR (Air-way hyperresponsiveness) between the microsatellite markers D6Mit16 and D6Mit13, also on mouse chromosome 6, and which includes the interleukin-5 (IL-5) receptor gene²⁴⁴. This locus was later confirmed in an airway hyperresponsiveness model (ICHS, Immediate cutaneous

hypersensitivity), where a LOD score of 2.5 in a backcross of F1 hybrids of A/J and C56BL/6 (B6) was determined for the microsatellite D6Mit105, close to the gene for the alpha-chain of the IL-5 receptor²⁴⁵. Three linkage analyses for IgE regulation have been performed in rat models. In the first one, four loci were identified, including the MHC class II locus RT1, in F1 and F2 backcrosses between LEW and BN rats, and in LEW.1N congenic rats injected with mercury dichloride²⁴⁶. In the second and third genome scans, using gold salts, three loci, *Atps1* on RNO20, *Atps2* on RNO10 and *Atps3* on RNO9 were identified in F2 progeny of BN and LEW strains^{170,247}.

AIMS OF THE STUDY

The aim of this thesis was to identify genes that regulate arthritis and IgE production in the rat, and to determine whether their human homologues influence rheumatoid arthritis and IgE production.

The specific aims of the study were:

- to define an optimal experimental model using different arthritis inducers in different strains and crosses, for genetic dissection of the previously identified arthritis susceptibility locus *Oia2* on rat chromosome 4
- to perform high resolution mapping of *Oia2* by production of rat chromosome 4 congenic and subcongenic strains in order to identify candidate genes
- to determine whether the human homologues for the arthritis-regulating genes, identified in the rat, associate with rheumatoid arthritis
- to characterize whether mapped arthritis-regulating genes in the rat regulate total IgE production in the rat and in humans suffering from atopic asthma

RESULTS AND DISCUSSION

Paper I. Mapping and functional characterization of rat chromosome 4 regions that regulate arthritis models and phenotypes in congenic strains

Rat chromosome 4 is dense in QTLs regulating arthritis as well as other inflammatory diseases such as EAE¹⁶⁵, IDDM^{163,167} and EAU¹⁶⁶ (Fig. 2). In addition, several of these QTLs overlap each other, which indicates that the diseases may be regulated by the same gene/-es. Eight arthritis-linked QTLs have been identified on rat chromosome 4 (Fig. 6). A QTL usually covers a large chromosome region and lacks exact position. In order to closer define *Oia2*, and the phenotype it regulates, it was essential to develop reproducible experimental systems. Our reserach group therefore established congenic strains for chromosome 4 and characterized these strains in four experimental RA models. By selective breeding a DA strain harboring genes from the arthritis-resistant PVG.1AV1 within a 70 centi-Morgan (cM) interval on chromosome 4 was produced. In addition, three subcongenic strains were developed, named R1, R2 and R3 (Fig. 6).

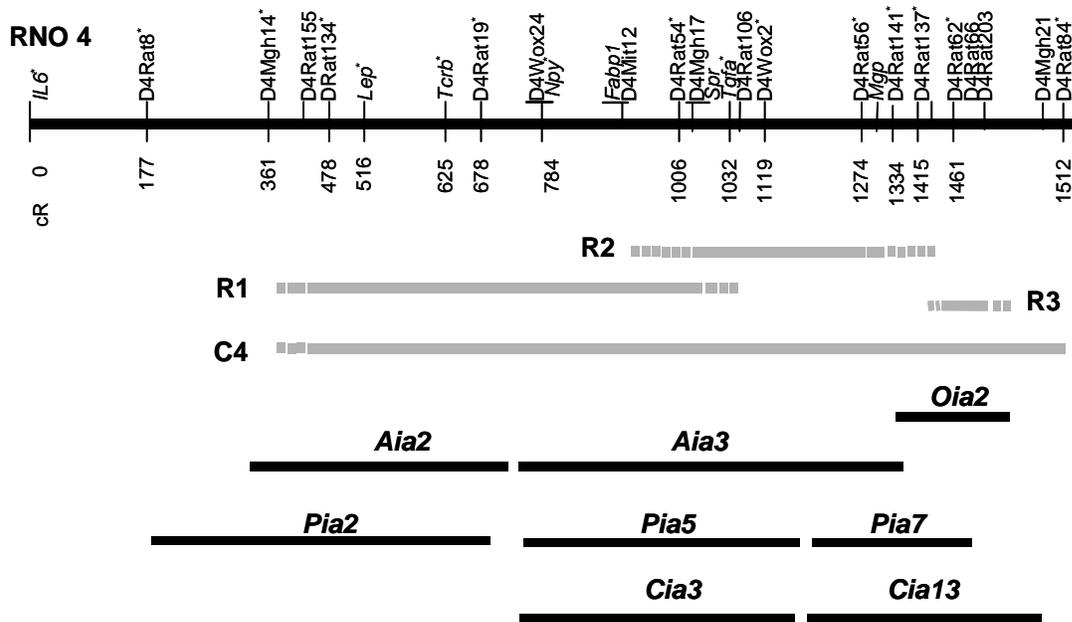


Fig. 6 Rat chromosome 4 (RNO4) aligned with eight arthritis regulating QTLs and the congenic intervals of the strains C4, R1, R2 and R3. Adapted and modified from Bäckdahl et al. *Arthritis Rheum* 2003¹⁹⁶.

The parental, congenic and subcongenic strains were tested for arthritis-induction by either of the adjuvants mycobacteria in IFA, rat collagen II, pristane or squalene in the experimental arthritis models AIA, CIA, PIA and SIA in IFA, respectively. In parallel, the rat strains were tested for arthritis-induction in OIA which was the optimal model for genetic dissection of *Oia2* (Paper II).

The 70 cM interval of the C4 congenic mediated arthritis protection in all four models, showing an intermediate arthritis phenotype in comparison to the parental strains DA and PVG.1AV1. When the interval was divided into three overlapping intervals, the R3 subcongene, harboring a 10 cM interval from the PVG.1AV1 rat and representing the QTL *Oia2*, showed decreased severity in CIA and SIA in comparison to the arthritis-prone DA strain. Of the other two subcongenic strains, the R2 subcongenic strain was tested in CIA and where males were protected from arthritis. This gender-specific regulation has earlier been described for the QTL *Pia5*¹²⁷ which overlaps the R2 interval. Similar to a previous report of *Pia5*, the R2 interval also regulated chronicity, as shown by accumulated arthritis severity. However, the chronicity regulated by the R2 interval is not gender-specific, whereas the chronicity regulated by *Pia5* has only been reported for males¹²⁷.

In addition to regulation of macroscopic arthritis, the R3 subcongene showed elevated levels of anti-CII IgG2b in comparison to DA and the other subcongenic strains, indicating a qualitative regulation of autoimmunity. Interestingly, the 10 cM R3 interval is homologous to human chromosome 12p13, which has been linked to both RA and MS^{85,86,248}.

Paper II. High resolution mapping of an arthritis susceptibility locus on rat chromosome 4, and characterization of regulated phenotypes

In this study, *Oia2* was fine-mapped using congenic and subcongenic strains harboring chromosome 4 alleles from PVG.1AV1 transferred onto the DA genome. By using 16 recombinant strains (Fig. 7), a high resolution mapping was performed of the QTL *Oia2*. The *Oia2* overlaps with *Pia7* and *Cia13*, and together these QTLs contain several genes involved in the immune system. Of particular interest was the Natural Killer cell gene complex (*NKC*), a suggested candidate gene cluster located within the chromosomal region of *Oia2* in the original genome-scan⁹². The *NKC* is known to regulate an aberrant NK cell activity of DA rats in natural toxicity²⁴⁹⁻²⁵¹, and thus our hypothesis was that the *NKC* regulates arthritis, since the DA rat is highly arthritis-prone.

Congenic strains were produced by reciprocal transfer of *Oia2*, and our group confirmed that *Oia2* with DA alleles conferred arthritis susceptibility when

transferred to PVG.1AV1 and LEW.1AV1, whereas *Oia2* from PVG.1AV1 and LEW.1AV1 conferred arthritis resistance to DA. This was true in homozygous and heterozygous forms. In addition, additional oil-induced phenotypes, besides macroscopic arthritis, were analysed and *Oia2* was shown to regulate levels of the acute-phase protein α -1 acid glycoprotein. Changes of lymphoid organs were analyzed in males of DA and the subcongenic strain R9 (Fig. 7), and the relative spleen weights were reduced in R9 but increased in DA. Finally, fine-mapping of the recombinants demonstrated that the *NKC* was situated outside the arthritis-regulating region. *Oia2* was then narrowed down to an approximated size of 1.2 Mb, harboring nine candidate genes: *C3AR1*, *Ribosomal protein L7*, a cluster of C-type lectins (*Clecsf6-10*), *C1s*, *C1r* and *CD163*, according to the Rat genome assembly as of 24th of October 2002.

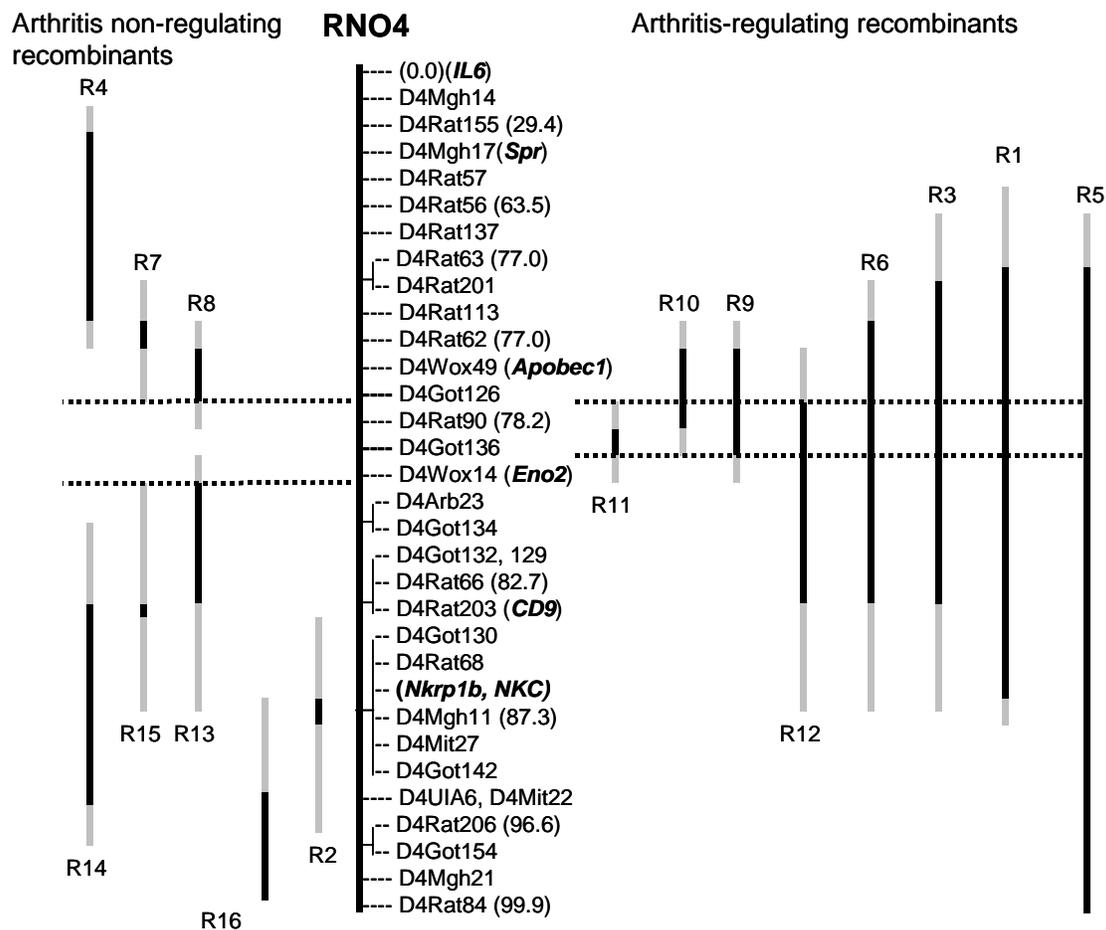


Fig. 7. Fine-mapping of *Oia2*. The figure shows the 16 recombinant strains, tested in OIA, aligned with RNO4. The arthritis-regulating region on RNO4 is located within a genomic interval, approximated to 1.2 Mb between the dashed lines. The positions of the microsatellite markers and genes, depicted in bold italics, are not to scale. Modified and adapted from Ribbhammar et al. *Hum Mol Genet* 2003¹¹².

Paper III. A gene complex encoding lectin-like receptors influences arthritis in rats and humans

In Paper III the aim was to identify the arthritis regulating gene(s) in *Oia2*. New microsatellite markers were developed and used for definition of the exact recombination points in the recombinant strains R8, R10 and R11 and the disease-regulating effect in *Oia2* could then be mapped to a 536 kb interval harboring a C-type lectin-like receptor complex designated Antigen Presenting Lectin-like receptor gene Complex (APLEC) (Fig. 8). In addition to these genes predicted in the rat genome (<http://www.ensembl.org>), search for evidence of other genes within this interval was performed by exon trapping on a PAC clone, which was identified by hybridisation of a probe containing the sequence of D4Rat90 previously mapped inside *Oia2*. The exon trapping identified no other genes than many so-called LINEs and SINEs, coding for mobile elements, such as ALU repeats, were trapped.

A new recombinant rat strain, designated R17 (Fig. 8), was produced and which contains only the APLR (APC lectin-like receptors), except for *Dcir4* and the promoter region and three first exons of *Dcir3*. The R17 recombinant showed protection against OIA already in heterozygous form.

The conclusion was that the arthritis-regulatory effect of *Oia2* is exerted by one or more lectin-like receptors.

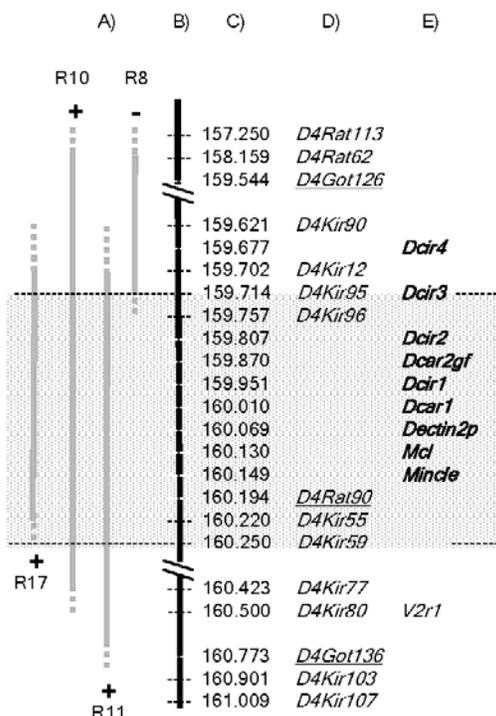


Fig. 8. Positional cloning of candidate genes within *Oia2*. The figure shows the OIA regulating interval and the APLEC gene complex.

A) Recombinant congenic strains. B) RNO4. C) Chromosomal positions (kb). D) Microsatellite markers. E) Genes of the APLEC (APC lectin-like receptor gene complex). Adapted from Lorentzen et al. Submitted to *Hum Mol Genet*.

In order to identify an arthritis-regulating gene(s), cDNA was sequenced for all the lectin-like receptors in different strains. Gene expression levels of these receptors in lymph nodes were then analyzed by RT-PCR and compared between DA and the arthritis-resistant strains PVG.1AV1 and R17. Hereby, a nonsense mutation could be identified in *Dcar1* (Dendritic cell activation receptor 1) in the DA strain, which in all probability was responsible for a reduced gene expression of *Dcar1* in the DA strain in comparison with PVG.1AV1, R17 and LEW.1AV1 strains.

The corresponding human gene cluster on 12p13.31 encodes five genes similar to the mutated rat gene *Dcar1*, including *DCIR* (Dendritic Cell Immunoreceptor). A total of 17 SNPs were tested for association with RA in patients, including matched healthy controls, and where the *DCIR* SNP rs1133104 showed relatively strong association with RA ($P=0.0076$). After stratification for seronegative (absence of rheumatoid factor, RF) and seropositive (presence of RF) patients, the same SNP showed strong association for seronegative patients versus all controls ($P=0.006$, $P_{\text{corr}}=0.011$).

Our conclusion was that the described lectin-like receptor genes may influence arthritis in both humans and rats.

Paper IV. Association of loci with IgE regulation in naive rats and in humans with atopic asthma.

The R3 recombinant strain, harboring an interval of 16.8 Mb on 4q42, has recently been reported to influence IgG isotypes in both CIA¹⁹⁶ (Paper I) and MOG-EAE²⁵², which may reflect that *Oia2* regulates the TH₁/TH₂ balance and affects both TH₁ and TH₂ types of diseases and influence total IgE levels. Here, I could demonstrate that *Oia2* regulates total IgE levels which differ between inbred rat strains, and where the R3 recombinant strain had intermediate levels in comparison with DA and PVG.1AV1. The recombinant strain R11, made congenic for a 1.2 Mb subregion, had very low IgE levels (Fig. 9).

In Paper III, it was shown that one of the candidate genes, the lectin-like receptor *DCIR* associates with RA. By analysis of plasma IgE levels in asthma patients an influence on IgE levels was detected from the *DCIR* SNPs rs2024301 (AT, AA and TT genotypes, $P=0.03$) and rs2377422 (TT, CT and CC genotypes $P=0.014$) assuming codominance (Fig. 10A and B, respectively). These SNPs were also associated with forced expiratory vital capacity (FVC), which demonstrates influence on asthma severity. Finally, a suggestive association of the marker from the *DCIR* gene with susceptibility for atopic asthma was detected.

The conclusion was that the rat 4q42 harbors several genes that regulate serum IgE levels, and that human *DCIR* regulates IgE in atopic asthma patients.

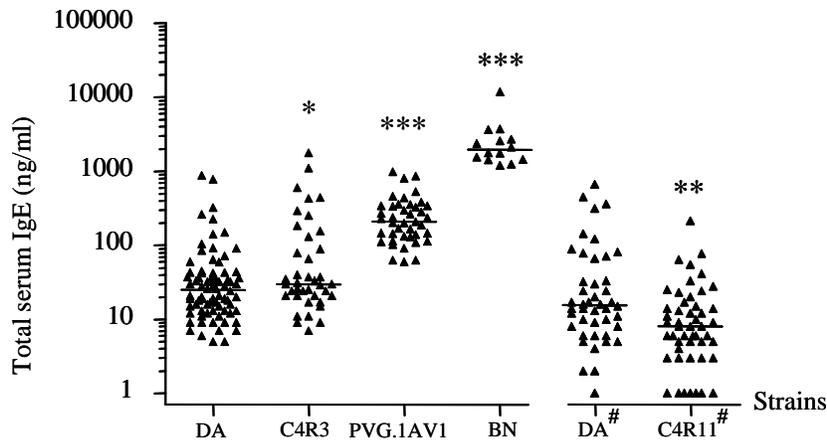


Fig. 9. Total serum IgE levels in naive DA (N=78), PVG.1AV1 (N=41), C4R3 (N=39), all analyzed in the same experiment, and naive DA (N=44) and C4R11 (N=49) both groups analyzed in the same experiment, and BN (N=14) analyzed separately. Median is included for each strain. *p<0.05, **p<0.01, ***p<0.001. Adapted from Ribbhammar et al. Paper IV.

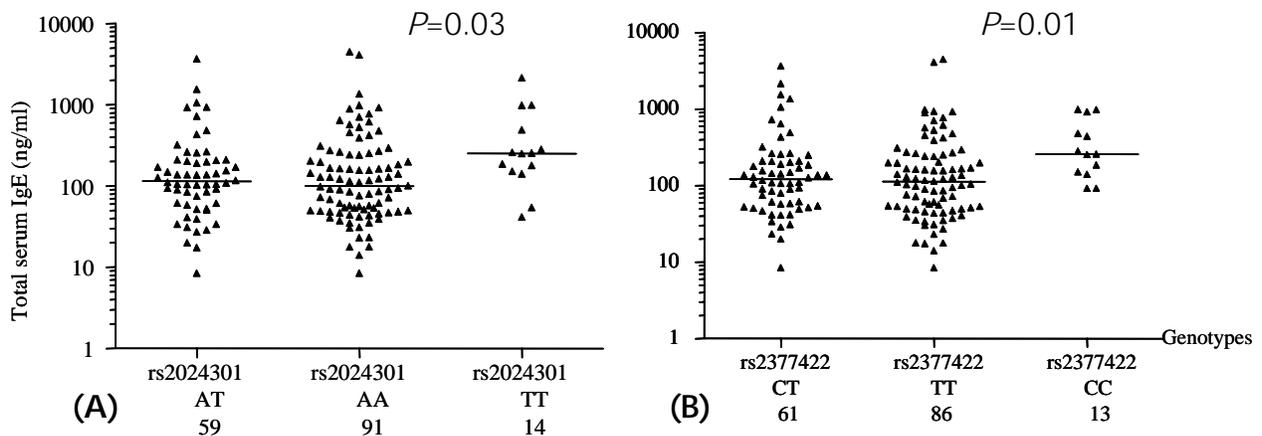


Fig. 10. Scatter plots showing total IgE levels in asthma patients in codominant model for single SNP analysis for the rs2024301 (A) and rs2377422 (B). Median is included. Adapted from Ribbhammar et al. Paper IV.

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

Our research group has identified a gene complex encoding C-type lectin-like receptors that influences RA and IgE. This gene complex, designated APLEC (APC lectin-like receptor gene complex) is localized on the distal part of rat chromosome 4 and is syntenic to the gene-dense human chromosomal region 12p13.31, previously linked to both RA and MS^{85,86,248}.

The way of identifying APLEC started as a genome-wide scan of the rat genome in the experimental model OIA (Oil-induced arthritis). By use of this model on F2 progeny of an intercross between the arthritis-prone DA rat and arthritis-resistant LEW.1AV1 rat, the susceptibility locus *Oia2* was identified⁹² on rat 4q42 (band 42 on the q arm of rat chromosome 4). Several candidate genes of immunological importance are harbored within this original QTL, and the strongest candidate gene was the *NKC* (Natural Killer cell gene complex). This gene was of special interest since the DA rat, was reported to have an aberrant NK cell-mediated natural toxicity (i.e. deficiency in eliminating allogeneic target cells), which is regulated by the *NKC*. This led us to the hypothesis that *NKC* regulates arthritis. However, arthritis susceptibility and the aberrant *NKC* alloreactivity were mapped in different crosses. Therefore, *Oia2* was replicated in an F1 backcross between DA and the alloreactivity-competent, arthritis-resistant PVG.1AV1 rat¹⁹⁰, i.e. the same parental strains used to map the *NKC*.

Fine-mapping of 16 congenic and subcongenic strains (Fig. 7) in OIA enabled isolation of a 1.2 Mb chromosomal region on rat 4q42 harboring 9 candidate genes. The *NKC* could then be excluded as being the arthritis-regulating determinant. Among the candidate genes, four genes coded for C-type lectin-like receptors. New microsatellite markers were designed and the receptor genes were concluded to be the only genes of interest. During this process *Oia2* was narrowed down to a size of 536 kb and within this QTL the complex of lectin-like receptor genes APLR was identified: *Dcir3* (except for the promoter region and the three first exons), *Dcir2*, the gene fragment *Dcar2gf*, *Dcir1*, *Dcar1*, the pseudogene *Dectin2p*, *Mcl* and *Mincle* (Fig. 8). Together with an additional lectin-like receptor, *Dcir4*, just outside the telomeric end of *Oia2*, they span a genomic region of 472 kb, almost the complete interval of *Oia2*. The remaining sequence contains mobile, non-coding elements in the form of LINES and SINES, and thus the conclusion was that the APLR regulates arthritis.

A new DA recombinant, R17, encoding only APLR, was completely resistant to OIA, and thus confirmed the arthritis-regulating ability of these genes.

Sequencing of the APLR genes revealed a nonsense mutation in the *Dcar1* gene in the arthritis-susceptible DA strain, causing reduced mRNA expression in comparison with other rat strains. The dysfunctional *Dcar1* is therefore likely to be the genetic determinant for arthritis-susceptibility in DA rats.

The function of the APLR are unknown. The extracellular part of the proteins contains a lectin-like domain with preserved amino acid residues involved in calcium-dependent carbohydrate binding, leading to their classification as group II members of the C-type lectin-like superfamily (Clecsf or Clsf) (C means Ca²⁺-dependent). The receptors are mainly expressed on the cell surface of neutrophils and APC. The Clecsfs have been implicated in several cellular activities such as cellular adhesion, migration, microbial pattern recognition, antigen uptake, T cell co-stimulation and signal transduction^{253,254}. The receptors *Dcir1* and *Dcir2* contain ITIMs (Immunoreceptor Tyrosine-based Inhibitory Motifs) in their cytosolic domains, indicating a cell-regulatory function, probably involving enzyme cascades leading to activation or inactivation of expression of immunoregulatory genes such as *NFKB*. The receptor *Mincle* has a positively charged amino acid in the transmembrane domain, suggesting an activating function via association with adaptor molecules carrying ITAMs (Immunoreceptor Tyrosine-based Activating Motifs). The other receptors do not contain any cytosolic signalling-like domains and it can be speculated that they may act as co-stimulatory molecules to either any of the APLRs, or other lectin-like receptors, such as the KLRs (Killer cell lectin-like receptors).

The gene products of APLR may form homodimers and probably also heterodimers upon cross-linking with a ligand, either with an APLR or a pathogen-recognition TLR (Toll-Like Receptor), and thus fine-tuning of the immune response. The APLR may also cluster, for example into tetramers, as does similar to the C-type lectin receptor DC-sign²⁵⁵. When comparing cloned sequences of *DCIR*²⁵⁶, *McP*²⁵⁷ and *Mincle*²⁵⁸ in other species, their strong resemblance in the structure between the species indicates that the genes in the APLR, similarly to the TLRs of the innate immune system, are rather conserved during evolution. They may primarily be involved in the innate immune response. In addition, there is a possibility that the APLR can act as signalling molecules in cell-cell interactions.

As for other lectin-like receptors, suggested ligands for the APLR would likely be sugar residues (mannose, fructose or galactose) on glycoproteins, lipids and lipoproteins.

The human homologues for the genes of APLR were tested for disease association by determination of SNP frequencies in RA cases and matched

healthy controls, where the SNP rs1133104 in *DCIR*, a homologue of the rat *Dcar1*, showed strong association with RA, and after stratification also showed association for seronegative (absence of rheumatoid factor) RA.

SNP frequencies for *DCIR* were also analysed in asthma patients and showed association with total IgE-levels as well as with impaired lung function capacity.

The newly identified APLEC was here demonstrated to exert immunoregulatory functions in both arthritis and atopic asthma, and possibly other inflammatory diseases. In future perspectives, a more detailed functional analysis, for example in single-gene recombinant strains for each of the APLEC encoding genes and their products, as well as their ligands, would be desirable. The information will clearly contribute to our understanding of the regulation of the immune system both in health and in disease.

SUMMARY IN SWEDISH

Åtskilliga helgenomanalyser har identifierat loci för kvantitativa egenskaper (QTLs) på kromosomband 4q42 i DA-råttor och som inverkar på inflammatoriska sjukdomar. Flera av dessa QTLs överlappar QTL-et *Oia2* (Oil-induced arthritis-2), som ursprungligen identifierades i en helgenomanalys i oljeinducerad artrit (OIA), en model som visar likheter med reumatoid artrit (RA) i människa.

När denna avhandling påbörjades utgjorde natural killer cell-genkomplexet (*NKC*) den viktigaste kandidatgenen i *Oia2*, eftersom *NKC* i DA-råttor kodar för en avvikande NK-cellmedierad toxicitet som kan misstänkas inverka på artrit och andra immunopatologier.

Huvudsyftet med denna avhandling var att identifiera gener i *Oia2* och som reglerar immunopatologier, eftersom detta kan ge ny kunskap om sjukdomsmekanismer som leder till RA och eventuellt andra inflammatoriska tillstånd i människa.

Kongena och subkongena stammar producerades genom att överföra kromosomala intervaller, som täcker *Oia2*-regionen, mellan DA-råttor och artritresistenta MHC-identiska PVG.1AV1- och LEW.1AV1-råttor. En ny kongen stam, DA.C4R3(PVG), som bär på PVG-alleler inom ett 16.8 Mb *Oia2*-intervall på DA-bakgrund, skiljde sig från DA både vad gäller artritbenägenhet och serumnivåer av total-IgE. Den var resistent mot OIA och visade skydd mot artrit inducerad av både collagen typ II (CIA) och squalene (SIA).

Genetisk finmappning av 16 kongena och subkongena stammar i olje-inducerad artrit demonstrerade att *NKC* är beläget utanför den artritreglerande regionen. Således kunde *NKC* uteslutas att utgöra *Oia2*. Råttor som var kongena för *Oia2* inom en 1.44 Mb region, och som betecknas DA.C4R11(PVG), var resistent mot OIA och hade lägre IgE-nivåer i jämförelse med DA. Detta tyder på att det större 16.8 Mb-intervallet i DA.C4R3(PVG) innehåller mer än en gen som reglerar nivåer av serum-IgE, en fenotyp som eventuellt korrelerar med astma.

Därefter producerades en *Oia2*-kongen stam, DA.C4R17(PVG), som var artritresistent och som definierar ett typ C lektinliknande receptorgenkomplex, betecknat Antigenpresenterande Lektinliknande receptorgenkomplex (APLEC). Sekvensering av APLEC cDNA från DA och PVG.1AV1 upptäckte en nonsensmutation i den gen som kodar för Dendritcellsaktiverande receptor 1 (*Dcar1*) i DA-råttan och RT-PCR visade significant reducerade mRNA-nivåer av *Dcar1* i lymfnoder från DA.

I människa testades de homologa APLEC-generna, belägna på kromosom 12p13, i fall-kontroll-material. Jämförelser av frekvenser av singel-nukleotid-polymorfier (SNPs) i Dendritcellsimmunoreceptorn (*DCIR*), som är nära besläktad med *Dcar1* i råttor, upptäckte association med såväl RA som med nivåer av

total-IgE i atopisk astma. Dessutom associerades *DCIR* även med forcerad utandningskapacitet (FVC) och som demonstrerar inverkan på svårighetsgraden av astma.

Sammanfattningsvis, inverkar gener som kodas av *APLEC* både på artrit och nivåer av total-IgE i både människa och råtta.

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Someone told me that people usually start reading the acknowledgements. There is a point of that. Because if it wasn't for you, mentioned in the following lines, none of these projects would have been completed. I am grateful for all of you for helping me in prosperity and adversity. I would also like to thank *Karolinska Institutet* and the *Rheumatology Unit, Karolinska University Hospital*, for giving me the opportunity to gain my Ph D education at such a international place, filled with enthusiasm, day and night.

However, the way to the finishing of this thesis has been anything but straight. Long time ago, I was unimpressionable convinced to become a managing director in a big company, making lots of money. After studying economics in Lund I reevaluated my future career plans and completed my education with the scientific subjects. Meanwhile, I worked in different parts of Sweden, among them at a stable in the aim to become a jockey, but something was always missing. I went to Umeå and studied molecular biology. Here, I would like to thank *Kerstin and Curt Framell*, who welcomed me, as the stranger I was, to live in their apartment, at my arrival in Umeå. Thank's to you I could begin my education and find a place to live.

I was very interested in marine biology at that time, and especially sharks fascinated me, but the closest I got to sharks was a diving license. Instead immunology and biochemistry caught my interest. This was something which enticed me to jump on my motorcycle "Lappelill" and move to Stockholm University and Karolinska Institutet. The rest is history, and the people I would like to thank are:

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