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Genetic Dissection of Experimental Arthritis in the DA Rat

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ABSTRACT

Rheumatoid arthritis (RA) is a chronic inflammatory disease affecting peripheral joints. Persistent inflammation causes cartilage deterioration with severe joint deformations as a consequence. The etiology is largely unknown but complex interactions between genetic and environmental factors contribute to the disease. The disease is also clinically heterogeneous which further hampers etiological investigations.

Analogs of certain disease pathways can be studied in experimental models mimicking RA. A more thorough characterization of such disease pathways may tell us of discrete disease subsets of RA.

Numerous experimental models for RA exists, both spontaneous and induced. The DA rat is remarkably arthritis prone, an intradermal injection of a mineral oil is sufficient to induce arthritis. The difference in arthritogenicity between the arthritis inducers depends on the genetic susceptibility threshold of each rat strain.

The general aim of this thesis was to by a genome – inducer approach identify a suitable experimental system to use for mapping arthritis susceptibility genes in the rat, and through this approach identify genes of equal importance in humans, ultimately to provide new insights for pathway characterization of RA.

I characterized the arthritis susceptibility in a set of recombinant congenic strains overlapping several arthritis regulating regions on rat chromosome 4, in five arthritis models; collagen type II-induced arthritis, pristane-induced arthritis, mycobacteria-induced arthritis, squalene-induced arthritis and oil-induced arthritis. All five induced arthritis-models were regulated by chromosome 4 genes. A 10 cM fragment that harbor the *Oia2* locus mediated arthritis down-regulation in collagen type II induced arthritis and squalene-induced arthritis. Oil-induced arthritis was completely prevented. Further fine mapping was continued in oil-induced arthritis. By using 18 *Oia2* intra-recombinant congenic strains the arthritis regulating interval was fine-mapped to 1.2Mb.

The arthritis regulating interval was further mapped to 600 kb that only harbor a C-type lectin gene complex denoted *Aplec*. Comparison of gene sequences identified a nonsense mutation in *Dcar1* in the DA strain as the most possible arthritis-regulating rat gene. The human homolog to rat *Dcir,* one of the other genes in the complex, was tested in a patient / control material. One SNP showed significant association to RA. Association was pronounced in RF-negative patients.

The genome – inducer approach was also applied in mapping of the arthritis regulating region, *Oia3,* on rat chromosome 10 in the F7 generation of an advanced intercross (AIL) between the arthritis susceptible rat strain DA, and the arthritis resistant PVG.1AV1. To chose the most appropriate arthritis model for linkage mapping in the AIL pristane-induced arthritis, squalene-induced arthritis and oil-induced arthritis was induced. Pristane–induced arthritis was the most appropriate for the population. *Aplec* and the newly identified arthritis regulating gene *Ncf1* was also mapped to determine the mapping resolution. Linkage mapping of *Oia3* identified two distinct quantitative trait loci (QTL), one at D10Rat13 at 97.2Mb, and an other at D10Got158 at 105.2Mb. The placement for the *Ncf1* gene, and the *Aplec* were both less than 100kb, 200kb surrounding the *Oia3* peak marker was considered as the confidence interval. The proximal *Oia3* QTL contains the Protein kinase C alpha gene together with a set of calcium channel voltage-dependent gamma subunit genes. The distal *Oia3* QTL contain a cluster of dendritic cell derived Ig-like receptors, among them the homolog to the human *CMRF35* gene previously associated to psoriasis.

In conclusion the search for appropriate experimental systems to map arthritis susceptibility regions, subsequent congenic mapping in oil induced arthritis, lead to the identification of *Aplec*, a C-type lectin complex, that codes for genes important in a number of immunological processes. The human homolog to rat showed association to RA in a patient / control material.

LIST OF PUBLICATIONS

I. **Bäckdahl, L**., Ribbhammar, U. and Lorentzen, J.C. (2003) Mapping and functional characterization of rat chromosome 4 regions that regulate arthritis models and phenotypes in congenic strains. *Arthritis Rheum*, 48, 551-9.

II. Ribbhammar, U., *Flornes, L.,***Bäckdahl, L**., Luthman, H., Fossum, S. and Lorentzen, J.C. (2003) High resolution mapping of an arthritis susceptibility locus on rat chromosome 4, and characterization of regulated phenotypes. *Hum Mol Genet*, 12, 2087-96.

III. Lorentzen, J.C. *Flornes, L. *Eklöw, C. ***Bäckdahl, L**. Ribbhammar, U. Dissen, E. Brookes, A. Klareskog, L. Padyukov, L. Fossum, S. A gene complex encoding lectin-like receptors influences arthritis in rats and humans *(Manuscript)*.

IV. **Bäckdahl, L**. Guo, J. P. Jagodic, K. Becanovic, M. Ohlsson, T. and Lorentzen, J.C Advanced intercross lines for high-resolution mapping of three experimental arthritis regulating quantitative trait loci in the rat *(manuscript)*.

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LIST OF ABBREVIATIONS

AIL	Advanced intercross line
Aplec	APC lectin like receptor gene complex
Aplr	APC lectin like receptors
BAC	Bacterial artificial chromosome
BDCA-2	Blood DC antigen -2
BN	Brown Norway
CFA	Complete Freund's adjuvant t
CII	Collagen type II
CIA	Collagen type II-induced arthritis
CpG	Cytocine Guanocine dinucleotides
cM	Centimorgan
CRD	Carbohydrate binding region
DA	Dark agouti
DC	Dendritic cell
DCIR	DC immunoinhibitory receptor
DCAR	DC immunoactivating receptor
Dectin-2	DC-associated C-type lectin -2
GPI	Glycose -6-phosphatase isomerase
HEV	High endothelial venule
HLA	Human leukocyte antigen
IFA	Incomplete Freund's adjuvant
Ig	Immunoglobulin
LOD	Logarithm of odds
LPS	Lipopolysaccaride
Mb	Megabase
MCL	Macrophage C-type lectin
MDP	Myramyl dipeptide
MHC	Major histocompatibility complex
MIA	Mycobacteria-induced arthritis
Mincle	Macrophage-inducible C-type lectin
Ncf1	Neutrophil cytosolic factor 1
OIA	Oil-induced arthritis
PAC	Plasmid artificial chromosome
PADI4	Peptidylarginine deiminase type 4
PD1	Programmed death receptor -1
PIA	Pristane-induced arthritis
PVG	Piebald Virol Glaxo
PTPN22	Protein tyrosine phosphatase nonreceptor 22
QTL	Quantitative trait Loci
RA	Rheumatoid arthritis
RF	Rheumatoid factor
RNO4	Rattus norvegicus chromosome 4
SIA	Squalene-induced arthritis
SLE	Systemic lupus erythematosis
SNP	Single nucleotide polymorphism
TNF	Tumor necrosis factor
ZAP70	ζ-associated protein 70

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Preface

Over the last century, the global health situation has gone through a profound transformation, thanks to the progress of medical science. Many of the pandemics and mysterious life-threatening diseases that used to torment mankind have been explained and either cured or eradicated. There are, however, medical enigmas that still remain unsolved. Millions are suffering from chronic illnesses: diabetes mellitus, multiple sclerosis, rheumatoid arthritis; doctors can only alleviate and ameliorate these diseases; there is no cure. Among these, rheumatoid arthritis is the most prevalent. There are common features among these diseases: They are immunity driven, have a genetic component, and are influenced by environmental factors. Their disease etiology is mostly unknown. They are often referred to as multifactorial diseases, caused by a complex interplay between genetic and environmental risk factors. Epidemiologists and geneticists alike have put great effort into unraveling some of the risk factors, but conclusive identifications of disease genes or environmental factors are limited. Some of the complexities can be alleviated by addressing questions in animal models, where genetic and environmental factors can be controlled and manipulated. This thesis is based on a series of mapping studies of genetic regions in the rat that influence experimental arthritis, where a C-type lectin gene complex was identified, and a gene within this complex, DCIR, was shown to be associated to rheumatoid arthritis in humans.

Introduction

The immune defense

Throughout evolution species have evolved through adaptation to the challenges of their specific environment. The evolving multi-cellular organisms, attacked by invading and aggressive organisms, were forced to develop some kind of defense system. The mammalian immune system consists of both evolutionary "older" and "newer" defending mechanisms working in cooperation. It can defeat effectively most invaders, from viruses to worms. The older innate immunity recognizes "invader typical" structures binding to its surface receptors, and some cells also present foreign structures to the evolutionary "new" adaptive immunity. The adaptive immunity consists of B- and T- lymphocytes, sharing the ability to genetically rearrange highly specific receptors able to target any invader. This adaptive recognition will be saved in the immunological memory and constitutes an advantage in the struggle to cope with the environmental pressure.

Autoimmunity

The most important ability of the immune system is to discriminate between self and non-self molecules. In autoimmune disease, this discrimination has been altered and certain endogenous proteins are mistaken as being foreign. The mechanisms of self-tolerance normally protect from potentially self-reactive lymphocytes. Two separate mechanisms for tolerance exist; central, and peripheral tolerance. Central tolerance occurs within the thymus during a positive and a negative selection process depending on MHC recognition. The peripheral tolerance occurs in the periphery and is mediated through processes such as clonal anergy, deletion or suppression.

Many autoimmune diseases are tissue-specific and usually the result of a response to an antigen expressed only in that organ. Other diseases are systemically manifested as is the case for systemic lupus erythematosis (SLE), with high titers of autoantibodies directed against DNA, histones, and anti-phospholipids that forms immune-complexes.

In Rheumatoid Arthritis (RA) immune responses specific for joint antigens have been difficult to demonstrate conclusively[1-3], although autoantibodies to non joint antigens have been identified such as IgG, filaggrin and citrullinated proteins[4,5]. Questions have been posed whether rheumatoid arthritis is in fact autoimmune mediated[6]. In certain strains of rats it is possible to induce chronic arthritis that well fulfill most of the RA criteria, with a single intradermal injection of a mineral oil, without antigen. It is puzzling although like many of the other unambiguous autoimmune diseases it seem mediated to a great extent by T-cells[7-10].

It is observed that RA patients often concur in families with other autoimmune diseases, indicating that the genetic predisposition is important to develop autoimmunity[11,12]. Different autoimmune diseases share genetic susceptibility predisposition[13,14]. This is true for certain autoimmunity prone animal strains as well, such as the non obese diabetic NOD mouse strain that is susceptible to a number of other autoimmune diseases[15-17] except arthritis, and the autoimmunity prone DA rat used in this thesis.

Rheumatoid Arthritis (RA)

Rheumatoid Arthritis (RA) is a chronic inflammatory disease affecting peripheral joints leading to joint deformation and impaired mobility. The disease is common among the Caucasian population with a prevalence of 0.5 -1%, Asian populations have a lower occurrence, 0.3%, whereas certain native north-American populations have as high prevalence as 7%[18]. The prevalence for females is three times higher than for males. It has a relatively late onset, most often between the ages 50-60[19-21]. The disease most often presents with symmetrical manifestations, and its progression involves destruction of cartilage, bone, and surrounding soft tissue. The etiology of RA is largely unknown, but most likely includes several disease pathways. The RA diagnosis requires the presence of at least four of the following seven criteria, and criterion 1-4 must have been present for at least six weeks[22].

Table 1. 1987 Revised criteria for the classification of rheumatoid arthritis

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

The joint synovium is the main disease target, where a rampant synovitis persists, and eventually the inflammation expands, affecting all neighboring tissue. The synovial lining layer first loses its two-cell layer appearance and is thickened. The tissue is infiltrated by macrophages, activated T-cells, B-cells, and dendritic cells[23]. Polymorphonuclear cells mainly neutrophils are found in abundance in the synovial fluid[24]. The massive infiltration of leukocytes is accompanied by proliferation of vessel endothelium, promoting the formation of high endothelial venules (HEVs)[25], thus further facilitating leucocyte influx from the circulation. Infiltrating leukocytes are memory T-helper cells, CD8+ T-cells, and B-cells. Macrophages are also abundant among the infiltrates. B-cells form germinal centers within the RA synovium, of which a proportion produce rheumatoid factor (RF)[26]. RF is an autoantibody that binds the Fc region of the immunoglobulin G (IgG) molecule[5], often forming pathogenic immune complexes. Within the RA synovium are also multinucleated giant cells, synoviocytes that grow but not divide. Influx of inflammatory cells to the synovial tissue through HEVs, upregulated proliferation of fibroblast-like synoviocytes, and recruitment of

macrophages from the bone marrow[27], creates a pannus that expands into the cartilage, and causes cartilage and bone destruction. In the pannus high levels of cytokines (IL-1, IL-6, and IL-15), inflammatory mediators (TNFα, PGE_2, and M-CSF)[28], and destructive enzymes like matrix metalloproteinase's (MMPs) are expressed, and together they have the capacity to irreversibly damage the cartilage and bone.

It is assumed that presence of activated T-cells in the synovium is essential for the RA pathogenesis, however there is little evidence for T-cell autoreactivity[6]. The importance of T-cells in the pathogenesis of RA has been demonstrated in animals where arthritis can be induced by transferring T-cells from diseased rats to a healthy[8,29,30]. The exact role of these T-cells in the arthritis etiology is to a large extent unknown. Certain MHC haplotypes are associated to RA[31] which argues for T-cell involvement. The proinflammatory cytokine IL-17 is upregulated in arthritic joints and is involved in cartilage destruction. IL-17 is produced exclusively by T-cells and is significantly down-regulated when regulatory T-cells are added to co cultures[32], indicating secondary pathogenic activities by T-cells in the joint. There are also studies indicating that arthritis development may be T-cell independent. Studies in mice have shown that *rag1* deficient mice still develop arthritis after collagen injection[33] although these mice lack mature B- and T-cells.

Great efforts has been put into understanding the molecular mechanisms for RA development, the findings may however be difficult to interpret since they may represent pathological processes downstream from the triggering event. Important clues of the etiology can be found by identifying risk factors. This could also give valuable insight for development of new treatments, and ultimately for disease prevention.

Risk factors in RA

Manifest RA is the outcome of a complex interaction of environmental and genetic factors, random events, and time.
The genetic contribution has been estimated by familial clustering. Epidemiological studies of environmental risk factors, on the other hand, have been contradictory and vague. Levels of certain hormones have been indicated as risk factors, since pregnancy and use of contraceptives lead to disease remission, although this effect has been shown to be associated more with postponement than disease prevention[34]. Weather and psychological stress are the most commonly perceived causes of disease flare among patients[35], although this is not the scientific consensus.
Smoking is the only environmental risk factor that has been verified in epidemiological studies[36,37].

Worth mentioning for this thesis are the observations that occupational exposure to certain mineral oils is associated to an elevated risk of developing RA[38]. In the same aspect, there are case reports of voluntary repeated injection of mineral oil into the skin later developing into multi manifestations of autoimmune disease characteristics[39].

Clinical heterogeneity of RA

RA is a heterogenous disease. When comparing two patients the molecular mechanisms contributing to disease could be very different. This feature reflects both the clinical outcome with regards to disease severity and how the patients respond to certain treatments. Anti-TNF treatment is one such example, where the response to therapy varies a lot between different patients with RA[40].

Identifying molecular markers specific for a subgroup of patients may not only improve disease diagnostics and treatment but also contribute to the understanding of specific pathways with common risk-factors within a subgroup. For example, there is a subgroup of individuals that are smokers, who are positive for rheumatic factor RF, and have at least one shared epitope allele, as explained below. These people are at high risk to develop RA[41]. There are healthy individuals with RF in the blood, but in the afflicted it can be used as a prognostic marker. RF positive RA patients often have a more severe disease than patients that do not have the RF-factor[26].

There are other autoantibodies important in RA. Anti cyclic citrullinated peptide (Anti-CCP) is a very RA specific type of autoantibody directed against citrullinated proteins. Anti-CCP is also associated with more severe disease[42]. Anti-BiP antibodies are found in sera from 60% of RA patients[43]. BiP is a chaperone and a member of the heat-shock protein family. Anti-A2/ anti-RA33 antibodies are directed against ribonuclein A2 involved in mRNA splicing and transport. They are not detected in more that 30% of RA patients but may be a marker for a cohort of patients with various rheumatic diseases[44]. Finding molecular markers for properly characterizing subgroups of patients, would also be a good tool to restrict some of the polygeneity of RA (many contributing genes), that hampers identification of genetic risk factors.

Genetic influence of RA

The inherited component of RA can be observed in the difference in concordance rates between monozygotic and heterozygotic twins, for most studies 12-15% and 2-4% respectively[45,46]. There is a recent meta-analysis using both Finnish and British twin data, in which a number of quantitative genetic methods were used to assess disease heritability and suggesting it to be 50-60%[47].

Determining the genetic component of RA has some difficulties, since RA susceptibility does not follow Mendelian laws, due to incomplete penetrance. There are also some practical difficulties in collecting the twin material. There is

an onset bias since RA has a late onset and the other homozygotic twin may not develop RA until many years later, and there is an ascertainment bias since other forms of polyarthritis may be mistaken for RA.

HLA is the only region conclusively linked to disease[48,49], and it is estimated to contribute to one third of the total genetic variance[50]. The association between RA and the HLA locus on chromosome 6p has been known for almost 20 years[51]. This region is one of the most gene-dense in the genome harboring more than 200 genes on ~3.5 Mb. The most important candidate genes are the human leukocyte antigen (HLA) class II genes. The association is particularly strong for HLA DRB1 alleles that code for a similar amino acid sequence, called the shared epitope[31], five peptides at position 70-74 in the third hypervariable region of DRB1 (QRRAA, RRRAA, QKRAA). Certain RA associated DRB1 alleles known to share this sequence; HLA-DRB1*0401 has QKRAA; *0404, *0405 and *0101 has QRRAA; and *1001 has RRRAA. This sequence is in the binding groove where HLA-DR present peptides.

DNA Markers:

- **SSLPs - Simple sequence length polymorphisms:**
  - Mini satellites: larger repetitive segments size ~20Kb - only on telomeres.
  - Micro satellites: dinucleotide repeats size<150 bp ~6.5 x 10^5 in the Human genome.
- **SNPs - Single nucleotide polymorphisms.**

Genome-wide scans in RA

Genetic linkage can be studied using multi-case families or trios where transmission of a genomic region is traced together with the outcome of a specific phenotype, such as RA. Genomic transmissions can be traced by site-specific polymorphic, short repeat sequences; microsatellites. In a genome-wide linkage analysis the microsatellite markers are evenly distributed in order to scan the entire genome. The region identified is often called a locus (for place) or loci in plural.

Aiming to identify genes involved in RA, four genome wide linkage analyses were performed 1998-2002; one in Japan; UK; US; and France (Table 2)[52-61].

The only region that showed genome-wide linkage was HLA. Therefore the Lander and Krygliak standard for genome-wide significant linkage ($p<0.000025$)[62], was abandoned for the non-HLA loci. Another approach was taken; nominal linkage ($p<0.05$), were reported with the intention to confirm with additional scans. A number of loci with linkage ($p<0.005$) were reported; one locus in the UK scan; three loci in the Japanese scan; 6 loci in the US study; and 4 loci in the French study. As seen in Table 2, many loci overlap between the scans.

Table 2. Genomic regions with linkage to RA

Analysis	Author	Year	Country	nr. of families	nr. of markers	p-value for HLA	Chromosomal interval* P<0.005 or *P<0.05*
Genome scan							
	Shiozawa et al.[52]	1998	Japan	41	358	0,008	1p36, 8q22, Xq27
	Cornélis et al.[53]	1998	France	97	309	2,5E-05	1p36, 2q33, 13q32, 18q22, < ***12p13*** *, 16p12, Xq27, 1q25, 2p13, 3q13, 3q27, 5q32, 6q22, 8p21, 12q21, 14q11, 16q24, 18q12,20p13, 21q22, 22q11, Xq11*
	Jawaheer et al.[54]	2001	USA	252	379	0,00005	1q43, 4q23,**12p12**, 16p12, **17q25 <** *3p21, 3q21-q26, 5p14, 5q11, 5q13, 5q21, 8p23, 8q11, 8q24, 9q34, 10q22-24,11q23, 12p11, 14q11, 14q21, 16q21-q23, 17p13, 17q21, 18q21, Xp22*
	MacKay et al.[55]	2002	UK	182	365	3E-06	6q16, < *1q43, 2q32, 3p25, 4q12, 7p14, 10q24, 14q11, 14q22, 16p13, 21p22, Xq22*
Meta analysis							
	Fisher et al.[56]	2003	4 scans	485	1367	2E-05	16p13-q12, < *6q15-23,* ***12p12-11*** *, 1q32-42, 1p36, 16q12-23, 2q34-35, 14q13-24, 8p22, 9q33, 4q13-24, 3q27, 5q11*
Replication scan							
	Jawaheer et al.[57]	2003	USA	256	379	3E-07	6p12, 9p21, 10q21
	combined	" 2001	'	508	379	4E-12	1p13, 1q43, 18q21, 12q13, 17p13
	Eyre et al.[58]	2004	UK	195	20 loci	NS	No linkage
	Os. y Fortéa et al.[59]	2004	France	88	1088	6E-05	1p36, 2q33, 13q34, 18q21, 20p13, < *1q32, 1q44, 3q21, 4p16, 5q34,* ***12p13*** *, 12q23, 16p12, 16q12, 18p11, 22q11, Xq26*
Linkage mapping for five homologous rat loci							
	Barton et al.[60]	2001	UK	200+100	33	-	**17q21-q25** (*Oia3*) p<0.001(300 families), *Oia2, Cia4, Aia2, Pia4* - no linkage.
Genome scan using SNPs							
	John et al.[61]	2004	UK	157	11245	0,00004	< *13q21, 21q21, Xp11, 6p11, 6q21*

* Region syntenic to rat loci in this thesis

Important to this thesis, syntenic regions 12p12; and 17q25 were identified in the US study. When subgrouping for RF seropositives, 12p12 was close to genome-wide significant linkage (p=0.00004). In the French study 12p13 was identified with nominal linkage (p=0.0077).

A meta-analysis was done from a compilation of all four genome scans. The analysis identified 16p13-q21 (p<0.0005), and 12 nominal loci, and among these the rat chromosome 4 syntenic region 12p12-11. Within the last year three new genome scans have been performed by the same groups from UK, US, and France. The UK repeat scan could not verify linkage to any of the 20 previously reported regions including linkage to HLA, and identified only new loci with nominal linkage. A French repeat scan could confirm 10 previously reported linkages, among these 12p13. The new US scan did not confirm any previously identified loci, but new regions with linkage were identified (p<0.005). One analysis was performed combining the new and old families to a scan with 508 families, which identified 6 loci with linkage (p<0.005).

One interesting initiative was a linkage study on human chromosomal regions that corresponds to five quantitative trait loci (QTLs)[60] in the rat that regulate

experimental arthritis (se Figure 4). The rat QTLs investigated were; oil-induced arthritis 2 (*Oia2*), oil-induced arthritis 3 (*Oia3)*, pristane-induced arthritis 4 (*Pia4)*, collagen-induced arthritis 2 (*Cia4)*, and adjuvant-induced arthritis 2 (*Aia2)*. 300 families were tested in the complete scan. The human region 17q21-25 syntenic to *Oia3* showed linkage ($p<0.001$). None of the other rat loci showed linkage in the English families.

A recent UK linkage study was performed using more than ten thousand SNPs instead of microsatellites. Despite dense genomic coverage, this scan could only replicate HLA linkage. Four additional new loci were identified at linkage ($p<0.05$).

Association analysis in RA, and other arthritic disease.

Whole-genome scans have the potential (theoretically at least) to detect all susceptibility linked loci in the genome. However linkage analyses in RA so far have been underpowered. A different strategy is to analyze association to disease in affected cases and unrelated non-affected controls to genetic markers. Single Nucleotide Polymorphisms (SNPs) are polymorphisms very abundant in the genome. The SNPs are present both in non-coding DNA and within exons of genes, where it may or may not alter the amino acid sequence, and consequently the gene product. Much effort has been put into association studies of RA during the last years. Apart from MHC class II, both class I, and class III have been associated to RA[57]. Many of the gene associations implied have not been reproducible in subsequent association studies. In the last years, several interesting associations have been reported.

The recently identified PTPN22 ($p=6.6 \times 10^{-4}$; replication study $p=5.6 \times 10^{-8}$) was found through a candidate gene study initiative of loci identified in the US genome wide scans[63]. The replication study was performed in affected families which strengthen the results further. PTPN22 is an adapter molecule involved in the T-cell signaling pathway. It was suggested that this polymorphism results in dysfunctional down-regulation of T-cell activation.

PADI4 was identified through a candidate gene approach of the chromosomal region 1q36, identified in the Japanese RA genome scan[64]. PADI4 is involved in citrullination of peptides[65]. However the strong association ($p=0.000008$ based on 830 affected and 736 unaffected) could not be repeated in two later associations in UK and Europe[66,67].

Recently a RUNX1 binding site in the cationic solute carrier SLC22A4 ($p=0.000034$, candidate gene approach) was found to be associated to RA[68]. The RUNX genes are mainly expressed in hematopoetic cells regulating various genes specific for hematopoiesis and myeloid differentiation[69]. RUNX1 binding sites have been associated with both PD1 alleles in SLE and RA[70-72], and SLC9A3R1 in psoriasis[73]. No repeat studies in RA have been reported as yet.

There are limitations and difficulties for “gene-hunting” in complex diseases in humans. Concerning linkage studies is the problem of lack of power; there are too few cases. It has been suggested that to detect the genetic variance in complex diseases with low relative risk but high prevalence in the population, the minimum affected families should be 5000[74], an almost unfeasibly large family material. Association studies on the other hand, where 5000 cases is a more practical test size, are hampered by most likely several SNP susceptibility variants for each disease locus. The susceptibility variants only mediate moderate effect which would not be possible to detect with linkage disequilibrium as small as that in patient control materials. This phenotype dilution could potentially be prevented by forming haplotype maps.

Understanding Complex diseases

It has proven to be a difficult challenge to identify the genes behind the genetic variance outside of HLA. The complexity of arthritis lies not only in intricate interactions between genes and environment, but also in elaborate genetic phenomena such as gene-gene interactions, epistasis, synergistic effects and epigenetic effects. For a complete understanding of the genetics of complex diseases, these effects clearly have to be delineated (Figure 1 and 2).

One hypothesis regarding the genetic component of RA is that it consists of a series of susceptibility polymorphisms with low penetrance and relatively small effects, that in response to certain environmental triggers, passes the threshold for disease and chronic inflammation in the joint develops[75].

Figure 1.

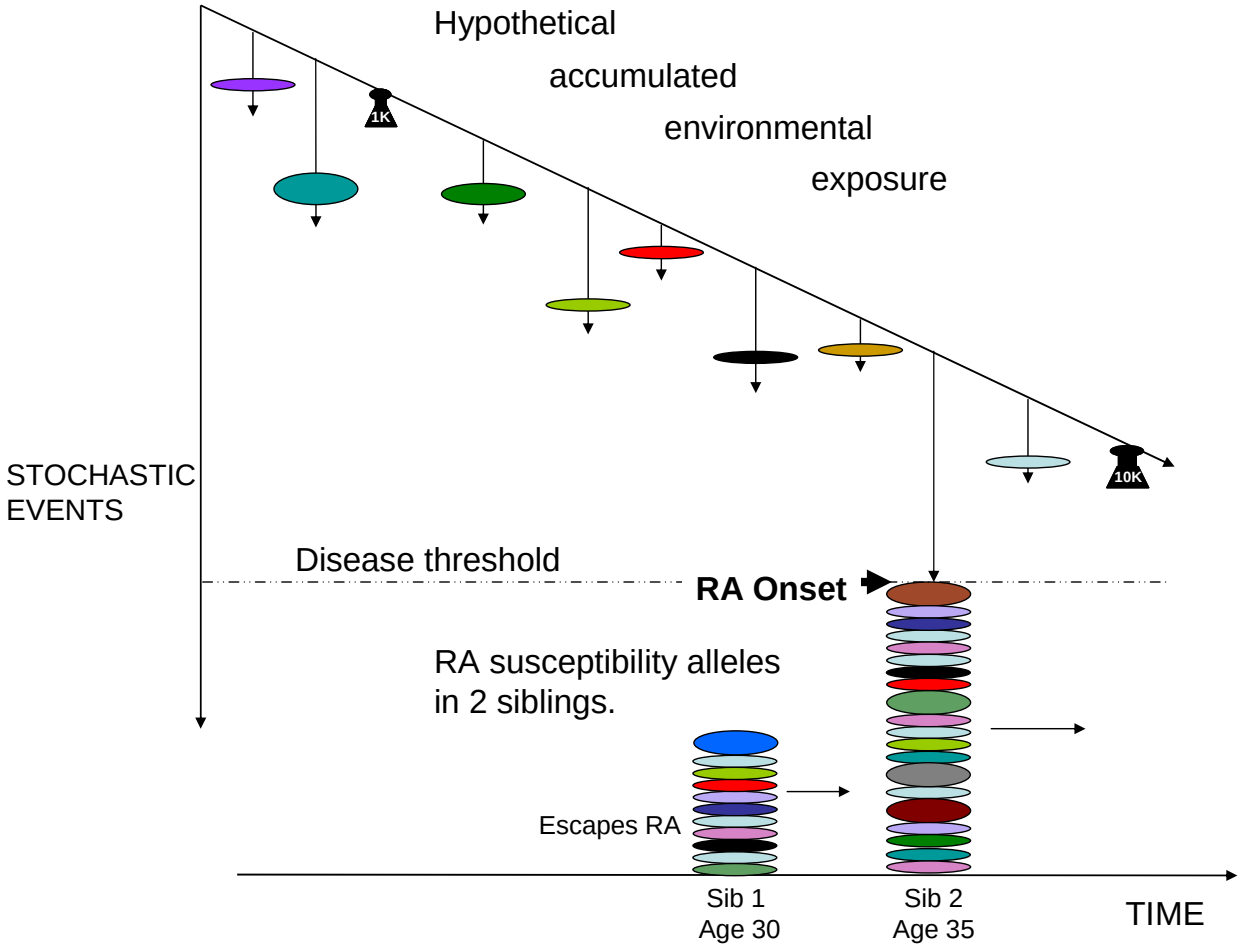
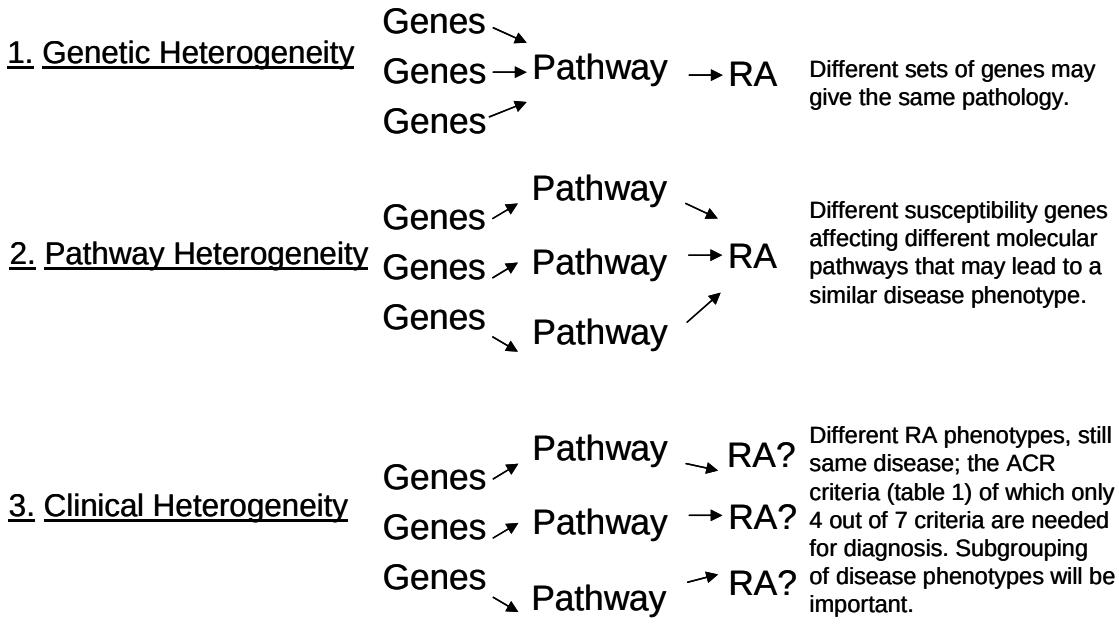


Figure 2. Factors that complicate identification of susceptibility genes



Special challengers in genetic heterogeneity

There are certain molecular and genetic mechanisms that contribute to the incomplete penetrance that is so closely associated to complex diseases:

"Epistasis" was described by Bateson 100 years ago, as one gene masking the effect of another. The term was redefined by Fisher 1918, as statistical interaction between genes – deviation from additive effects[76].

"Locus heterogeneity" is explained by multiple allelic variants at a specific disease locus.

"Synergistic modifiers" are two alleles that in combination contribute to more extreme genetic effects than additive, one plus one[77].

"Epigenetic modifications" are when one allele is shut off through chromatin modifications, making one allele transcriptionally silent. Imprinting is one such effect causing only maternal-specific, or paternal-specific allele-expression[78,79].

RA is a late-onset disease more or less unaffected by natural selection. RA susceptibility allelic mutations with late-effects occurring randomly in each generation may aggregate within a population, since they do not affect reproductivity[80]. Trying to understand the human genetic diversity brings up questions regarding the allelic structure of disease. How is the allelic structure of RA - common or rare variants[81,82]? What argues for many common variants instead of rare is the heritability, which is remarkably low in heterozygotic twins compared to homozygotic, which suggests common alleles acting epistatically[80]. The most likely scenario is a combination of both, however identifying evolutionary new rare alleles will be very difficult.

Animals can provide etiological clues!

Animal models provide a means to control environmental factors and manipulate the genetic contribution to the susceptibility to inflammatory diseases such as arthritis. By using inbred animal strains the polygenetic heterogeneity can be constrained, and the environmental exposure is controlled. Surgical and immunological manipulations are possible to perform, avoiding many of the ethical issues of human research. One other benefit of using animal models for genetic research is the possibility to selectively modify the genome e.g. produce congenic, knock-out or transgenic strains and linkage mapping.

Experimental models for RA

There exists a spectrum of different animal arthritis models. Since RA is a multifactorial and heterogeneous disease, no animal model could mirror all features of RA, but each model may contribute to new insights into certain pathways leading to arthritis.

Spontaneous arthritis

Some species may develop arthritis spontaneously, for instance dogs[83]. There have been reports on erosive arthritis found on bones of Jurassic dinosaurs[84]. A number of spontaneous arthritis models in mice exist, due to gene techniques.

DBA/1 male mice spontaneously develop a mild arthritis with the incidence 60-90% [85], under a specific condition: if they share cage with one or more other males (if caged with a female there is no disease development). Arthritis development is hormone dependent, since arthritis does not develop after castration, but arthritis susceptibility can be restored by testosterone treatment[86].

The K/BxN mouse model originates from a TCR transgene recognizing bovine RNase. To introduce TCRα-chain-null mutation[87], this TCR transgenic strain was crossed with NOD line[88,89]. Quite unexpectedly the mice carrying the transgenic TCR start to develop severe arthritis around 3 weeks of age. It was also established that serum from these mice could transfer disease to healthy or B- or T- cell deficient mice[90]. The autoantigen was identified to be the ubiquitously expressed glycose -6-phosphatase isomerase (GPI).

ZAP70 is an important signal transduction molecule in T-cells. Mice carrying a point-mutation in ZAP70 have altered selection of T-cells in the thymus that leads to positive selection of auto-reactive T-cells and as a result spontaneous arthritis develops[91].

Mice transgenic for human TNFα develop spontaneous arthritis after over-expression of TNFα[92]. This arthritis is both T-cell and B-cell independent.

Induced Arthritis

Arthritis can be provoked in certain arthritis-prone animal strains by an intradermal injection of arthritogenic substance. The disease triggers can be divided in to two groups (Table 3), containing either antigen emulsified in adjuvant or the adjuvant component alone. Although the second category of arthritis inducers only activates the immune system nonspecifically (i.e. no immunogenic substance is added), the manifested joint inflammation is very similar to antigen-induced arthritis (Figure 3).

Table 3. Examples of structurally different arthritogenic substances of different origin.

	Type of Structure	Molecule	Origin
Antigen + adjuvant:	Peptides	Collagen type II*	Cartilage
	Peptides	Collagen type VI*	Cartilage
	Peptides	COMP*[a]	Cartilage
Adjuvants:	Peptidoglycans	Myramyl dipeptid (MDP)*	*M. Tuberculosis*
	Polysaccarides	β-glucan	*S. cerevisiae*
	Metylated DNA	CpG* (cytosine guanine dinucleotides)	Bacterial
	Glycolipids	Lipopolysaccaride (LPS)*	Gram-negative bacteria
	Lipids:	$C_{30}H_{50}$ (squalene)	Eucaryotic
		$C_{16}H_{34}$ (hexadecan)	
		$C_{17}H_{36}$ (heptadecan)	
		IFA (mineral oils + emulsifier)	
		$C_{43}H_{52}N_2O_2$ (Avridine)*	
		$C_{19}H_{40}$ (Pristane)	Plants

* Arthritogenic only together with complete or incomplete Freund's adjuvant (CFA or IFA).
[a]Cartilage oligomeric matrix protein
Table Adapted from ref. 96.

Microbial and bacterial molecules are recognized by structure specific receptors on cells from the innate immunity; dendritic cells, macrophages, neutrophils etc. Oils are most likely not recognized by oil specific receptors but may provoke endogenous signals that innate immunity responds to[93]. Adjuvant molecules trigger antigen presenting cells in particular, who interpret adjuvants as danger signals[94]; thus inducing cytokine production, and upregulated antigen presentation. When administered with antigen the adjuvant serves as a depot, loading antigen to the T- and B-cells for a long time[95]. This adjuvant capacity appears to coincide with arthritogenicity. The arthritogenic substances exhibit different arthritis-inducing capacity. The arthritis susceptibility is dependent on the genetic background. This is illustrated in the different susceptibility thresholds displayed by different inbred rat strains (Figure 3)[29,30,96-109].

Figure 3. The arthritis susceptibility threshold.

	F344	E3	PVG.1AV1	LEW	LEW.1AV1	DA.1H	DA
MDP	0.8			5.0	6.8		11.8
Pristane	0	0.2	0.5	7.4	11.0		9.7
β-glucan			2.5		5.5		7.9
Avridine		0	0	6.3	9.0		11.0
Mycobacteria			0	0.6-3	1.5		1.5-8.5
Bovine collagen type II	0			6.5			12.4
Rat collagen type II	0	0	0	0	3.6	11.4	12.6
Squalene			0	0	0.3	5.4	9.8
Incompltete Freund's adjuvant	0	0	0	0	0	3.2	6.1
Olive oil							0

Combinations between disease inducers and genomes (inbred rat strains) results in arthritis severity and incidence transformed to a 0-16 susceptibility score scale. The inducers and genomes are tentatively ranked along the Y and X axis, respectively, according to published data. Where there are more publications than one for the specific combination data are median score from different publications, except for Mycobacteria where the range is displayed, references 29, 30, 96-109. The figure illustrates some of the published data showing influences from MHC genes (DA, DA.1H) and non-MHC genes (DA, PVG.1AV1, LEW.1AV1) for some of the arthritogenic substances.

The DA rat

Dark Agouti (DA) rats are susceptible to a number of autoimmune diseases; experimental autoimmune encephalomyelitis[110], experimental allergic neuritis[111], experimental allergic uveitis[112], and experimental autoimmune thyroiditis[113]. Among the inbred rat strains the DA rat is conspicuously arthritis-prone (Figure 3). It is the only strain susceptible to oil-induced arthritis and other arthritis inducers provoke very severe disease in DA compared to other susceptible strains. This disease susceptibility is mediated by both MHC and non-MHC genes (Figure 3), as is displayed by the milder disease seen in DA.1H, which is a DA strain with different MHC[108], and by the more severe disease seen in LEW.1AV1, which has DA alleles in the MHC. PVG.1AV1 is also DA-MHC congenic, but is still resistant or develops extremely mild arthritis in response to most arthritis inducers. The intriguing difference in susceptibility between the MHC identical strains is explained by non-MHC influences that could be either oligogenic or highly polygenic and demands to be uncovered.

Table 4. Characteristics of RA and some selected rat models

	RA	MIA	OIA	SIA	PIA	rCIA
Bone and cartilage erosions	+++	+++	- / +	++	+++	+++
Enthesopathy and new bone formation	+ /-	+++	-	-	++	+++
T-cell infiltration in the joints	+++	++	+	++	++	+
Circulating COMP	+++	nd	nd	nd	+++	+++
Symmetric involvment of peripheral joints	+	-	+	+	+	+
Chronicity (sustained or relapsing joint infiltrations and erosions)	+++	-	-	-	+++	+++
Rheumatoid factor	+++	-	nd	nd	++	nd
MHC association	yes	yes	yes	yes	yes	yes
Gender preponderance	Females	Females	Females	No/Females	Females	Females

+++ (pronounced) ++ (clearly present) + (barely significant) - (not present) nd = not done
Adapted from reference 121.

Collagen type II -induced arthritis (CIA); Antigen in adjuvant

Collagen type II-induced arthritis is the most common arthritis model, used for both pharmacological, and pathological research. It was first described in 1977 by Trentham[114]. In rats, both heterologous (human, bovine porcine and chicken) and homologous (rat) collagen type II dissolved in either Freund's complete or incomplete adjuvant is used. Collagen type II induced arthritis, but not type I or III.

CIA is inducible in both mice and rats, although disease-induction in mice requires complete Freund's adjuvant, heterologous collagen type II, boosting and arthritis develops after 60 days[115]. DA rats, DA-MHC congenic LEW.1AV1, and BN.1AV1 rats develop CIA after injection of rat collagen type II[108,116]. Bovine and porcine collagen type II are used to induce CIA in other strains.

Collagen-induced arthritis develops 10-14 days after injection of collagen type II in incomplete adjuvant in arthritis prone rats. Collagen type II -induced arthritis in both rats and mice displays pronounced bone and cartilage erosions (Table 4), symmetrically distributed, presence of rheumatoid factor, both T-cell and B-cell involvement, and autoantibodies to collagen type II. In fact it is possible to transfer CIA from one mouse to another only by injecting arthritic donor serum indicating that CIA is partly driven by autoreactive B-cells[117].

Other cartilage derived arthritogenic auto-antigens are cartilage oligomeric matrix protein (COMP)[118], collagen type IV, and collagen type VI[119]. These antigens are also depending on emulsification in adjuvant.

Mycobacteria-induced arthritis (MIA)

The first established experimental RA model[120], often designated classical adjuvant arthritis, is induced by an intradermal injection of complete Freund's adjuvant. Complete Freund's adjuvant consists of heat-killed mycobacteria in mineral oil. This combination induces arthritis in mice, hamsters[121] and rats with many features similar to RA, but probably even more similar to spondyloarthropathies with periostitis, ankylosis, and many other extra-articular manifestations like pervasive inflammatory infiltrates into other organs like spleen, liver, bone marrow, meninges, skin, and eyes[122,123]. One disadvantage with MIA is the relative irreproducibility, severity and incidence varies greatly between experiments, as seen in Figure 3.

The myramyl dipeptide (MDP) is a mycobacteria derived adjuvant molecule identified in an effort to find the arthritogenic component contributing to mycobacteria induced arthritis[124].

Pristane -induced arthritis (PIA)

The plant oil pristane (2,6,10,14-tetramethylpentadecan), present in chlorophyll and therefore omnipresent in the diet, is a potent arthritogenic oil that, though it only activates the immune system nonspecifically, induces an arthritis that clinically resembles RA (Table 4). Pristane–induced arthritis is chronic, joint specific and T-cell regulated[30]. The disease is manifested 2-3 weeks after injection and persists as active lesions for a long time, sometimes disappearing only to reappear again later. Large pannus formations are evident, with presence of neutrophils and macrophages. There is severe cartilage destruction without bone formation. The disease is most likely driven by auto-reactive T-cells since disease parameters were reduced after treatment with antibodies against αβTCR[30]. The chronic phase was also altered after T-cell treatment, with lesser pannus formations and other clinical signs.

Pristane–induced arthritis can also be induced in mice, but with some effort. Disease appears after two intraperitoneal injections 50 days apart, requires a triple dose, but it is not joint specific and does not resemble PIA in rats. Pristane injection to BALB/cJ mice induces the production of Lupus-associated auto-antibodies and immune complex mediated glomerulonephritis[125,126]. This autoantibody production however, is not exclusive to PIA; it has been detected for SIA and OIA as well[127]. BALB/cAn mice on the other hand have been reported to develop plasmacytomas after pristane injection[128].

Squalene-induced arthritis (SIA)

Certain rat strains injected with the cholesterol precursor squalene develops arthritis that fulfills four of the ACR criteria requested for RA diagnosis. Joints are affected symmetrically, and bone and cartilage erosions are noticeable. The squalene-induced arthritis is characterized by T-cell infiltrations in the synovium. Transfer experiments of lymph node cells from a sick to a healthy rat, suggest that squalene-induced arthritis is T-cell dependent. T-cell depletion has been done and disease parameters were reduced after treatment[29]. The specific time windows where arthritogenic T-cells are generated have been analyzed in transfer experiments and it has been demonstrated that cells from draining lymph nodes could transfer disease at day 8 but not day 5 after squalene injection. Non draining lymph node cells were also shown to be able to transfer SIA just before disease onset at day 11[129].

Squalene is one of the constituents of MF59, one of the few adjuvant approved for vaccinations in humans, commonly used in Influenza vaccine, as well as in clinical trials of vaccines against CMV, HIV, herpes simplex virus and hepatitis B. Much controversy in the last years has been raised concerning the safety for human use of this adjuvant. The adjuvant mixture was possibly used by the US military when vaccinating soldiers fighting in the Gulf war, and it has been implicated that squalene injections could have been the cause of the pseudo-rheumatic disease, Gulf War Syndrome[130,131].

Oil-induced arthritis (OIA)

Incomplete Freund's adjuvant (IFA) is used as vehicle in many different immunization protocols to induce experimental inflammatory diseases. IFA consists of 85% mineral oils (Bayol F) and 15% of the emulsifier Arlacel A. The often used adjuvant can induce arthritis in DA rats[132]. Oil-induced arthritis develops approximately 14 days after an intradermal injection of IFA, a mild monophasic joint inflammation in the hind paw and ankle is manifested, sometimes with progression to front paws. Disease subsides before day 45, with limited erosions, and no ankylosis or other functional disorders. Joints show minimal hyperplasia of the synovia but infiltrations by polymorphonuclear cells in the joint space and subintimal tissues occur. Pannus tissue and marginal erosions are manifested on day 29[132]. Disease is T-cell dependent since anti-αβTCR antibodies ameliorate disease[133], and serum could not transfer disease[7]. Disease is influenced by both MHC and non MHC genes[108].

Mapping susceptibility genes in experimental arthritis

Genetic linkage was first discovered in the beginning of the 20th century by Morgan and colleagues[134]. They observed that certain phenotypes were inherited together but sometimes they were not, a phenomenon called partial linkage. They combined thoughts about partial linkage and the idea of crossing-overs. They assumed that the crossing over was a random event, so that two genes far apart will be separated by a cross-over more often than two genes closer together. By calculating the frequencies by which two "linked" phenotypes were separated they could determine their genetic distance from each other on the chromosome. This in combination with genetic markers instead of visible phenotypes is the basis for genetic linkage mapping.

Genome scans: identifying QTLs

Linkage mapping

The first step in trying to find genes contributing to disease is to isolate genomic regions that are coinherited with the disease phenotype. This is done through linkage mapping. The mapping population is crucial for the linkage analysis. It is produced by intercrossing an arthritis prone inbred rat (most often the DA rat), and a resistant inbred rat, such as F344, E3, LEW.1AV1, PVG.1AV1, BN, or ACI. The progeny from the first intercross is heterozygous state, and is denoted FI. The mapping population is the generation derived from interbreeding within the heterozygous population. This population is denoted F2. A QTL can be mapped in F2 populations using linked genetic markers. The polymorphic marker will be in linkage when one of the two alleles is coinherited with a certain phenotype. Linkage is measured in LOD which is the logarithm of odds:

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

Lander and Krygliak[62] proposed criteria for genome wide linkage LOD 4.3, and suggestive linkage 2.8. Permutation test is an alternative approach to determine significant linkage[135].

Experimental arthritis genome scans

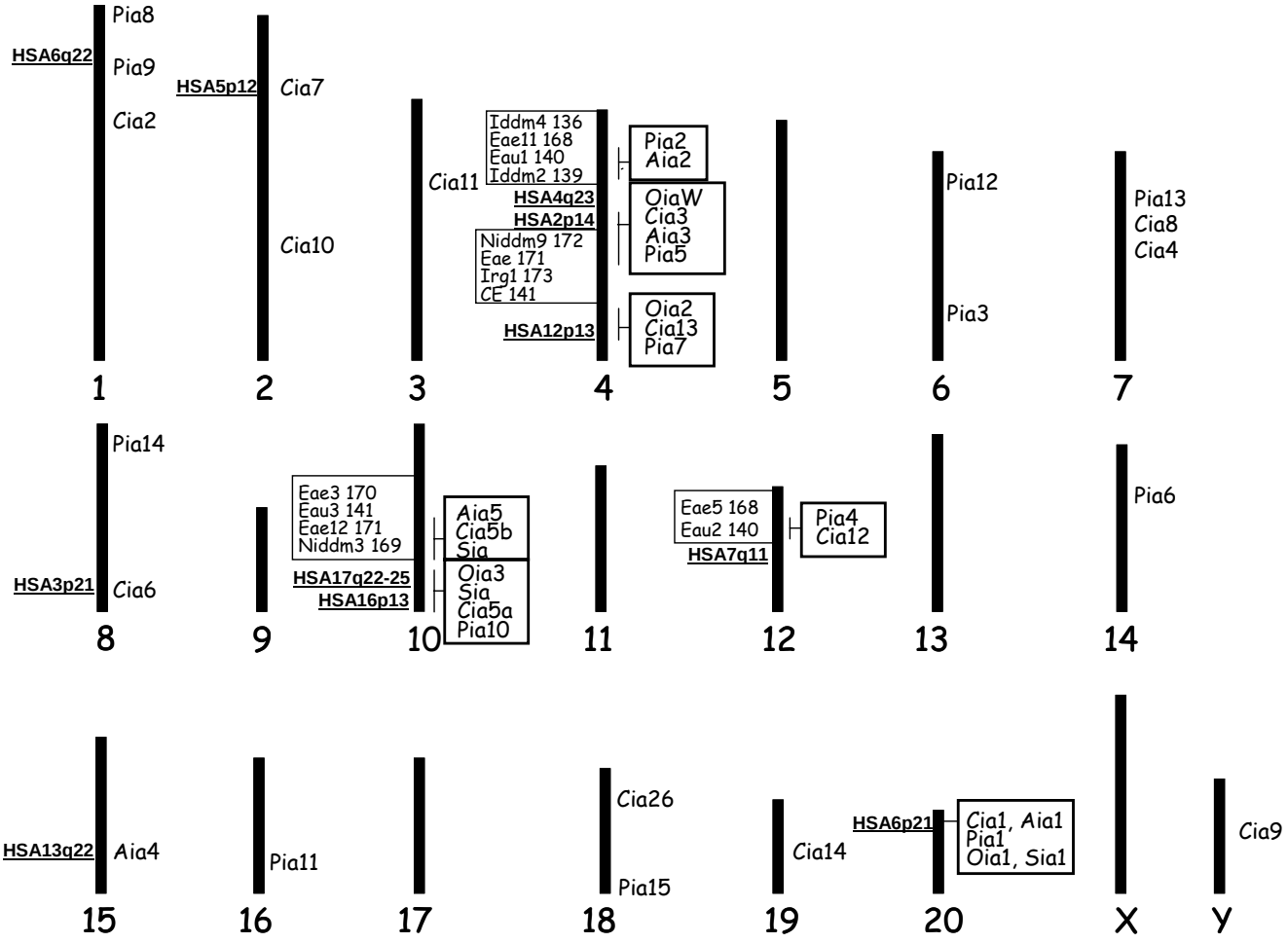
To investigate the genetic influence in experimental arthritis, 10 original genome scans have been performed, using the DA rat as the susceptible strain while the resistant strains differ (Table 5). The analyses were derived from three research groups. More than 20 arthritis-linked quantitative trait loci (QTLs) have been identified so far. Five different arthritis models have been used; MIA, PIA, SIA, OIA and CIA (either bovine, rat, or porcine Collagen type II). The QTLs often overlap, most likely because the used arthritis models have common susceptibility alleles, and at least to some extent due to common modes of induction, i.e. the use of adjuvant oil in all models. Four overlapping QTL regions; chromosome 4, 10, 12 and 20 (figure 4 and table 5), have been identified by all three research groups.

From the genome scans it is evident that chromosome 4 harbors many arthritis regulating regions in fact of all identified loci in experimental models in the rat 40% harbor on RNO4. A centromeric QTL seem to regulate both MIA susceptibility and PIA onset (Kawahito, Vingsbo table 5). In a second region near 4q31 four models are overlapping Cia5, Pia5, and two suggestive QTLs OiaW, and Pia3. Interestingly these regions also overlap with QTLs identified in rat models of other inflammatory diseases such as; diabetes type I, encephalomyelitis, carrageean exudates, and uveitis[136-141]. The telomeric region harbors three overlapping arthritis QTLs Oia2, Pia7, and Cia13. The homologous region in human 12p13 harbors susceptibility genes for both RA[53,54,56] and multiple sclerosis (MS)[142].

RNO 10 also harbors many overlapping arthritis regulating QTLs. One or possibly two QTLs have been identified in each of OIA, CIA, PIA, and SIA. Uveitis, and encephalomyelitis regulating region overlap[140,143]. The telomeric part of the region overlaps with the human syntenic region 17q22-q25 that has been associated to many autoimmune diseases such as RA, MS, ankylosing spondylitis, osteoarthritis and psoriasis[54,60,144-152].

Chromosome 12 harbors Pia4/Cia12. Pia4 in a recombinant inbred backcross gave an impressive LOD 53. This QTL was pursued in later mapping in congenic strains.

Figure 4. Arthritis regulating regions in the DA rat.



Experimental arthritis regulating regions are placed to the right of the chromosome[107,153-167], described in detailed in Table 5. Other overlapping inflammatory disease regulating loci are viewed to the left of the chromosomes[139-141,168-173]. Homologous Human RA regulating loci are underlined and are described in Table 2.

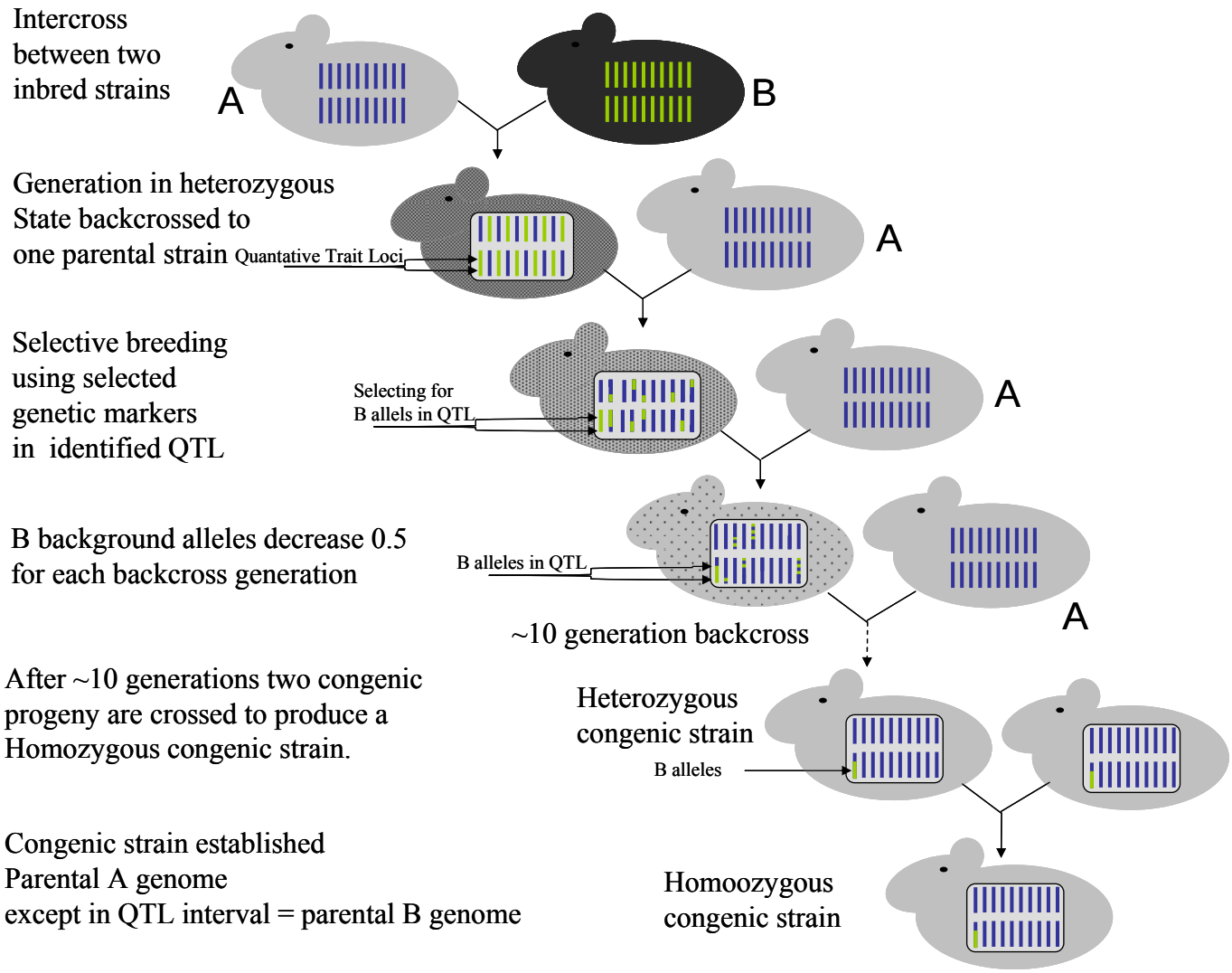
Table 5, Experimental arthritis regulating QTLs in the rat and genome scans performed

	Author	Year	Inducer	Strains	nr animals	markers	QTLs identified	Comments
Genome scan	[153]Remmers et al.	1996	B CIA	DA x F344	370	98	Cia1, Cia2, Cia3, Cia4, Cia5, Cia6*	370 of 502 F2.
""	[154]Dracheva et al.	1999	B CIA	DA x F344	370	96	Cia4/Cia8	Refined map repeat scan
""	[155]Griffiths et al	2000	P CIA	DA x BN	360	159	Cia11, Cia12, Cia13, Cia14* Ciaa3	CII antibody titers QTL Ciaa3
""	[156]Furuya et al.	2000	R CIA	BB(DR) x BN	120	187	Cia2*, Cia7*, Cia6*, Cia13, Cia15, Cia16* Cia17, Cia18*, Cia19	Ciaa4* Ciaa5*, Ciaa3
Genome scan	[157]Gulko et al.	1998	R CIA	DA x ACI	327	86	Cia7	
""	[158]Meng et al.	2004	R CIA	DA x ACI	327	86	Cia5, Cia7, Cia19, Cia25, Cia26	scan males and females sepateded Cia26 in epistasis with Cia7
2 genome scans	[107]Kawahito et al.	1998	B CIA	DA x F344	80 + 80	150	Cia1, Cia3	From Remmers et al. 80 severe and 80 mildly affected rats.
""	""	""	MIA	DA x F344	80 + 80	150	Aia1, Aia2, Aia3*	From 546 F2 80 severe and 80 mildly affected rats.
Genome scan	[159]Lorentzen et al.	1998	OIA	DA x LEW.1AV1	189	140	Oia2, Oia3,	Oia2 p= $3x10_{13}$
Assosciation	[160]Jansson et al.	1999	OIA	DA(DA x PVG.1AV1)	24	1	Oia2	Backcross
Genome scan	[161]Vingsbo-lundberg et al.	1998	PIA	DA x E3	153	330	Pia2, Pia3, Pia4, Pia5, Pia6	
Genome scan	[163]Olofsson et al.	2003a	PIA	DA (DA x E3)	650	263	Pia7, Pia4, Pia1, Pia14, Pia10, Pia15, Pia12, Pia13	Stratified for Pia4 homozygotes
2 genome scans	[164]Olofsson et al.	2003b	PIA	DA (DA x E3)	650	238	Cia11, Cia3=Pia5, Cia13=Pia7, Cia20=Pia3,	
	""	""	R CIA	DA (DA x E3)	364	238	Cia12=Pia4, Ciaa7=Pia6	
Genome scan	[165]Lu et al.	2002	PIA	LEW.1F x E3	181		Pia9, Pia4, PiaX*, Pia11	
Genome scan	[162]Nordquist et al.	2000	PIA	DA(DA x DXEC)	131	330	Pia8, Pia7, Pia3, Pia1,	Pia7 and Pia1 significant only after stratification
Genome scan	[166]Olofsson et al.	2002	PIA	DA x E3	153	323	Apr1, Pia4, Cia17, Pia6, Apr2/Ciaa5, Cia15	Mapping acute phase responses
""	[167]Wernhoff t al.	2003	PIA	DA x E4	153	323	15 QTLs	mapping rheumatoid factor

Congenic strains: isolating QTLs and fine mapping

The next step after a QTL has been identified by linkage analysis, is to fine–map the interval. The linkage identified QTLs and have large confidence intervals often exceeding 20 cM in size, far from the resolution where identifying susceptibility genes are possible. Fine-mapping has often been performed by mapping in congenic strains. The production of a congenic strain is described in figure 5 and is based on marker assisted selection of individuals carrying the desired alleles in the QTL interval (B alleles in Figure 5). Undesired B alleles outside the QTL are removed by consecutive breeding (> 10 generations) onto the desired background (A).

Figure 5. To produce a congenic strain



Speed congenics is a strategy that more rapidly reduces the undesired background alleles through marker assisted selection of individuals with the least B alleles. This speeds up the background contamination cleanup to 6-7 generations[174].

Table 6 is a summary of all congenic studies published in experimental arthritis so far in the rat[97,99,100,163,164,175-180]. Congenic mapping in three different arthritis models identified a 20 cM interval on RNO4 (R3) that mediate protection CIA, OIA, and SIA and down regulate (not significant) PIA (paper II). In another strain combination DA.F344 a larger interval (~60 cM) influenced arthritis OIA, PIA, CIA, and MIA[100,175]. This region also harbors genes influencing MOG induced encephalomyelitis, where DA alleles in the 20 cM interval enhanced disease severity (R3)[137].

Chromosome 10 is an equally interesting region, which possibly harbors two QTLs. Our group produced a set of overlapping congenic strains by backcrossing susceptibility alleles (DA) on to a resistant genome (LEW.1AV1)[176]. This identified that the *Cia3* region (46 cM) could transfer arthritis with the increased incidence from 25% to 80% in females and from 0% to 60% in males. This susceptibility interval was also further mapped in three recombinants to a 24 cM interval *Oia3b* where the susceptibility increase 73% was conserved from the 46 cM interval. A third recombinant *Oia3c* ~15 cM of the telomeric end mediated a susceptibility increase of 59%, which could indicate a second QTL with the effect 73-59% outside of Oia3c. Other studies have produced similar results, identifying arthritis protection from resistant alleles to the telomeric end of chromosome 10 (~22 cM), for both OIA, PIA and the female population in CIA and MIA. A larger interval 80cM, was protective in all four models, also indicating more than one QTL[100,175,177].

Fine-mapping the *Pia4* locus on RNO12 has been productive. The QTL had impact on susceptibility in a DA (DA x E3) backcross. A DA.E3 Pia4 congene was tested both in CIA and PIA. The congene had an ameliorating effect in PIA where arthritis was decreased with 90%, and in CIA with 50%[163,164].

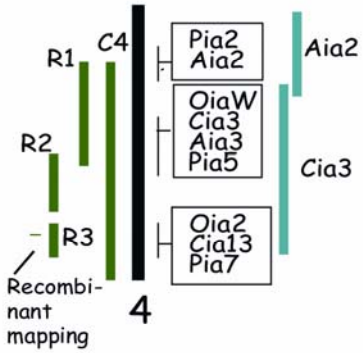
The MHC congenics can have a complete protective effect in CIA and OIA and are significantly protective in MIA and PIA. MHC alleles from E3 in PIA however showed a weaker protection.

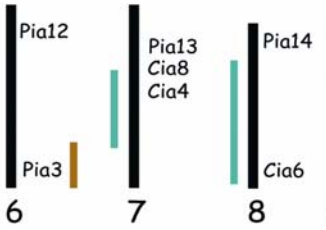
Congenic mapping has proven to be a very rewarding technique since it recently has identified the susceptibility genes underlying two arthritis QTLs in the rat; the *Ncf1* gene from *Pia4* on chromosome 12[179], and the *Aplr* complex from *Oia2* on chromosome 4 (paper III of this thesis). Here have a series of very small recombinant intervals been used to transfer the phenotype expression. The *Ncf1* gene was one out of two genes on a 300 kb fragment that still had the PIA protective phenotype preserved. There was no difference in expression between the two genes but the second gene had no polymorphisms whereas *Ncf1* had two. After comparing polymorphisms in more than 20 different strains including wild rats, the conclusion is that the susceptibility was linked to one of these polymorphisms that contribute to lower oxidative burst in DA rats since *Ncf1* is a part of the NADPH oxidase complex[181].

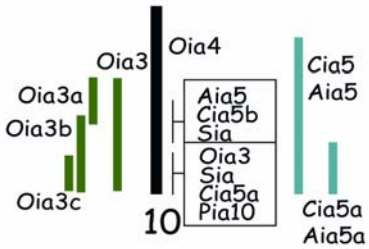
The mapping of *Oia2* and ultimately identifying the C-type lectin like complex *Aplr* as the arthritis regulating gene complex will be discussed more in detail further on in this thesis.

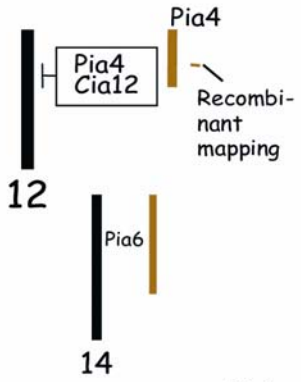
Table 6.

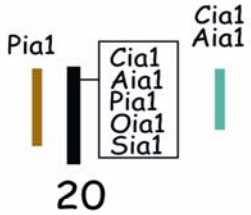
RNO	Author	Congene name	Inducer	Effect
4	[175]Joe et al. 2001	DA.F344Aia2	MIA	Males protective
		DA.F344Aia3	MIA	Females protective
	[100]Remmers et al. 2002	DA.F344Cia3	b CIA	Females protective
		DA.F344Cia3	PIA	Protective
		DA.F344Cia3	OIA	Protective
	[97]Bäckdahl et al. 2003	DA.PVG C4	PIA	Protective
		DA.PVG C4	r CIA	Protective
		DA.PVG C4	SIA	Protective
		DA.PVG R1	PIA	No effect
		DA.PVG R1	r CIA	No effect
		DA.PVG R2	r CIA	weak protection in males
		DA.PVG R3	PIA	Not Protective
		DA.PVG R3	r CIA	Protective
		DA.PVG R3	SIA	Protective
	[99]Ribbhammar et al. 2003	**18 R3 recombinants**	**OIA**	**OIA protection mapped to 0.8 Mb**
	[164]Olofsson et al. 2003b	DA.E3 Pia7	r CIA	More amelioration in CIA than PIA
		DA.E3 Pia7	PIA	
	[163]Olofsson et al. 2003a	DA.E3 Pia7	PIA	50% amelioration
6	[180]Olofsson et al. 2003	DA.E3Pia3+Pia4	PIA	E3 alleles in pia3 + Pia4 more severe
7	[100]Remmers el al. 2002	DA.F344Cia4	b CIA	No effect
		DA.F344Cia4	PIA	Females protective
		DA.F344Cia4	OIA	Males protective
8	[100]Remmers el al. 2002	DA.F344Cia6	b CIA	No effect
		DA.F344Cia6	PIA	No effect
		DA.F344Cia6	OIA	Males protective
10	[176]Holm et al. 2000	LEW.DAOia3	SIA	79%
		LEW.DAOia3a	SIA	30% Mediate susceptibility
		LEW.DAOia3b	SIA	73%
		LEW.DAOia3c	SIA	59%
	[177]Joe et al. 2000	DA.F344Cia5	b CIA	Large congene p<0.001
		DA.F344Cia5a	b CIA	Short congene p = 0.002
	[100]Remmers el al. 2002	DA.F344Cia5	b CIA	Protective
		DA.F344Cia5	PIA	Protective
		DA.F344Cia5	OIA	Protective
		DA.F344Cia5a	b CIA	Females protective
		DA.F344Cia5a	PIA	Protective
		DA.F344Cia5a	OIA	Protective
	[175]Joe et al. 2001	DA.F344Aia5	MIA	Protective
		DA.F344Aia5a	MIA	No effect
12	[163]Olofsson et al. 2003a	DA.Pia4	PIA	90% amelioration
	[164]Olofsson et al. 2003b	DA.Pia4	r CIA	More pronounced amelioration in
		DA.Pia4	PIA	PIA than CIA (CIA 50%)
	[179]Olofsson et al. 2003c	**Recombinant mapping in PIA to 300 Kb; 2 genes Ncf1/Gtf2i**		
14	[178]Wester et al. 2003	DA.E3Pia6	PIA	Less chronicity p<0.001
20	[100]Remmers el al. 2002	DA.F344Cia1	b CIA	Complete protection
		DA.F344Cia1	PIA	Protective
		DA.F344Cia1	OIA	Complete protection
	[175]Joe et al. 2001	DA.F344Aia1	MIA	Protective
	[163]Olofsson et al. 2003a	DA.Pia1	PIA	Reduced severity day 12-19 <0.05









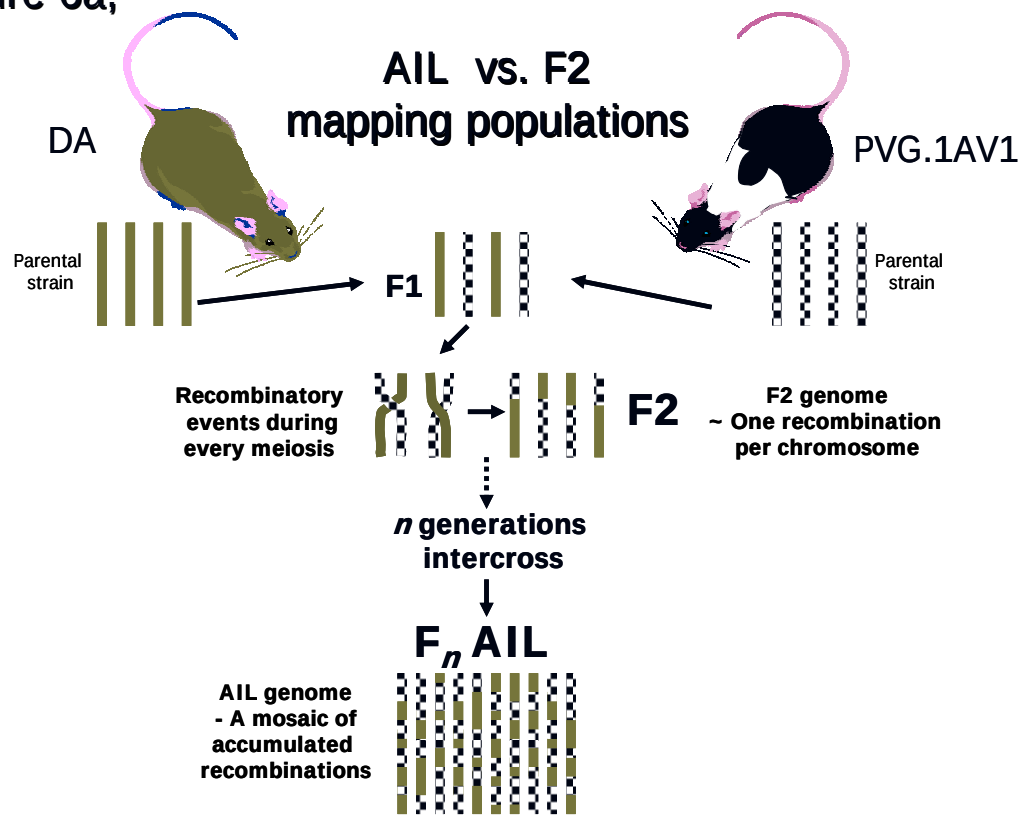


Alternative strategies in QTL fine-mapping

Like RA, experimental arthritides are complex diseases, affected by multiple susceptibility factors where genetic phenomena such as epistasis and epigenetics are difficult to delineate. Congenic mapping is limited to investigating fixed regions. It does not give the possibility to look for genetic interactions between additional loci, without for that reason producing double or multiple congenics. However there are other mapping methods available.

There are different fine-mapping strategies, where accumulation of recombinations is a central element to reduce the confidence interval (CI). One such strategy is selective genotyping, where only intra-QTL recombinant F2 individuals are selected for phenotyping. Another is to test the QTL recombinant progeny in backcross to add recombinations in the interval. Both methods need large samples sizes[182]. A third strategy to gain aggregated recombinations for each chromosome is to produce an advanced intercrossed line (AIL)[183] (Figure 6a). It is important to maintain a large number of breeding pairs, and that the breeding is random controlled, i.e. brother sister mating is avoided to prevent homozygous chromosomes. The confidence interval of a QTL can then be reduced, theoretically by a factor of t/2, were t is the

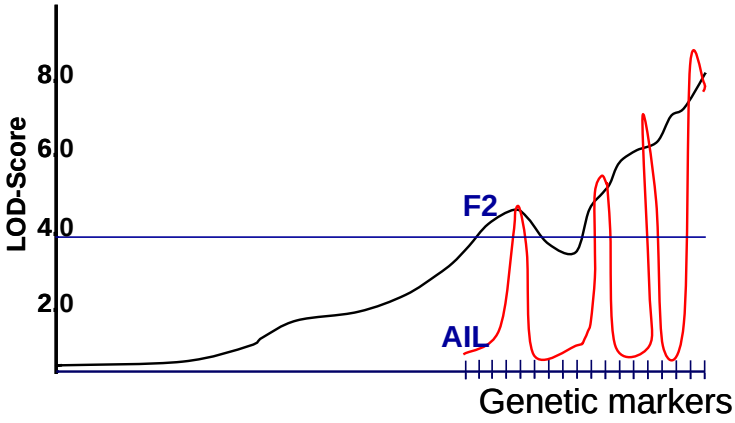
Figure 6a,



number of generations of its advanced population (Figure 6b). AIL may well provide sufficiently good mapping resolution to narrow a QTL to <20 candidate genes.

We initiated analysis in AIL (Paper IV), a densely recombinant experimental population, to enable high-resolution fine-mapping on a genome wide scale. The F7 AIL is the seventh intercross generation, which stems from an initial cross between arthritis susceptible rat strain DA, and the arthritis resistant strain PVG.1AV1 with the DA MHC haplotype[184].

Figure 6b, Comparison of mapping resolution in AIL versus F2



QTL mapping software

The two softwares used in this thesis are MAPMAKER and R/qtl. MAPMAKER was the first publicly available mapping-software[185]. With this program it is possible to perform linkage maps (multipoint analysis), and interval mapping is done by MAPMAKER/QTL (wilcoxon rank-sum test). R/QTL has many more features[186,187]. It enables single- or two- QTL mapping using Haley-Knott regression, EM-algorithm, Kruskal-wallis for Nonparametic testing, and provides other models such as binary trait and two-parts models. Multiple-QTLs can be tested with multiple imputation. It is also possible to calculate LOD-threshold by permutation tests, and to investigate additive effects and epistatis.

From QTL to gene

A cornerstone in phenotype to genotype research is the presence of genetic markers in the QTL interval, either microsatellites or SNPs. However there are few SNPs identified in the rat, consequently we have had to rely on the occurrence of satellite markers in our regions of interest. The release of the draft BN rat genome sequence has enabled straightforward identification of new markers[188]. In fact the microsatellites are spaced ~100kb through the genome. The rat genome sequencing project has taken experimental genetic research on a giant leap into the 21st century. Procedures that would have taken months or years to accomplish in the lab can now be performed on a coffee-break in silico.

.
Before the rat genome was sequenced it was more difficult to proceed into positional cloning after a QTL interval had been narrowed to ~1 cM. Genes harboring the interval had to be identified by either chromosome walking or exon trapping. In chromosome walking the QTL flanking markers sequences had to be identified on bacterial artificial chromosomes BACs, or plasmide artificial chromosomes PACs from a rat genome library[189]. The identified BAC or PAC was then sequenced and a new marker in different placements hopefully in the direction of the second flanking marker was designed. A contig was then produced from overlapping BACs or PACs between by walking along the sequence produced between the two original satellite sequences. In exon trapping BACs or PACs are also used to identify expressed RNA within the genomic fragment harboring the satellite sequence. The identified micro satellite sequence positive BACs or PACs are cut into smaller pieces and cloned into a mammalian expression vector. This will enable mRNA expression from the BACs or PACs in mammalian cells. Expressed exons are harvested and sequenced which will identify expressed genes within the PAC hence within the QTL[190].
Identifying the susceptibility gene still demands sequencing however, in order to delineate the causal polymorphism by sequencing both the susceptible and resistant strain. The identified polymorphisms are often determined in a variety of different inbred strains from disease susceptible to resistant as well as outbred strains or wildtype rats. It is also important to verify the functional effect of the polymorphism identified by functional testing of the candidate genes. This could be done by "knock-in" technique or by gene targeting, but this is only feasible in certain species[191].

The aim of this thesis:

The overall goal of this thesis was to identify genes that regulate arthritis in the rat, and to determine if the homologous human genes influence rheumatoid arthritis.

Specifically, I aimed:

- to determine combinations between arthritis inducers and rat genomes (strains and crosses) that result in experimental arthritis suitable for genetic mapping.

- to use the experimental arthritis systems for positional mapping of arthritis regulating genes on rat chromosome 4 and 10 regions that were previously linked to arthritis susceptibility.

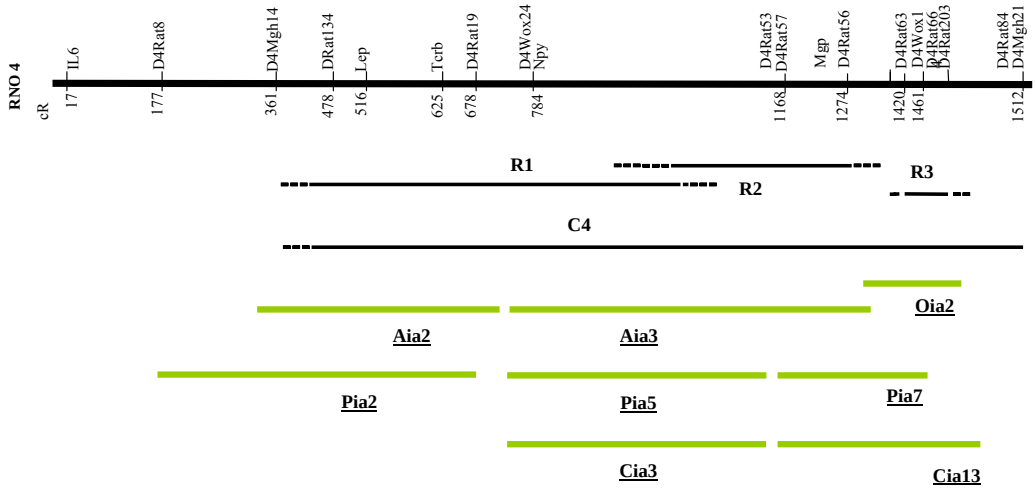
- to determine if human homologs of the identified arthritis-regulating rat genes associate with rheumatoid arthritis.

Results and discussion

Mapping and Functional Characterization of Rat Chromosome 4 Regions that Regulate Arthritis Models and Phenotypes in Congenic Strains (Paper I)

The distal part of rat chromosome 4 is a region harboring many overlapping QTLs that regulate various inflammatory diseases[136-141,153,155,159,161,162,192], eight of these QTLs regulate arthritis and they constitute 40% of the total number of arthritis regulating QTLs in the rat. The first mapped QTL was Oia2, and our research group had initiated the production of congenic strains for this region. This provided a basis for mapping of one or several QTLs, which was my initial aim. Therefore we aimed to determine the common genetic regulation of chromosome 4 alleles in four different arthritis models using congenic strains.

Figure 7. Rat chromosome 4, congenic strains; C4, R1, R2, R3, and eight arthritis regulating QTLs.



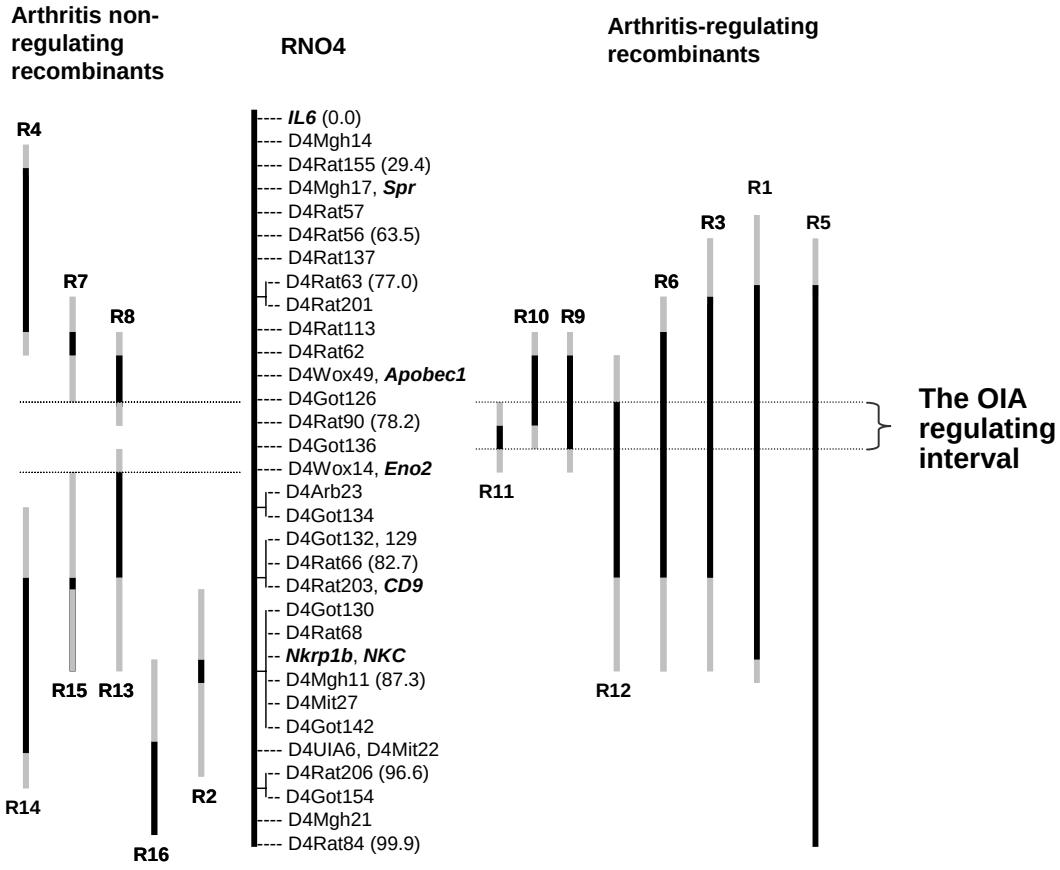
By selective breeding we produced a DA strain that harbors genes from arthritis resistant PVG.1AV1 within a 70 cM interval on chromosome 4 (C4 in Figure 7). In addition, three subcongenic strains were developed, that represent different QTLs within this interval (R1-R3). The congenic strains were tested in four different experimental arthritides induced by either collagen type II (antigen dependent), mycobacteria (bacteria), pristane or squalene (two non immunogenic oils). The 70 cM interval mediated arthritis protection in all 4 RA models i.e. collagen-induced arthritis (CIA), mycobacteria-induced arthritis (MIA), pristane induced arthritis (PIA), and squalene-induced arthritis (SIA). When the interval was divided into three overlapping intervals (R1, R2, and R3 in Figure 7), the 10 cM R3 subcongene representing the QTL *Oia2* did downregulate SIA and CIA. Genes in R3 also caused a change in anti-collagen antibody isotype-levels towards a pattern similar to that of

PVG.1AV1. The subcongenic R2 indicated a gene regulating arthritis in males located in a 20 cM interval overlapping with the QTL *Pia5*.
The QTL *Oia2* regulates arthritis induced both by the non-immunogenic immunostimulant squalene, and by cartilage collagen. In CIA, it also skews anti-collagen isotype profiles, suggesting qualitative regulation of autoimmunity. Interestingly the homologous human chromosomal region 12p12-p13 has also been linked to RA, suggesting that genetic and functional dissection of this locus will give clues to disease pathways leading to joint inflammation.

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

The congenic strains used in paper I, were evaluated also in oil-induced arthritis (OIA), and arthritis development was completely prevented in C4 and R3. Given that the congenic strains displayed complete protection against OIA but only partial downregulation in SIA and CIA, further mapping of this interval was performed in OIA.
In paper II reciprocal transfer of RNO4-intervals establishes that overlapping *Oia2* alleles from DA confer arthritis susceptibility, whereas LEW.1AV1 and PVG.1AV1 alleles confer resistance.
Oia2 was then fine-mapped using 18 Oia2 Subcongenic strains with PVG.1AV1 alleles introduced on DA genome. We were able to fine-map *Oia2* in oil-induced arthritis (OIA) to an interval of approximately 0.8 cM (Figure 8). This interval harbored gene sequences similar to *C3AR1*, Ribosomal protein L7, a cluster of C-type lectins *(Clecsf6, Clecsf8, Clecsf9, and Clecsf10), C1s, C1r* and *CD163,* according the Rat genome assembly from 24 of October 2002.
Alleles in *Oia2* regulated arthritis in an additive fashion, and determined arthritis incidence, severity, and day of onset, in both males and females. Besides macroscopic joint-inflammation, *Oia2* also regulated other oil-induced phenotypes, including lymphoplasia and plasma levels of the inflammation marker α1-acid glycoprotein. Our results demonstrate transformation of multifactorial arthritis into a disease specifically triggered by adjuvant-oil exposure, which displays dichotomous phenotypes, and maps as a monogenic trait.

Figure 8, The arthritis regulating interval determined in 18 *Oia2* recombinant congenic strains



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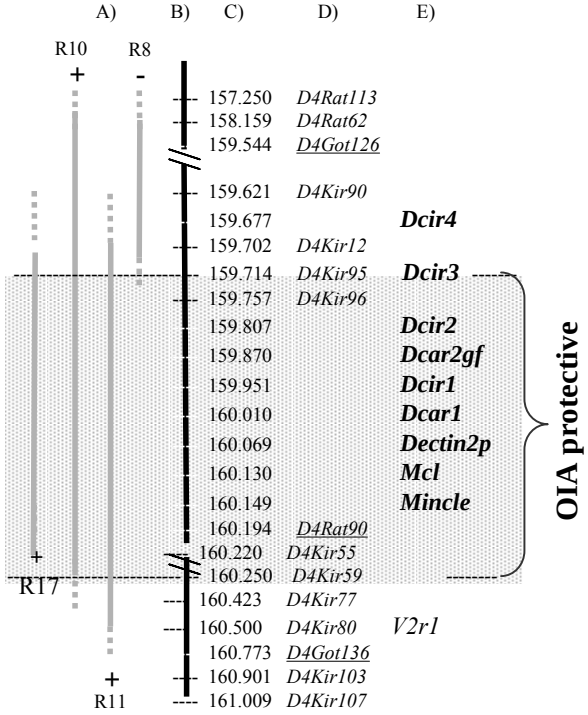
A gene complex encoding lectin-like receptors influences arthritis in rats and humans (Paper III)

In paper III we aimed to identify the arthritis regulating gene(s) in *Oia2*. We first fine-mapped the disease regulating effect in *Oia2* to a C-type lectin gene complex designated APLEC (antigen presenting lectin-like receptor complex.) The encoded receptors belong to the group II C-type lectin-like receptor family expressed mainly on antigen presenting cells (APC). A 600kb congenic strain with PVG.1AV1 alelles in *Aplec* confirmed protection against oil-induced arthritis. Sequence analysis looking for polymorphisms between DA and PVG.1AV1 identified a nonsense mutation in *Dcar1*. Polymorphisms in *Dcir1*, and *Dcir2* did not effect expression levels. *Dcir3*

had identical sequences but did differ in expression levels. *Mincle* differed both in sequence and expression levels but results strongly indicate *Dcar1* as the determining gene since the polymorphism leads to an aberrant protein.

The corresponding human gene cluster is located on chromosome 12p13.31 and encodes three genes similar to the mutated rat *APLEC* encoding gene, including dendritic cell immunoreceptor (*DCIR*). Three SNPs in *DCIR* and one in *MINCLE* were tested. Association of *DCIR* with rheumatoid arthritis was established after determination of single nucleotide polymorphisms in patients and matched controls. The SNP rs1133104 is located in the 3' UTR region of *DCIR*. The *DCIR* SNP showed association with RA (p=0.008). After stratification for rheumatoid factor the association for seronegative patients versus all controls was still clear (p=0.006, p_{corr}=0.01). We conclude that the described lectin-like receptor genes influence arthritis in both humans and rats.

Figure 9. The *OIA* regulating interval and the *Aplr* complex



A) Recombinant congenic strains
B) Rat chromosome 4
C) Physical position (Mb)
D) Microsatelite marker
E) *Aplr* gene

Identification of Candidate Disease Genes by High-Resolution Linkage Mapping of Arthritis Regulating Rat Chromosomal Intervals in Advanced Intercross Lines (Paper IV)

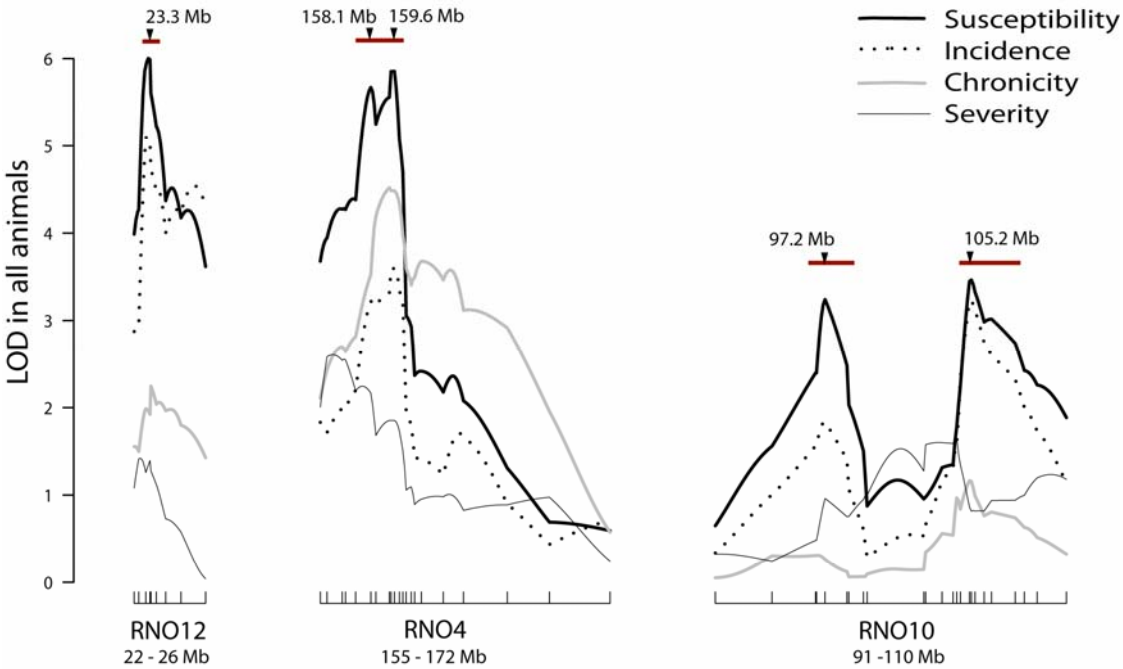
The previous genetic mapping studies (paper I-III) in this thesis were based on congenic mapping. In paper IV I employ and evaluate a fine-mapping tool that is complementary to congenic mapping, and initiated analysis in an advanced intercross line (AIL), a densely recombinant experimental populations that facilitates fine-mapping on a genome wide scale.

The chromosomes of each individual in the experimental population are a unique patchwork of short fragments from either of the founder strains. The F7 AIL is the seventh intercross generation that stems from an initial cross between arthritis

susceptible rat strain DA, and the arthritis resistant strain PVG.1AV1 with the DA MHC haplotype. Differentially arthritogenic oils were used to trigger joint inflammation in F7 progeny. A high incidence, 57 %, was recorded in 422 pristane-injected animals.

All 422 rats were subsequently genotyped over three genomic regions that have documented impact on arthritis, a previously identified 20Mb QTL, *Oia3,* on rat chromosome 10[176], and the two newly identified arthritis susceptibility genes/complexes; the *Ncf1*gene on chromosome 12[179], and the *Aplec* complex on chromosome 4 (Paper III). The chromosomal region upstream of *Aplec* was also fine mapped to search for additional susceptibility genes. All three chromosomal regions were analyzed for arthritis susceptibility, arthritis severity, incidence of arthritis, day of arthritis onset, and chronic arthritis. They were also scanned for genetic interactions and additive effects.

Figure 10. Pristane-induced arthritis in 422 F7(DAxPVG.1AV1) animals



Maximum LOD scores were recorded only 100 kb from *Ncf1* on chromosome 12, and only 77 kb from the *Aplec*. Additional QTLs were identified near *Aplec*, including one that regulates chronic arthritis in females (Figure 10). Novel QTLs were also defined on the chromosome 10 terminus, including two loci involved in mutual epistatic control of chronicity. All other identified loci showed additive effects with at least one other QTL. Two additive locus on chromosome 10 were identified. *Pia29*

(Table 7) harbor *Prkca*, a gene that has been associated to MS[146]. A second QTL on chromosome 10, *Pia30*, showed maximum LOD scores 50 kb telomeric of an Ig-like receptor gene cluster that was previously implicated in human psoriatic arthritis, i.e. PSORS2[151]. Similar to *Aplec*, this mapped gene cluster encodes multiple activating and inhibiting receptors expressed by key cells of the immune system[193]. Since SNPs in *DCIR*, one of the human homologs to one of the genes in *Aplec* associate with RA, we suggest that the newly identified sets of candidate disease genes represent important etiopathogenetic clues, especially in light of the accompanying information on genetic interactions.

Table 7. Candidate disease genes in mapped QTLs on RNO10

QTL	Position (Mb)	Marker	LOD	Gene	Gene Name
	96.7	D10Rat15	2.39		
	96.81			LOC360649	s.t. Ac2-210 (Putative ribosomal protein L22)
	96.89			LOC363689	s.t. ribosomal protein S23
	96.9			LOC287772	s.t. proteasome 26S non-ATPase subunit 12
	97,00			LOC287773	s.t. mouse helz (Helicase with zinkfinger)
	97.11			Cacng1	calcium channel, voltage-dependent, gamma subunit 1
Pia29	97.15			Cacng4	voltage-dependent calcium channel gamma-4 subunit
RNO10 ►	97.2	D10Rat13	3.49		
97.2 Mb	97.31			Cacng5	voltage-dependent calcium channel gamma-5 subunit
	97.38			Prkca	protein kinase C, alpha
	97.74			LOC287774	s.t. beta-2-glycoprotein I (ApoH)
	97.88			LOC28777	s.t. MGC33887 (hypothetical chromosomal segregation ATPase)
	98.14			LOC36369	s.t. 1700001M19Rik protein (putative exonuclease)
	98.3			Axin2	axin2
	98.4	D10Mco15	2.48		
	104.6	D150F	2.33		
	104.5			LOC287803	s.t. tweety homolog 2
	104.75			LOC287805	s.t. retinoic acid inducible protein 3
	104.79			LOC303664	s.t. triggering receptor expressed on myeloid cells 5
	104.87			LOC363701	s.t. dendritic cell-derived Ig-like receptor 1
	104.95			LOC287809	s.t. leukocyte mono-Ig-like receptor2
	105.01			LOC363702	s.t. Slc25a5
	105.02			LOC287813	s.t. Igsf7 protein
	105.09			LOC303666	s.t. dendritic cell-derived Ig-like receptor 1
	105.1			LOC287811	s.t. Adenine nucleotide translocator 2 (ANT 2)
	105.11			LOC360655	s.t. dendritic cell-derived Ig-like receptor 1
	105.14			LOC363703	Ribosomal protein L6
Pia30	105.14	D10Got155	3.21		
RNO10 ►	105.15	D10Got158	3.23		
105.2 Mb	105.21			LOC287818	s.t. DC-derived Ig-like receptor 2
	105.24			LOC363704	s.t. GTPase Rab37
	105.25			LOC59114	ERM-binding phosphoprotein s.t. mouse slc9a3r1
	105.27			LOC303669	s.t. RIKEN cDNA 1110028N05 (Putative acetyltransferase)
	105.28			LOC303670	s.t. FLJ00021 protein (putative aminoacidtransporter)
	105.34			LOC287915	s.t. mouse grin2c
	105.36			Fdxr	adrenodoxin reductase
	105.37	D10Got157	3.11		
	105.38			LOC303671	s.t. human delta 5 fatty acid desaturase
	105.41			LOC287819	s.t. Jackson shaker
	105.42			LOC287820	s.t. mouse otop2 otopetrin
	105.43			LOC287821	s.t. mouse otop3 otopetrin
	105.44			LOC287822	s.t. CG8841-PA
	105.46			LOC360656	s.t. paraneoplastic antigen
	105.49			LOC303673	s.t. Ict1 protein
	105.9	D10Rat7	2.98		

Genes and positions were retrieved from NCBI rat genome assembly 2 at http://www.ncbi.org.
s.t. Similar to

Concluding remarks

The primary focus of this thesis has been to fine-map the *Oia2* locus. This QTL was previously identified in a F2 (DA x LEW.1AV1) intercross[159]. The *Oia2* locus harbored the natural killer cell gene complex (NKC). DA rats are deficient in eliminating allogeneic target cells, and the deficiency in alloreactivity had previously been mapped to the NKC[194] in a DA x PVG intercross. Our group formulated the hypothesis that NKC genes could be the arthritis-regulating gene(s) in*Oia2*. In this hypothesis, DA would carry the allele that leads to arthritis susceptibility, and to aberrant NK cell mediated natural cytotoxicity. In contrast, LEW and PVG would carry alleles that lead to arthritis resistance, and to normal NK cell mediated natural cytotoxicity. However, PVG alleles had not been evaluated in arthritis and the hypothesis could be rejected if the alleles were found not to down-regulate arthritis. To challenge the hypothesis, our group therefore tested if *Oia2* could be confirmed in a DA (DA x PVG.1AV1) backcross[160]. Indeed *Oia2* was confirmed in the DA x PVG.1AV1 strain combination. Thus, the hypothesis was still valid when my studies were initiated. In addition to *Oia2* eight other arthritis linked QTLs have been assigned to rat chromosome 4 (Figure 4). Since the QTLs identified by genome wide linkage analysis usually cover a large chromosome region and do not constitute an exact position, it was essential to establish experimental systems that allow a closer definition of the chromosomal regions, and of the phenotypes they regulate. The first two studies were concurrently performed (Paper I and II).

Reciprocal strains for *Oia2* determined the Oia2 effect in DA, LEW.1AV1 and PVG.1AV1 C4 alleles /genome combinations. The congenic strain C4 and R3 (Figure 7), were then tested in additional experimental arthritis models to determine the most suitable model for genetic mapping. OIA was the most appropriate, since arthritis was completely prevented in the congenic strains. In addition R1 and R2, two recombinants that overlap arthritis linked QTLs centromeric of *Oia2*, were also tested but showed no significant regulation. OIA was selected for recombinant-mapping. We were intrigued to find that NKC could be discarded as being *Oia2* since NKC-harboring recombinants showed no protection. Finally, *Oia2* was mapped to 0.8 cM using 18 overlapping recombinant strains. This mapping was later refined to *Aplr* (paper III). One human homolog of a gene in this complex did present significant association to RA in a patients/control material. Speculating that there may be additional susceptibility QTLs close to *Aplr*, we performed more mapping in PIA (paper IV). The presence of additional QTLs was confirmed and novel QTLs in the

Oia3 interval on chromosome 10 was identified. In addition linkage to *Ncf1/Pia4*[179] was reproduced.
Two QTLs were identified on chromosome 10. The QTL *Pia29* harbors 11 genes (Table 7) among which the most interesting are coding for Ca^{++}-channel subunits, and the protein kinase C α-subunit (*Prkca*) gene. Could this mean that *Aplr* and *Pia29* are involved in the same pathway since *Aplr* code for Ca^{++}-dependent receptors? Are certain arthritogenic pathways Ca^{++}-dependent? It is known that Ca^{++}-levels promote phagocytosis, an important mechanism in both degradation and antigen-uptake[195]. Although the direct connection is rather questionable since Ca^{++} is involved in a majority of processes. Nevertheless *Prkca* is involved in Ca^{++}- induced regulation mainly activated by antigen receptors in T cells, B cells and mast cells[196]. Protein kinase C together with diacylglycerol regulate a wide range of gene transcription programs as well as responses to chemokine and antigen receptors, thereby regulating lymphocyte adhesion, migration, differentiation and proliferation.

The QTL *Pia30* (Table 7) harbors a gene complex encoding immunoregulatory leukocyte receptors, just like the *Aplec*. However the *Pia30* gene cluster encodes receptors belonging to the immunoglobulin superfamily (IgSF). These genes have not been characterized in the rat, but the human homologs are Single V-like Ig domains. One representative molecule within the complex is *CMRF-38A* expressed on monocytes, macrophages granulocytes and subpopulations of B- and T-cells[151]. Its expression on lymphocytes is altered after mitogen exposure. Some have immunoreceptor tyrosine based inhibitory motifs (ITIMs) in their intracellular domains. Others have positively charged amino acids (aa) in the transmembrane regions (TM), which can associate with adaptor molecules that have immunoreceptor tyrosine-based activating motifs (ITAMs). Thus, the receptors in the complex can transduce opposing signals, as have previously been suggested for C-type lectin-like leukocyte receptors encoded from *APLEC* and from the natural killer cell complex (*NKC*)[197].

The human homolog to the rat *Aplr* complex represents one of the first complex disease regulating genes originally identified in an experimental model also to be associated to the corresponding human disease homolog.
A number of genes have been identified in mice or rats by genetic mapping. Apart from the previously mentioned *Ncf1*, other genes in other complex diseases have been cloned and identified in animals but have not yet been associated with the corresponding human disease; *lyp* phenotype loci gene in type 1 diabetes prone BBDP rats has been identified as Immune-Nucleotide (IAN)-related gene[198]. The

bphs locus for pertussis response in mice was identified as the histamine receptor 1 (H1)[199].
Some human disease genes have been conclusively identified; CTLA-4 a multi autoimmunity disease causing gene[200], ADAM33 in asthma[201], CARD15 in inflammatory bowel disease[202], CDPD1 in systemic lupus erythrematosis[70], and PTPN22 in aRA[63].

The Aplr-complex

In this thesis *Oia2* is mapped to a newly discovered gene complex[203]. The genes encode receptors collectively referred to as APC lectin like receptors (*Aplr*) to indicate their preferential although not exclusive expression on APCs, i.e. B cells, neutrophils, Mϕ and DC[204]. So far, there is limited information on the functional roles of *Aplr*. Carbohydrates are plausible *Aplr* ligands since the receptor extracellular structure forms a carbohydrate binding region (CRD) with preserved amino acids required for carbohydrate binding in a calcium dependent manner.
Challenge with exogenous adjuvants may lead to APC activation by endogenous signals. Such signals related to cellular stress, endoviral infection or necrosis have been referred to as natural adjuvants[93]. It appears likely, however, that *Aplr* may influence the largely undetermined interplay between APC and T cells in arthritis development[205].

In the rat, seven *Aplr* encoding genes are contained within a 472 kb chromosomal region and designated the APC lectin like receptor gene complex (*APLEC*). Similar to the *NKC* encoded killer cell lectin-like receptors the *APLEC* have structural features indicating that they regulate leukocyte reactivity by opposing signaling functions[206,207]. Thus, rat *Dcir1* and *Dcir2* carry immunoreceptor tyrosine-based inhibitory motifs (ITIMs) in their cytoplasmic domains, predicting inhibitory function through recruitment of protein tyrosine phosphatases to phosphorylated ITIMs following receptor ligation[208]. Functional studies of mouse *Dcir1* did indeed demonstrate ITIM-dependent inhibition of B-cell receptor mediated Ca^{++} mobilization and protein tyrosine phosphorylation. Conversely, *Mincle* has a positively charged amino acid in the transmembrane domain, suggesting activating function through association with adapter molecules carrying immunoreceptor tyrosine-based activating motifs (ITAMs). Some of the APLR carry neither ITIMs nor charged transmembrane (TM) residues suggesting roles other than signaling. However, human *BDCA-2* and mouse *Dcar* have been shown to activate calcium mobilization and protein tyrosine phosphorylation, although they lack a positively charged amino acid in the TM domain, indicating as yet unidentified signaling motifs,

or signaling through heterodimer formation. Altogether, the evidence for inhibitory and activating signaling functions indicate that *Aplr* may function by fine-tuning leukocyte responses through the computation of multiple opposing signals[209].

The following section explains some of the findings concerning the homologous genes in the *Aplr* complex, either from mouse or humans:

DC immunoinhibitory receptor (*Dcir*): Inhibitory receptor expressed on CD14+ derived DC, neutrophils, B-cells and monocytes/ MΦ. *Dcir* is co-expressed with MHC class II, indicating it to be expressed on APC. DCIR expression decrease after LPS or CD40L stimulation, hence expression is down-regulated upon maturation. This down-regulation is also observed in blood neutrophils after stimulation with pro-inflammatory cytokines such as TNF, IL-1α, Il-4, and GM-CSF[210-213].

DC immunoactivating receptor (Dcar): *Dcir* and *Dcar* are highly homologous in their CRD domains. These receptors are identified structurally and functionally capable as paired C-type lectin receptors expressed on APC, although their function is not known. DCAR is expressed in DC, and to a lower extent on in Monocytes, and MΦ[214].

DC-associated C-type lectin (Dectin)-2: Expressed in Monocytes, DC and MΦ. *Dectin-2* is indicted to be involved in UV irradiation-induced tolerance, and may interact with ligands on T-cells[215,216].

Blood DC antigen (BDCA)-2:. BDCA-2 has not been identified in the rat, but is located close to the human APLEC. In human it is expressed mainly on $CD11c^{-}$ $CD123^{bright}$ plasmacytoid DC. Expression is down-regulated by IL-3. Interestingly, ligation of BDCA-2 suppress INF α/β production[217].

Macrophage C-type lectin (MCL): There is some evidence that MCL, one of the genes in *Aplec,* is engaged in antigen capture through receptor mediated endocytosis leading to antigen presentation and induction of T cell responses[218].

Macrophage-inducible C-type lectin (Mincle): It is expressed mainly on MΦ. Expression is induced by proinflammatory cytokines, INF-γ TNF-α, and IL-6. It has been suggested that a transcriptional target of IL-6 regulate Mincle expression[219].

On the background that APLR may influence APC in several ways, many of the *APLEC* genes can now be arthritis regulating, and none of the investigated receptors can be excluded from potential disease regulation, either alone or in concert. However, the rat *Dcar1* gene does stand out as a strong candidate, with the early nonsense mutation rendering it non-functional in the DA strain, and with the low mRNA expression levels probably reflecting nonsense mediated decay[220].

Through analyses of big case-control material we established association of a *DCIR* SNP with seronegative but not with seropositive RA. However this result does not prove that *DCIR* has functionally important variations, neither does it exclude that other *DCIR* alleles, or other APLR, may influence arthritis. But is does provide a strong suggestive indication that sequences in this gene cluster influence human RA.

The search for experimental systems to map arthritis susceptibility regions and subsequent genetic dissection of experimental arthritis in the rat has led to the identification of *Aplec*, a complex of immunologically important receptors. Additional functional studies of the *Aplec* encoding receptors is of importance, as well as producing single-gene congenics harboring each *Aplr* gene, to investigate the arthritis protective phenotype of each of these gene.
Further association studies of the human homologs to rat *Aplr* genes in RA and possibly other complex diseases is also an important assignment in the future.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Reumatoid artrit (RA) är en kronisk inflammatorisk sjukdom som främst drabbar perifera leder och leder till allvarlig nedsättning av rörelseförmåga och lidande.

Sjukdomsuppkomsten är till största deled okänd, men en komplex inblandning av gener och miljöfaktorer anses bidra till dess uppkomst. Att identifiera sådana faktorer försvåras av att sjukdomen även är kliniskt heterogen, det vill säga två människors RA kan vara mycket olika, både med avseende på svårighetsgrad, svar på behandling och systemiska manifestationer.

Mycket av den komplexitet RA uppvisar kan begränsas med hjälp av att göra studier i experimentella modeller, tex råtta. Både miljö- och genetiska faktorers påverkan kan kontrolleras avsevärt.Vissa inavlade råttstammar är väldigt benägna att utveckla en RA-lik artrit efter en injektion av ett artritogent ämne. Vissa oljor ger artrit i råttor, så även microbacteriella substanser och broskprotein. Denna artrit beror på den genetiska bakgrunden hos den artritkänsliga råttstammen. DA råttan är mycken artritbenägen, medan PVG och LEW råttor inte är det. Genom att korsa DA råttor med PVG och sedan korsa avkomman får man en population av djur som är en blandning mellan de två. Vilka gener som kommer från den ena eller andra ursprungsstammen kan följas med hjälp av genetiska markörer. Efter att dessa djur injicerats med t.ex en mineralolja utvecklar vissa djur artrit. Genom att koppla en viss region på kromosomen till utfallet av sjukdom kan vissa artritkopplade regioner identifieras.

Denna avhandling innehåller en serie studier av kongena råttstammar där man genom selektiv avel har flyttat en kromosomal region från en artritresistent råttstam till en artritkänslig råtta, och undersökt hurvida denna region kan nedreglera eller förhindra artrituppkomst eller ej. Den nya råttstammen kommer att ha DA gener i hela genomet förrutom i det avsedda intervallet där det (i detta fallet) finns PVG versioner av generna. Fem olika artritogena substanser udersöktes; kollagen, squalen, olja, mycobacterie, samt pristan. Modellen för olje-inducerad artrit befanns mest lämpad för vidare finmappning av den kromosomala regionen. Det kromosomala intervallet minskades genom att testa 18 olika rekombinanta kongener, med olika versioner av kortare PVG intervall än den ursprungliga kongenen. Från artritstudier i dessa stammar kunde ett kort intervall (endast 0.8cM) indentifieras, och efter vidare analys av dessa stammar kunde ett komplex av gener kallat Aplec identifieras. Genom att analysera polymorfismer i DCIR (en human homolog till Dcir i råtta och vidare en av generna i Aplec) i ett patientmaterial med RA patienter och friska kontroller så kunde det påvisas att DCIR är kopplad till RA. Detta är den första gången en tidigare

identifierad rått-artritgen uppvisat koppling mellan den mänskliga versionen av samma gen och RA.

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"Writing is good, thinking is better. Being smart is good, being patient is better"

From Siddhartha
By Hermann Hesse

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