Thesis for doctoral degree (Ph.D.) 2009

Genetic Studies of Arthritis on a Novel C-type Lectin Receptor Gene Complex

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Stockholm 2009

Published by Karolinska Institutet. Printed by Larserics Digital Print AB.

ISBN 978-91-7409-335-3

To my family

ABSTRACT

Rheumatoid Arthritis (RA) is a chronic, systemic autoimmune syndrome primarily affecting peripheral joints. The etiology of RA is largely unknown but complex interactions between genetic and environmental factors contribute to the disease. Arthritis models more or less resembling RA are used to search for clues.

Our laboratory recently identified the antigen presenting lectin-like receptor complex (*APLEC*) as a genetic determinant for susceptibility of DA rats to arthritis induced by incomplete Freund´s adjuvant oil (IFA). The extent to which *APLEC* would influence other RA models and other immunity-related traits was unclear, and functional knowledge was lacking.

In this thesis, I developed an *APLEC* congenic strain (designated R17) with DA background but carrying *APLEC* from an arthritis-resistant rat strain (PVG). Any observed phenotypic difference between DA and R17 rats will thus depend on genetic variation in *APLEC*.

By comparing DA and R17 rat in six different RA models, it is demonstrated that *APLEC* regulates several RA models and phenotypes, sometimes depending on sex. A possible mechanism might be regulation of autoimmunity because in collagen-induced arthritis DA and R17 rats differed in their serum levels of auto-antibodies and transcripts for pro-inflammatory cytokines, including IL-17. The much higher IL-17 mRNA levels in DA rats were subsequently demonstrated also in oil-induced arthritis (OIA, induced by IFA), and it was observed that the transcripts appear up to 10 days before disease onset in DA rats. Since IFA is a non-immunogenic stimulator of the immune system, we next examined innate immunity related phenotypes.

Following infection with *S. aureus* and *Herpes Simplex Virus*, survival rates indeed differed between DA and R17 rats. Furthermore, bone marrow macrophages from the two strains also responded differently when stimulated *in vitro* with a panel of microbial agents, e.g. in terms of IL-6 and IL-10 secretion. Altogether, these data suggest that genetic variation in *APLEC* may play a role in many inflammatory diseases.

In pristane-induced arthitis, we searched for evidence of genetic interactions between *APLEC* and other genes, while at the same time performing fine-mapping of the arthritis-regulating QTLs *Pia4*, *Oia3*, and *Oia2* (which harbors/equals *APLEC*). By high-resolution mapping in G7 advanced intercross line (AIL), novel arthritis-regulating QTLs with limited sets of candidate disease genes were identified, as well as putative genetic interactions, possibly including *APLEC*.

Altogether, our results provide incentive for further studies e.g. on the role of *APLEC* in various inflammatory diseases in the rat, and in other species. In humans, it should be interesting, e.g. to use the results in this thesis to perform guided genetic studies on the human *APLEC* encoded C-type lectin-like receptors, which are expressed on leukocyte subsets, including macrophages and dendritic cells (i.e. DLEC/ BDCA-2, DCIR, DECTIN-2, MINCLE and MCL).

LIST OF PUBLICATIONS

I. Liselotte Bäckdahl, Jian Ping Guo, Maja Jagodic, Kristina Becanovic, Bo Ding, Tomas Olsson and Johnny C. Lorentzen.
Definition of arthritis candidate risk genes by combining rat linkage-mapping results with human case control association data.
Annals of Rheumatic Diseases, 23 Dec 2008 [Epub ahead of print]

II. Jian Ping Guo, Liselotte Bäckdahl, Monica Marta, Linda Mathsson, Johan Rönnelid and Johnny C. Lorentzen.
Profound and Paradoxical Impact on Arthritis and Autoimmunity of the Antigen Presenting Lectin-like Receptor Complex.
Arthritis & Rheumatism. 2008 May; 58(5):1343-1353

III. Line M Flornes, Jian Ping Guo, Erik Dissen, Michael R. Daws, Johnny C. Lorentzen and Sigbjørn Fossum.
APLEC receptors influence IL-17 response in oil-induced arthritis.
Manuscript

IV. Jian Ping Guo, Margareta Verdrengh, Andrej Tarkowski, Stefan Lange, Eva Jennische, Johnny C. Lorentzen* and Robert A. Harris*.
The rat antigen presenting lectin-like receptor complex influences innate immunity and development of infectious diseases.
Accepted by Genes and Immunity

* These authors contributed equally to the work.

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LIST OF ABBREVIATIONS

ACPA	Anti-citrullinated protein antibodies
AIA	Adjuvant-induced arthritis
AIL	Advanced intercross line
APCs	Antigen-presenting cells
APLEC	Antigen presenting lectin-like receptor complex
BM	Bone marrow
C5	Complement component 5
CFA	Complete Freund's adjuvant
CI	Confidence interval
CIA	Collagen-induced arthritis
CII	Collagen type II
CLRs	C-type lectin-like receptors
CNS	Central nervous system
CRD	Carbohydrate recognition domain
Csk	C-terminal Src tyrosine kinase
DC	Dendritic cells
Dcar	Dendritic cell immunoactivating receptor
Dcir	Dendritic cell immunoreceptor
DC-SIGN	DC-specific ICAM3-grabbing nonintegrin
Dectin	Dendritic cell associated C-type lectin
DLEC	Dendritic cell lectin
DLN	Draining lymph node
DTH	Delayed type hypersensitivity
EAE	Experimental autoimmune encephalomyelitis
GIA	β-glucan induced arthritis
HLA	Human leukocyte antigen
HSV	Herpes simplex virus
IFA	Incomplete Freund's adjuvant
IFN-γ	Interferon-γ
Ig	Immunoglobulin
IL	Interleukin
IRF5	Interferon regulatory factor 5
ITAM	Immunoreceptor tyrosine-based activating motif
ITIM	Immunoreceptor tyrosine-based inhibitory motif
KLRs	Killer cell lectin-like receptors
LD	Linkage disequilibrium
LOD	Logarithm of odds
Mcl	Macrophage C-type lectin
MHC	Major histocompability complex
Mincle	Macrophage inducible C-type lectin
MS	Multiple sclerosis
MΦ	Macrophage

NKC	Natural killer cell gene complex
OIA	Oil-induced arthritis
OR	Odds ratio
PADI4	Peptidylarginine deiminases citrullinating enzyme 4
PAMPs	Pathogen-associated molecular patterns
PIA	Pristane-induced arthritis
PPRs	Pattern recognition receptors
PTPN22	Protein tyrosine phosphatase non-receptor 22
QTLs	Quantitative trait loci
RA	Rheumatoid arthritis
RFs	Rheumatoid factors
RNO	Rat chromosome (Rattus norvegicus)
S. aureus	Staphylococcus aureus
SE	Shared epitope
SIA	Squalene-induced arthritis
siRNA	Small interfering RNA
SLE	Systemic lupus erythematosis
SNPs	Single nucleotide polymorphisms
STAT4	Signal transducer and activator transcription 4
TGF-β	Transforming growth factor-β
Th cells	T helper cells
T1D	Type 1 diabetes
TLRs	Toll-like receptors
TNF-α	Tumour necrosis factor-α
TRAF1	TNF receptor-associated factor1

1 INTRODUCTION

1.1 IMMUNE RESPONSE AND AUTOIMMUNITY

Our bodies are constantly exposed to pathogens such as bacteria, viruses, fungi and parasites. To stay healthy we have developed several lines of immune defense mechanisms to protect us from these pathogens. Our first line of defense is the innate or non-specific immune system, which is made up of several distinct components. Firstly, we have physical barriers such as skin and epithelial linings that can prevent entry of these foreign agents and prevent infection from becoming established. Next, if these barriers are broken and pathogens breach for example the epithelium, there are phagocytic cells and other cell types available to control or destroy the infectious organisms. One of the most important of these cell types is macrophages, which can express pattern recognition receptors (PPRs) such as toll-like receptors (TLRs) and C-type lectin-like receptors (CLRs) to recognize evolutionarily conserved structures on pathogens. Through recognition of pathogens, macrophages become activated, and can engulf and destroy the invaders. Furthermore, macrophages can serve as antigen-presenting cells (APCs) to process and present foreign antigens during activation of the second line of defense, the adaptive immune system.

The adaptive immune system is characterized by T cells specific for antigens derived from the invading pathogens, and by B cells producing antibodies that bind to these antigens. T cells and B cells, once primed, initiate a highly efficient response to the pathogens and immunologic memory to encountered antigens is also developed. The innate and adaptive immune systems co-operate to fight off invading pathogens.

For a functional immune response, not only immune cells but also proteins such as cytokines have an important role. As communicators between immune cells, different cytokines have distinct properties, e.g. pro-inflammatory or anti-inflammatory. Common pro-inflammatory cytokines include tumour necrosis factor (TNF)-α, interleukin (IL)-1β, interferon (IFN)-γ, IL-2 and IL-17 that are involved in the initiation and amplification of immune responses. In contrast, IL-4, IL-10 and transforming growth factor (TGF)-β are considered as typical anti-inflammatory cytokines which control the pro-inflammatory cytokine response. The cytokine IL-6 has pleiotropic functions, being both pro- and anti- inflammatory (Haddad et al., 2005; Park and Pillinger, 2007; Tilg et al., 1997; Xing et al., 1998).

During an immune response the immune system needs to distinguish self from non-self antigens. Evolution has thus created an intrinsic mechanism denoted self-tolerance to avoid lymphocytes from self-reactive actions. Self-tolerance can be divided into two categories-central and peripheral tolerance. Central tolerance occurs within bone marrow (B cells) and thymus (T cells), in which T cells and B cells are selected by undergoing positive and negative selections. Both selections are dependent on major histocompability complex (MHC) recognition. But central tolerance does not ensure deletion of all autoreactive cells and some self-reactive cells do reach the periphery. In this case peripheral tolerance acts to minimize the risk of harmful autoimmune response. The peripheral tolerance includes the suppression of autoreactive cells by regulatory T cells and the generation of hyporesponsiveness (anergy) in lymphocytes which encounter antigen in the absence of co-stimulatory signals. Central and peripheral tolerance mechanisms are powerful in synergy. However, sometimes self- tolerance can be broken and self-reactive cells start attacking endogenous cells, tissues and organs, and the consequence of this could be the development of an autoimmune disease.

Approximately 3-5% of the populations worldwide suffer from autoimmune diseases (Cooper and Stroehla, 2003). Normally autoimmune diseases are divided into two classes: organ- or tissue-specific and systemic. An organ-specific autoimmune disease is one in which the immune response is directed toward antigens in a single organ. Examples are multiple sclerosis (MS) and type I diabetes (TID). In systemic autoimmune diseases the immune system affects many different organs, tissues and cells of the body. Examples include rheumatoid arthritis and systemic lupus erythematosis (SLE). Most autoimmune diseases are complex diseases, which tend to be chronic and manifest clinically heterogeneous conditions with more or less unclear pathogeneses.

1.2 RHEUMATOID ARTHRITIS (RA)

RA is a chronic, systemic autoimmune disease primarily affecting peripheral joints and leading to joint deformation and ultimately disability. In RA, multiple joints are usually inflamed in a symmetrical pattern and the symptoms in the course of disease include fever, fatigue, pain, stiffness in joints, and weight-loss. In addition, RA may be associated with serious extra-articular disease involving cardiovascular, renal and pulmonary functions, as well as the mucosa of the mouth and eyes. Altogether, the disease may lead to a 5-15 year shorter lifespan for patients with RA, if proper treatment is not provided (Callahan and Pincus, 1995). According to the American College of Rheumatology 1987 revised criteria (Table 1), a diagnosis of RA requires that criterion 1-4 must persist for more than 6 weeks and at least four of the seven criteria have to be fulfilled. RA is thus a heterogeneous disease with a range of clinical manifestations, and with differences in disease severity and progression. Therefore RA is often regarded as syndrome representing a collection of diseases rather than a single disease.

Table 1. The American Collage of Rheumatology classification criteria of RA

1. Morning stiffness > 1 hour
2. Arthritis of three or more areas
3. Arthritis of hand joints
4. Symmetry of arthritis
5. Rheumatoid nodules
6. Rheumatoid factors
7. Radiographic changes

Patients fulfilling at least four of the seven criteria are classified as having RA.

RA affects approximately 0.5-1% of populations worldwide (Silman and Pearson, 2002), and the prevalence varies between different ethnic groups. Some native American-Indian populations have the highest recorded occurrence of RA with a prevalence of 6.8% (Silman and Pearson, 2002), whereas Asians and some Africans have a lower occurrence 0.2-0.3% (Shichikawa et al., 1999; Zeng et al., 1997) and 0.003-0.29% (Brighton et al., 1988; Silman et al., 1993b), respectively. More women suffer from RA than do men, with a ratio of about 3:1. The disease can start early in life, but the peak disease onset is between the age of 50 and 60 (Silman and Pearson, 2002).

1.2.1 Pathology of RA

The synovium is the primary site of pathology in RA. In established RA the synovial membrane is much thickened with massive and persistent infiltration of inflammatory cells. The infiltrating cells produce a variety of enzymes, cytokines and chemokines. This leads to increase of synovial fluid volumes and cellularity. As the disease

progresses synovial blood vessel proliferation, hyperplasia and angiogenesis allows the pannus to develop and grow. The pannus heavily infiltrated with inflammatory cells further invades and erodes contiguous bone and cartilage, eventually causing cartilage and bone destruction.

1.2.1.1 The immune cellular response in RA

In RA, joint inflammation is characterized by a complex cellular network, where T cells, B cells, macrophages, dendritic cells, neutrophils, and synovial fibroblasts all play an important role in disease pathogenesis.

Macrophages (MΦs) are numerous in the inflamed joint, both in synovial tissue and synovial fluid. Besides possessing broad proinflammatory and destructive capacities, and contributing to inflammation and joint destruction both in the acute and chronic phases of RA, they can also function as antigen-presenting cells that contribute to the initiation of RA (Kinne et al., 2000). Furthermore, activation of the monocytic lineage in RA is not only restricted to synovial macrophages, but extends to circulating monocytes and other cells of the mononuclear phagocyte system, including precursors of the monocytic lineage in the bone marrow (Kinne et al., 2000). **Dendritic cells (DCs)** are the major antigen-presenting cells of the immune system and have been found in synovium and joint fluid in RA, often at the center of a cluster of T cells. These DCs express MHC II, the costimulatory molecules CD40, CD80, CD86, adhesion molecules such as DC-SIGN and chemokine receptors such as CCR7. DCs can polarize T cells into different Th subsets, e.g. Th1, Th2 and Th17 depending on e.g. the cytokine environment. Dendritic cells may be central to the pathogenesis of RA and could also be logical targets for treatment (Lutzky et al., 2007; Sarkar and Fox, 2005). The effects of **T cells** in RA has been well described (Cope, 2008). Substantial evidence for T cells being involved in RA pathogenesis includes the presence of synovial T-cell infiltrates, the effects of T-cell-derived cytokines, evidence for T-cell-dependent arthritis and successful disease transfer with T cells in experimental arthritis. Different T-cell subsets have been identified in the inflamed joint, including cytotoxic ($CD8^+$) T cells, T helper (Th, $CD4^+$) cells and T regulatory (Treg) cells. T-cell responses were previously typically classified as either Th1 or Th2, based on their relative expression levels of cytokines, especially IFN-γ and IL-4. Although neither Th1 nor Th2 cytokines are present at high levels in the RA joint, IFN-γ consistently predominated over IL-4 and RA has been viewed as a Th1-driven disease (Lundy et al., 2007). Recent studies in mouse models has questioned the role of Th1 cells in RA and

identified a new T-helper subset - Th17, which are characterized by production of the highly inflammatory cytokine IL-17 (Furuzawa-Carballeda et al., 2007; Lubberts et al., 2005; Nakae et al., 2003a). The effector function of Th17 cells makes them a strong candidate for mediating joint pathology. Although **B cells** are a minority in inflamed joint they are still important in RA. That is, autoantibodies such as rheumatoid factors and anti-citrullinated protein antibodies, produced by autoreactive B cells may be involved in pathogenesis of RA. Furthermore, B cells can act as APCs that mediate synovial $CD4^+$ T cell activation and secretion of pro-inflammatory cytokines. Successful B cell depletion therapy using anti-CD20 monoclonal antibodies provides further evidence for a role of B cells in RA pathogenesis (Bugatti et al., 2007). Other inflammatory cells in RA include **Neutrophils** which are cells with high phagocytic capacity that are mainly present in the synovial fluid in RA. Infiltration of joints by immune cells requires the local production of chemoattractants, and activated neutrophils are capable of secreting powerful chemotactic factors such as IL-8. Furthermore, activated neutrophils can secrete many cytokines present in RA synovial fluid, such as TNFα and IL-1β (Edwards and Hallett, 1997). **Fibroblasts,** which in the inflamed joint are hyperplastic and invasive, and are the major source for production of matrix-degrading enzymes such as matrix metalloproteinases -1 (MMP1) and proinflammatory cytokines, leading joint cartilage and bone destruction (Muller-Ladner et al., 2007).

1.2.1.2 Autoantibodies in RA

A number of autoantibodies have been described in RA, both in their clinical associations and as disease biomarkers. Of these only two different groups of autoantibodies - rheumatoid factors (RFs) and anti-citrullinated protein antibodies (ACPA), are used in clinical practice due to their high sensitivity and specificity (Mewar and Wilson, 2006).

RFs are present in 60% to 80% of RA patients, but can also be detected in up to 5% of healthy individuals and in a substantial proportion of other systemic diseases such as Sjogren's syndrome and many systemic infectious diseases (Steiner and Smolen, 2002). IgM and IgG RFs were found abundantly in inflamed synovial tissues, and can form immune complexes by binding to the Fc region of the immunoglobulin G (IgG) molecule. These immune complexes can cause inflammation by activating complement or through cytokine production following ligation of Fcγ receptors on macrophages (Edwards and Cambridge, 1998). The correlation between RF levels and disease

severity supports a role of RF in RA pathogenesis. **ACPA** are antibodies directed towards citrullinated antigens, and they show a remarkable specificity for RA (approximately 98%), and can be detected in up to 70% of the RA patients (Hoffman et al., 2005; Schellekens et al., 2000). Studies have shown that ACPA can precede the onset of clinical symptoms of RA by several years and are present in early RA (Rantapaa-Dahlqvist et al., 2003). ACPA levels correlate with disease erosive and progressive status (Agrawal et al., 2007; Vencovsky et al., 2003). Furthermore, citrullinated proteins are localized in RA synovial tissue and ACPA can potentially be produced in rheumatic joints (Baeten et al., 2001; Reparon-Schuijt et al., 2001; Vossenaar et al., 2004). These findings strongly suggest that the citrullination of proteins, in particular intra-articular citrullinated proteins, may play a role in the pathogenesis of RA and contribute to RA development and progression.

1.2.1.3 Cytokine network in RA

In RA it is well known that numerous cytokines are expressed and are functionally active in synovial tissues, and that an imbalance between pro- and anti-inflammatory cytokine activities leads chronic inflammation and joint damage (McInnes and Schett, 2007). The cytokine network in RA is complex, where cytokines can work alone or in synergy, with different effects on different cell types. A number of cytokines have been described with proinflammatory activity in RA synovitis, including TNFα, IL-1β, IL-6, IL-7, IL-12, IL-15, IL-18, IL-23, IFN-γ, and IL-17 which in the past few years has been suggested to have a prominent role in the pathogenesis of RA (Lubberts, 2008; Paradowska et al., 2007). In contrast, other cytokines such as IL-4, IL-10 and IL-13, function mainly as anti-inflammatory molecules. Although these anti-inflammatory cytokines are present in rheumatic joints, in active RA their levels are apparently too low to neutralize the deleterious effects of proinflammatory cytokines (Isomaki and Punnonen, 1997).

1.2.2 Etiology of RA

The etiology of RA is largely unknown despite extensive research worldwide for many years. But it is believed that RA results from complex interactions between genetic and environmental factors. Many of these disease risk genes have low penetrance and remain unknown.

1.2.2.1 Genetic risk factors in RA

The genetic contribution in RA is supported by evidence from twin studies. That is, monozygotic twins display excess disease concordance (12-20%) when compared with dizygotic twins (4-5%) (Silman et al., 1993a). Further evidence is that the prevalence of RA in siblings compared with that in general population (relative risk to siblings λ_s) has been described to be between 2 and 17 (Seldin et al., 1999). It has been estimated that the genetic contribution to RA susceptibility is approximately 60% (MacGregor et al., 2000). These results demonstrate that genetic factors have a strong influence on RA susceptibility. The most important genetic risk factor associated with RA is *HLA-DRB1*, with the major effects detected in ACPA-positive RA. In Swedish Epidemiological Investigation of Rheumatoid Arthritis (EIRA) study, the odds ratio (OR) for developing ACPA-positive RA associated with *HLA-DRB1* shared epitope (SE) alleles was 5.6 [95% CI 4.2-7.2]. Compared to *HLA-DRB1*, other genetic risk factors have lower OR (e.g. *PTPN22* R620W A allele, OR 1.6 [95% CI 1.3-2.1] in ACPA-positive RA, EIRA) (Kallberg et al., 2007).

HLA The association of human leukocyte antigen (HLA) genes, particularly the *HLA-DRB1* gene, with RA susceptibility were first described in 1976 (Stastny, 1976). It still remains the best described genetic risk factor contributing to RA. The HLA gene cluster is located on chromosome 6 (6p21.3) and it was clearly demonstrated that different *HLA-DRB1* alleles are associated with RA in a wide range of populations (Orozco et al., 2006). These alleles encode MHC class II molecules that are characterized by the presence of a conserved short amino acid sequence (QKRAA, GRRAA, RRRAA) at position 70–74 in the third hypervariable region (HVR3) of the molecule, defined as the shared epitope (SE) (Gregersen et al., 1987). This region forms part of the peptide binding pocket in the DR heterodimer (Brown et al., 1993). Some *DRB1* alleles lacking the SE have been suggested to be protective against development of RA (Mattey et al., 1999). Furthermore, accumulating data from the past few years have shown that the *HLA-DRB1* SE is associated only with ACPA- positive RA but not with ACPA- negative RA (Huizinga et al., 2005; Kallberg et al., 2007; Klareskog et al., 2006b).

Even though the well known HLA region has the strongest genetic effect in RA, it is estimated that the degree of familial risk due to the HLA genes is only about 30% (Orozco et al., 2006). Thus, non-HLA genes contribute substantially to RA susceptibility.

PTPN22 One of the non-HLA genes that associated with RA is the protein tyrosine phosphatase non-receptor 22 (*PTPN22*). The first reported association of *PTPN22* with RA was published in 2004 (Begovich et al., 2004), and the study was later replicated and confirmed in many ethnical populations (Pierer et al., 2006; Seldin et al., 2005; Steer et al., 2005; van Oene et al., 2005; Zhernakova et al., 2005). The *PTPN22* gene is located on chromosome 1p13 and encodes the intracellular protein lymphoid tyrosine phosphatase, which is a negative regulator of T-cell activation through binding to C-terminal Src tyrosine kinase (Csk) (Hill et al., 2002).

The *PTPN22* association concerns the minor allele of the non-synonymous SNP rs2476601 (C1858T, R620W), and the consequence of this amino acid substitution may result in an increased risk of inappropriate T-cell activation due to less efficient binding to Csk (Begovich et al., 2004). T allele frequencies in different ethnical populations displays a geographic difference where the frequency decreases from Northern to Southern Europe, from around 12.5% in the Swedish and Finnish populations to around 2.5-7.4 % in the Spanish and Italian populations (Begovich et al., 2004; Gregersen et al., 2006). Interestingly, the T allele is almost absent in African American and Asian populations (Orozco et al., 2006). Furthermore, the *PTPN22* C1858T polymorphism is not unique to RA but common to a number of autoimmune diseases, including T1D (Ladner et al., 2005; Zheng and She, 2005), Graves' disease(Velaga et al., 2004) and SLE (Kyogoku et al., 2004; Orozco et al., 2005). There is no evidence of association of *PTPN22* with MS (Begovich et al., 2005; De Jager et al., 2006). Moreover, *PTPN22* is associated almost exclusively with the anti-CCP–positive RA, and the *PTPN22* C1858T allele appears to contribute RA mainly when *HLA-DRB1* SE alleles are present (Kallberg et al., 2007; Kokkonen et al., 2007; Plenge et al., 2005).

TRAF1 and C5 A locus between TNF receptor-associated factor1 (*TRAF1*) and complement component 5 (*C5*) in the chromosome 9q33–34 region, has recently been identified as a genetic risk factor for RA, particularly being associated with ACPA-positive RA, in a genome-wide scan association study (Plenge et al., 2007). The association of *TRAF1* and *C5* with RA was also simultaneously reported in another study using a candidate gene approach (Kurreeman et al., 2007), and these reports were replicated in different populations (Kurreeman et al., 2008; Zervou et al., 2008). The protein encoded by TRAF1 is a member of the TNF receptor-associated factor 1 protein family that mediates signal transduction from various receptors of the TNF receptor superfamily, including the receptor for TNFα (Wajant et al., 2001). Since

TNF blockade has powerful effects in the treatment of RA, all genes related to TNF pathways are of potential importance for RA susceptibility. *C5* has a suggestive role in arthritis due to observed high C5a levels in synovial fluid of RA patients (Hogasen et al., 1995) and that C5a receptor-deficient mice are resistant to experimental arthritis (Grant et al., 2002). However, the single SNP (SNP14/rs10818488) showing strongest association with RA is located in the intergenic region between *C5* and *TRAF1* (Kurreeman et al., 2007). So far there is no solid data pointing out one distinct gene in the TRAF1-C5 locus and more studies are necessary for further identification of the functionally important variations.

Other non-HLA genes have also been suggested to confer RA susceptibility, for example signal transducer and activator transcription 4 (**STAT4**) on chromosome 2q33. An association between a common haplotype located in the third intron of *STAT4* and susceptibility to RA and SLE has been reported (Remmers et al., 2007). The association was replicated in several independent Caucasian RA and SLE populations, and also in Korean and Japanese RA populations (Kobayashi et al., 2008a; Lee et al., 2007a; Orozco et al., 2008; Remmers et al., 2007). In a recent study a suggestive association of Interferon regulatory factor 5 (**IRF5**) with ACPA negative RA was observed in both Swedish and Dutch cohorts (Sigurdsson et al., 2007). However, two other previous studies did not observe association between IRF5 and RA in Swedish, French and Argentinian populations, although these studies were not stratified for ACPA status (Garnier et al., 2007; Rueda et al., 2006). Interestingly, an association between RA and Peptidylarginine deiminases citrullinating enzyme 4 (**PADI4**) was originally reported in a Japanese case–control study as a fine-mapping study of a linkage region on chromosome 1p36 (Suzuki et al., 2003), and has been replicated in Japanese, Korean and Chinese RA studies (Fan et al., 2008; Ikari et al., 2005; Kang et al., 2006). Thus it must be considered a confirmed association in Asian populations. In contrast, it has been difficult to demonstrate an association with PADI4 in Caucasian populations (Lee et al., 2007b; Plenge et al., 2005). PADI4 is one of several peptidylarginine deiminase enzymes that catalyze the conversion of arginine residues into citrulline (Vossenaar et al., 2003), and this may be related to the production of anti-citrulline antibodies that are a characteristic of a major subset of RA (Schellekens et al., 2000).

1.2.2.2 Environmental risk factors in RA

As mentioned above not only genes but also environmental factors contribute to RA susceptibility. The most prominent environmental factor consistently associated with an increased risk of RA is smoking (Silman et al., 1996; Stolt et al., 2003). One important aspect of smoking as a risk factor contributing to RA is its involvement in gene-environment interaction. Smoking has greater impact on RA in individuals being carriers of HLA-DRB1 SE alleles (Mattey et al., 2002). Moreover, the interaction between smoking and HLA-DRB1 SE alleles further confers a higher risk for RF-positive RA (Mattey et al., 2002; Padyukov et al., 2004) and ACPA-positive RA (Klareskog et al., 2006a; Pedersen et al., 2007; van der Helm-van Mil et al., 2007).

Infectious agents such as viruses, bacteria, and fungi have long been suspected risk factors for RA, but so far there is no conclusive evidence to support the hypothesis. It is speculated that the individuals who are genetically susceptible to RA may develop the disease due to infectious agents initiating pathogenic T cell activation. Some activated T cells could cross-react with self antigens and thus trigger pathogenic processes that result in RA (molecular mimicry) (Fujinami et al., 2006; Holmdahl et al., 2001; Kobayashi et al., 2008b). Another speculation is that infections lead to significant activation of APCs. These activated APCs could potentially activate preprimed autoreactive T cells, which can then initiate autoimmune disease (bystander activation of autoreactive T cells) (Fujinami et al., 2006; Holmdahl et al., 2001). These theories are interesting in the light of RA models that are induced by injection of agents which activate the innate immune response, e.g. components from bacteria, yeast.

Influences of other environmental factors on RA, such as exogenous hormones and diet, have also been suggested. Hormones are believed to influence RA based on the fact that females are more prone to develop RA than are men, with a peak onset at 50-60 years of age. One hypothesis for the sex influence is that androgens protect younger men developing RA, and loss of estrogen at menopause increases the risk for RA development for women (Carlsten, 2005). The application of hormones as contraceptives and for treatment of post-menopausal complications has been suggested to have protective effect in RA (Brennan et al., 1997; D'Elia et al., 2003).

A number of dietary factors have been suggested to be of relevance for RA, such as a diet containing high levels of antioxidants or omega-3 fatty acids which are thought to ameliorate severe RA (Remans et al., 2004). Interestingly, alcohol consumption was recently demonstrated to have a protective effect in RA development (Kallberg et al.,

2009), and it has also been shown that alcohol prevents mice from development of destructive arthritis induced by collagen (Jonsson et al., 2007).

1.3 GENETIC APPROACHES TO STUDY COMPLEX DISEASES

Complex diseases such as RA are polygenic diseases. The challenge with genetic studies of complex diseases is that no single genetic factor is neither necessary nor sufficient for disease development, since the genes or genomic regions regulating a complex disease usually exert a magnitude effects, they are so-called quantitative trait loci (QTLs). Common approaches to identify QTLs include linkage and association studies. Before giving details of two mapping strategies some common genetic concepts are introduced.

1.3.1 Genetic markers

A genetic marker is a DNA sequence from a known location that allows the discrimination of individuals by the variation(s) or polymorphisms at that position. All mapping strategies are dependent on the possibility to distinguish alleles by genetic markers. The most commonly applied markers are **microsatellites** and single nucleotide polymorphisms (**SNPs**). Microsatellites are genomic regions containing tandem repeats of di-, tri- or tetra- nucleotides. The number of repeats is highly polymorphic and with a relatively high frequency (one microsatellite in 10 kb) in the genome, and they are easily analysed by PCR amplification and gel separation. SNPs are biallelic variations at a single nucleotide position and they contribute to the majority of the genetic variability, with wide distribution in the genome. Despite most SNPs being neutral with no or little effect on the phenotypes, SNPs are still nowadays the first choice as genetic markers due to high density (one SNP every 1 kb) in the genome and ultra high-throughput genotyping capabilities.

1.3.2 Genetic linkage and linkage disequilibrium (LD)

The term genetic linkage is used to describe the relationship between different genetic variants, and genetic variants that are inherited together are considered to be linked.

Linkage disequilibrium (LD) refers to a situation in which some combinations of alleles or genetic variants occur more, or less, frequently in a population than would be expected by chance. Whether two alleles will be in LD is determined by the recombination frequency of the region. Two alleles that are located relatively close to each other could be separated by recombination during meiosis and consequently in

weak LD. In contrast, two genetic variants that locate several kb apart could be in high LD due to no recombination events occurring to separate them (Wall and Pritchard, 2003).

1.3.3 Quantitative trait loci (QTLs)

Quantitative trait loci (QTL) are the genomic regions that are linked to or associated with a particular trait. The effects of each QTL, for a particular trait, vary in degree. Knowing the number of QTLs (QTL mapping) may tell us about the genetic architecture of a trait, e.g. a trait may be controlled by many QTLs with small effect, or by a few QTLs with large effect.

QTL mapping is used to identify a specific genomic region that is significantly more likely to co-occur with a phenotypic trait than would be expected by chance. The significance of a QTL is presented by the base 10 of the likelihood ratio (logarithm of odds, LOD) scores:

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

A LOD score of 3 implies a 1000-fold higher chance of presence than absence of a QTL in the studied population at a specific genomic location.

1.3.4 Linkage analysis

Linkage analysis is the identification of co-inheritance of a genomic region(s) and a disease phenotype within families by using genetic markers. It is based on the idea that if a genomic region(s) contains risk alleles, it will more often be carried by affected members in the families. Linkage analysis can be performed in a pedigree by searching for genome regions shared by affected individuals. Although the advantage of linkage analysis would be of broad chromosomal coverage and enabling the identification of QTLs in an unbiased fashion, its disadvantage is lack of enough power to identify QTLs displaying small or moderate effects due to a few recombination events in limited generations in a pedigree and the inability to collect large numbers of families with multiple affected individuals (Lander and Schork, 1994).

1.3.5 Association studies

Association studies identify disease gene(s) by comparing the allele frequencies between large number of patients and non-related healthy controls. While linkage analysis is based on the limited recombination events that occur within the families in limited generations, an association study takes advantages of the historical recombinations that have occurred in the entire population during many generations (Zhao et al., 2003), and the possibility to collect large numbers of subjects. Compared to linkage analysis, an association study provides better power and precision to detect risk alleles. There are two main approaches that can be applied to map disease gene(s) in association studies: a candidate gene approach and a genome-wide association approach. A **candidate gene approach** is based upon pre-existing knowledge of a genetic risk factor(s), e.g. from its function or prior evidence of association in other populations or in animal models, or from the identification of association with a related disease. This type of study can be conducted by using a limited number of genetic markers. In contrast to the hypothesis-driven candidate gene analysis, the **genome-wide association approach** is hypothesis-free, with the advantage that no previous information about genetic candidate risk factors is required. This permits a comprehensive scan of the genome in an unbiased fashion and thus has the potential to identify novel genetic risk factors and to evaluate the known or 'expected' risk factors. However, it will also come with the price of scanning thousands of samples and utilizing hundreds of thousands of SNP markers located throughout the human genome.

1.4 GENE MAPPING IN ANIMALS

Identification of disease-regulating genes may give insights into the pathogenesis and provide new strategies for disease prevention and therapy. Due to the incomplete penetrance of risk genes, and variable clinical presentation and environmental effects in autoimmune diseases, identification of causal genes is difficult in the heterogeneous human populations. Animal studies, as a comparative mapping strategy, provide opportunities to both control and manipulate genetic and environmental effects, and clinical, immunological and functional studies can be performed with less ethical issues than in humans. The idea is that both phenotypes and genomic organization tends to be evolutionarily conserved between species. Once diseases risk gene(s), especially those that are conserved and shared between species, are identified in animal models, the

results can then be evaluated in related human diseases, possibly leading to identification of new disease risk genes and/or pathways.

Many animal models more or less resemble human diseases. Rat and mouth models are particularly useful for the genetic study of human diseases, as rats and mice are easy to maintain and handle. Mice also have the advantage of transgenic and knock-out technologies. There are multiple rodent inbred strains for study of complex diseases; for example, at least six inbred rat strains (DA, LEW, F344, PVG, BN and E3) with different degrees of susceptibility are applied in experimental arthritis models. These inbred strains enable genetic segregation possible to be followed, thus making it easier to perform linkage analysis. Congenic strains can be created to study specific genes on different backgrounds. In this chapter, several strategies of genetic approaches in animal models are discussed in the context of my main research area - experimental arthritis.

1.4.1 Genome wide scans – identification of QTLs

QTL mapping strategies in animals usually consist of two steps: identification of QTLs in F2 intercrosses or backcross (BC) and fine-mapping QTLs into small genomic regions. As the differences in susceptibility to experimental arthritis depend on genetic background in different inbred strains, by crossing one susceptible strain (most often, DA rats) with a resistant strain (e.g., PVG, F344 and E3), the heterozygous F1 can be produced. An F2 or BC cross can then be obtained by either intercrossing of F1 individuals or backcrossing of F1 individuals to one of parental strains (Fig.1).

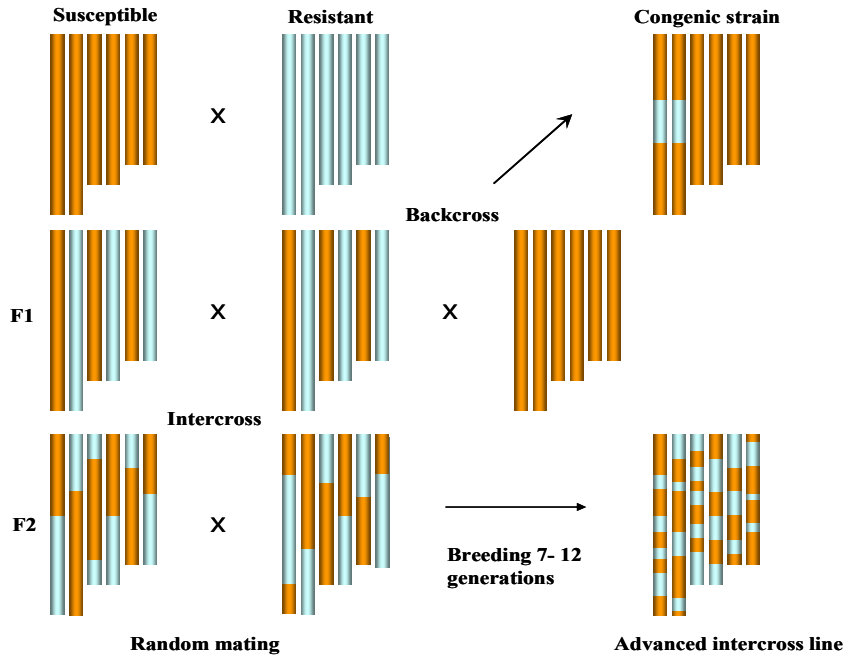

Figure 1. Breeding strategies for genetic mapping in experimental populations. For QTL mapping, an intercross between two parental inbred strains (one susceptible and another one resistant) will create a heterozygous F1 generation. An F1 intercross will produce an F2 population that can be applied for whole genome scans. To create congenic strains, F1 individuals are back-crossed to one of the parental strains for at least 9 generations. In each generation an individual with the fragment of interest is selected by genotyping and used for further back-crossing. To obtain an AIL, F2 individuals are intercrossed for several generations with avoidance of brother-sister mating in each generation.

A QTL analysis can be performed in F2 or BC populations by making use of their advantage that a few recombination events in the F2 or BC generations results in linkage to large genomic intervals and allows a genome-wide scan with a limited number of genetic markers.

To map experimental arthritis QTLs many genome scans have been performed using different F2 crosses in which the parental resistant strains varied in different arthritis models. To date, more than 60 arthritis-regulating QTLs have been identified in RA models regarding different traits such as onset, severity and chronicity (Joe, 2006) (Fig.2). The QTLs often overlap in different models and several of these loci also overlap with QTLs of other autoimmune disease models such as type I diabetes, encephalomyelitis and uveitis (Cornelis et al., 1998; Jawaheer et al., 2001; Sun et al., 1999). This indicates that the genetic variations in these QTLs may have a general regulatory function and regulate a broad range of inflammatory diseases.

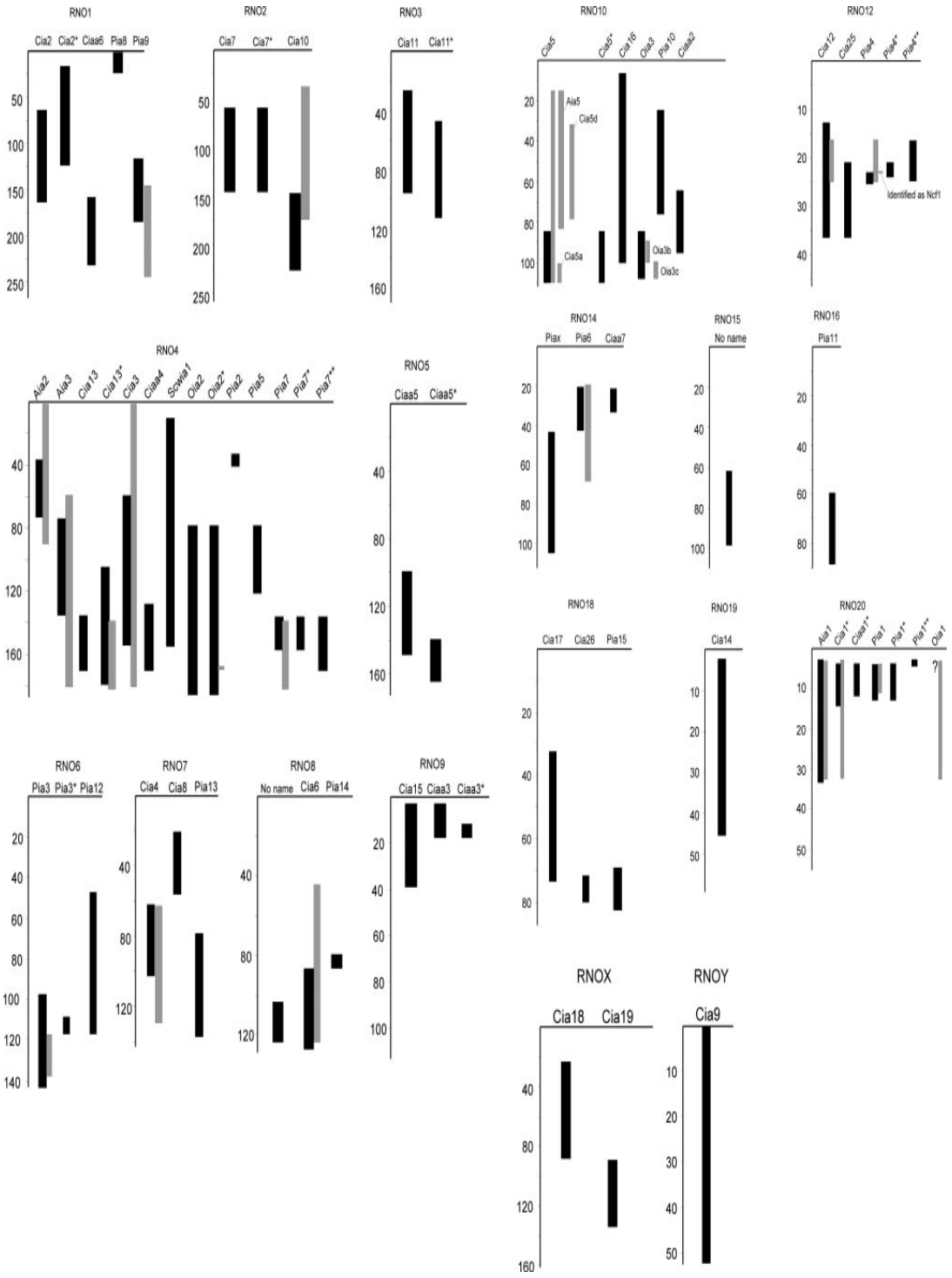

Figure 2. Physical locations of experimental arthritis quantitative trait loci (QTLs) on the rat genome [adapted from (Joe, 2006)] . Each panel represents the experimental arthritis and related QTLs defined on one rat chromosome. The *x*-axis for each panel represents the length of the corresponding rat chromosome (in Mb). Black bars represent the locations of experimental arthritis QTLs identified by linkage analysis. Gray bars represent the locations of experimental arthritis QTLs mapped by congenic strains.

From the results of genome scans it is evident that rat chromosome 4 (RNO4) harbors many arthritis-regulating QILs. Our research group identified the QTL oil-induced arthritis-2 (*Oia2*), located at the telomeric region of RNO4 (4q42) (Backdahl et al., 2003; Lorentzen et al., 1998; Ribbhammar et al., 2003). *Oia2* overlaps with *Pia7* (Nordquist et al., 2000) and *Cia13*(Griffiths et al., 2000). The originally defined *Oia2*/*Pia7*/*Cia13* regions contained many genes that exert important functions in immune system, including two gene complexes that encode C-type lectin receptors, i.e., the natural killer cell gene complex (*NKC*) and the antigen presenting lectin-like receptor complex (*APLEC*). Its syntenic region on human chromosome (HSA) 12p13 reportedly harbors susceptibility genes for both RA (Cornelis et al., 1998; Jawaheer et al., 2001) and MS (Jagodic et al., 2005; Xu et al., 2001).

RNO10 also harbors many arthritis-regulating QTLs. Among these QTLs, the *Oia3* locus was identified in our group (Lorentzen et al., 1998), and it overlaps with *Cia5* (Remmers et al., 1996). The *Oia3/Cia5* loci are localized on the telomeric part of RNO10. Besides experimental arthritis, the *Oia3/Cia5a* region has also been linked to experimental autoimmune encephalomyelitis (EAE) (Dahlman et al., 1998; Jagodic et al., 2001), Type I diabetes (Jacob et al., 1992) and hypertension (Kreutz et al., 1995). Furthermore, the genes in the human homologous genomic region may also be important for immune regulation, since HSA 17q22-q25 reportedly links to and associates with multiple inflammatory diseases such as RA (Barton et al., 2001; Jawaheer et al., 2001), MS (Barton et al., 2004), ankylosing spondylitis (Reveille, 2004) and type I diabetes (Vaessen et al., 2002). This indicates that there are one or several genes within the genomic region that affect general aspects of immune function and inflammation. .

In this thesis the fine mapping of *Oia2* and *Oia3*, and the studies of *APLEC* in different immune-mediated diseases will be discussed in detail.

1.4.2 Confirmation and fine mapping of QTLs in congenic strains

The QTLs identified in an F2 cross usually encompass large confidence intervals (CI) from 10 to 30 cM, containing hundreds of genes, far from pin-pointing specific susceptibility genes. Moreover, the QTL mapping represents a statistical measurement of linkage possibility, and the genomic location of a QTL need further confirmation before large efforts are put into gene identification. To confirm and fine map a QTL, one approach is mapping in congenic strains.

A congenic strain is an inbred strain in which a chromosomal fragment of interest, for example including a QTL, has been transferred from a resistant strain (donor) onto the genome of a susceptible strain (recipient), or *vice versa*. By back-crossing donor strain onto the recipient strain, coupled by marker assisted selection of individuals carrying the desired alleles in the QTL, for at least nine generations, and in each generation the contamination of genome background will be reduced 50%, thus this will theoretically give a 99.81% ($1-0.5^9$) purity of the recipient genome background (Wakeland et al., 1997; Weil et al., 1997) (Fig1). An alternative is the speed congenic approach, where the selection of the congenic fragment is coupled not only by markers in the QTL but also by markers evenly dispersed across the genome to check the extent of background contamination. Due to random allelic combinations the reduction of background contamination will follow a normal distribution in each back-crossing generation. By selecting the founders in each generation with the least background contamination, a 99.90 % pure background can be obtained in five generations (Wakeland et al., 1997).

By comparing the phenotypic difference between the congenic and the parental recipient strain, which differ only in the transferred region, the effect of the QTL can be assessed. Moreover, by further back-crossing the congenic strain to the recipient strain to create recombinations in the transferred genomic fragment, subcongenic (recombinant) strains within the region can be selected. These recombinant strains carry part of the originally transferred chromosomal region, and the correlation between phenotypes and genotypes in certain recombinant strains allows narrowing-down of a QTL.

To date, by using the congenic mapping approach two rat arthritis QTLs have been fine mapped down to a single gene or single type of genes . One is the *Ncf1* gene in *Pia4* on RNO12 (Olofsson et al., 2003). Another one is *APLEC* in *Oia2* on RNO4 (Backdahl et al., 2003; Lorentzen et al., 2007; Ribbhammar et al., 2003). Thus, fine mapping in congenic strains has proven to be a very powerful approach. However, the mapping strategy has some limitations. From a technical point of view, to achieve a small congenic fragment, a large breeding population is needed to obtain the recombinants. For a smaller recombinant it is more difficult to obtain polymorphic markers. Another obstacle is the possibility of phenotype loss in congenic strains. There are some explanations for this outcome: e.g. i) Initial QTL linkage was false positive; ii) A gene-gene (additive or epistatic) interaction with another QTL or within the QTL has been disrupted by creating congenic or sub-congenic strains.

Among these issues, the loss of gene-gene interactions is important aspect since multiple genes, each having a moderate effect, can interact and produce a complex trait and usually these interactions are extremely difficult to predict (Schadt, 2006).

1.4.3 High resolution mapping in AIL

To achieve high resolution mapping of a QTL and to detect gene-gene interactions, there are different mapping strategies available based on the principle that the accumulation of recombination events will reduce linkage disequilibrium and thus reduce confidence intervals. One mapping strategy applied in our laboratory is the creation of an advanced intercross line (AIL) (Fig.1). An AIL is generated by random intercrossing of two inbred strains while avoiding brother-sister mating for several generations, resulting in the accumulation of many recombination events and enabling high resolution that can discriminate between closely situated QTLs (Darvasi and Soller, 1995). Theoretically, with an appropriate AIL the confidence interval of a QTL is to be $t/2$ times smaller than the same-size F2 progeny, where t is the number of AIL generations (Darvasi and Soller, 1995). Therefore, an AIL is a powerful approach for high resolution mapping of QTLs.

To date, this approach has been successfully applied to refine QTLs in several studies. One example is the fine mapping of mouse *Cia2* and *Cia27* loci. These QTLs were originally identified in mice (DBA/1 x FVB/N) F2 progeny. By using the (DBA/1 x FVB/N) 11-12th generation of an AIL in a linkage analysis, the confidence intervals of two QTLs were refined from 40 and 43Mb to 12 and 4.1, Mb, respectively (Yu et al., 2006). Another example is rat *Eae5*, which was originally identified in two F2 intercrosses, (DA x BN) and (E3 x DA). Linkage analysis performed in the 7th generation of an AIL origination from the DA and PVG.1AV1 narrowed-down the *Eae5* to an approximately 1.3 Mb region with a small number of candidate genes including the *Ncf-1* gene (Becanovic et al., 2006).

Gene-gene interactions can be either additive or epistatic. Interactions with additive effects mean that the final effects depend on cumulative genetic contribution that drives the phenotype in the same direction. Epistasis occurs when the genotype at one locus affects the phenotypic expression of the genotype at another locus (Goodnight, 2000). Epistatic interactions between multiple loci can be either synergistic (positive) or antagonistic (negative) (Azevedo et al., 2006; Bonhoeffer et al., 2004).

In animal models it may be difficult to detect epistatic interactions in F2 crosses. The explanations for this would be: i) confidence intervals in F_2 mapping are typically large (10-30 cM), and contain hundreds of genes; ii) a single QTL may comprise several adjacent QTLs (Iraqi et al., 2000; Wang et al., 2003). In contrast, the AIL approach enables high resolution and precision in genetic mapping, and is thus a powerful method to investigate gene-gene interactions. So far several studies have been successfully conducted to assess the occurrence of additive and epistatic effects, e.g. *Eae22* was identified as an epistatic region in an AIL G10 cross (Jagodic et al., 2005), and three epistasic arthritis loci within the *Cia5* QTL were identified in mice in a partial advanced intercross (Johannesson et al., 2005).

1.5 EXPERIMENTAL ANIMAL MODELS

1.5.1 Animal models for RA

There are many animal models for RA, most being rodent models. The arthritis either develops spontaneously or is induced with external stimuli. Spontaneous arthritis models include MRL-lpr/lpr mice (Pataki and Rordorf-Adam, 1985), SKG mice (Sakaguchi et al., 2003), and DNase II x IFN-IR deficient mice (Kawane et al., 2006).
Induced arthritis models in rats have several advantages compared to mice. For example, rats are more susceptible to induction of arthritis and the diseases are more similar to that of RA (Holmdahl et al., 2001). Furthermore, rats easily develop arthritis not only through the immunization of antigen but also by the injection of adjuvants alone that do not contain antigen. This enables parallel studies of different disease pathways in the same species and strain. The following sections describe some rat models for RA which are applied in this thesis.

1.5.1.1 Oil-induced arthritis (OIA)

OIA is induced by injection of incomplete Freund's adjuvant (IFA). IFA consists of 85% mineral oil and 15% of the emulsifier mannide monooleate Arlacel A. Each component can induce arthritis in DA rats (Kleinau et al., 1991).
OIA is an acute, monophasic disease with symmetric inflammation of peripheral joints. The disease onset is approximately 12-14 days after an intradermal injection of IFA. There is no significant gender preponderance, but it was observed that males have a slightly more severe disease course than females (Ribbhammar et al., 2003). Usually the disease subsides before 45 days, with limited erosion, and no ankylosis or other functional disorders. OIA is T-cell dependent since anti-αβTCR antibodies ameliorate

the disease (Holmdahl et al., 1992), and passive transfer of $CD4^+$ T cells from arthritic lymph nodes induces arthritis in naïve, irradiated DA rats (Kleinau and Klareskog, 1993; Svelander et al., 1997). Concerning cytokines expression, IL-1β mRNA level was elevated in draining lymph nodes (DLNs) in DA rats injected with IFA (Svelander et al., 2001).

1.5.1.2 Squalene-induced arthritis (SIA)

SIA is induced by injection of squalene ($C_{30}H_{50}$), which is an endogenous cholesterol precursor present in all mammals. It has been used as a adjuvant component for vaccinations in humans, being commonly used in influenza vaccines as well as vaccines against CMV, HIV, anthrax and herpes simplex virus (HSV) (Podda and Del Giudice, 2003). Anti-squalene antibodies have been reported in individuals who have received anthrax vaccine (Asa et al., 2002; Matyas et al., 2004) and in Gulf War veterans, some of whom developed Gulf War Syndrome characterized by a presence of pseudo-rheumatic symptoms, e.g. muscle and joint pain, fatigue (Asa et al., 2000). But these results are controversial.

SIA is characterized by T-cell infiltration in the synovium and the presence of bone and cartilage erosions (Carlson et al., 2000). It has been shown that injected squalene mostly accumulated in draining lymph nodes, and not in the joints, and most of the squalene is present within the cells rather than in the extracellular matrix (ECM) (Holm et al., 2002). Furthermore, following *in vitro* stimulation with conA the draining lymph node cells could transfer arthritis to naïve irradiated DA rats (Holm et al., 2002).

In addition to DA rats, the inbred LEW.1AV1 rats also developed arthritis following injection of squalene. A female preponderance in SIA has been reported for LEW.1AV1 but not for DA rats (Carlson et al., 2000).

1.5.1.3 Pristane-induced arthritis (PIA)

PIA is induced with the lipid component pristane ($C_{19}H_{40}$) resulting in development of severe arthritis that fulfills almost all the criteria of RA (Holmdahl et al., 2001). Pristane is present in plant chlorophyll and therefore occurs in daily diet as well. In rats, PIA is a chronic joint-specific disease with a female preponderance, and is characterized by T-cell infiltration in the joint, presence of serum rheumatic factors (RF), and pronounced bone and cartilage erosions (Vingsbo-Lundberg et al., 1998; Vingsbo et al., 1996). Splenocytes from pristane-primed rats produced high amounts of IFNγ and TNFα, but not IL-4, when restimulated *in vitro* with Con A (Holmberg et al.,

2006). The disease onset is slightly later than in OIA and SIA. In common with OIA and SIA, no specific antigen is applied for induction of PIA. Interestingly, despite lack of the application of specific antigen , PIA has been shown to be a T-cell-driven disease and to be dependent on MHC class II molecules (Holmberg et al., 2006). The disease can be transferred with αβCD4$^+$ T cells to naïve recipients (Holmberg et al., 2006). More recently it was demonstrated that the autoantigen heterogeneous nuclear ribonucleoprotein (hnRNP-A2, RA33) was highly over-expressed in the joints of rats injected with pristane. Over-expression coincided with the appearance of anti-RA33 Abs and preceded the onset of clinical symptoms of PIA by several days. These data suggest hnRNP-A2 to be a target for autoimmunity in PIA (Hoffmann et al., 2007).

1.5.1.4 Adjuvant-induced arthritis (AIA)

AIA was the first model for RA (Pearson, 1956). It is induced by the injection of complete Freund's adjuvant (CFA) that contains heat-killed *Mycobacterium tuberculosis* in IFA. The clinical and pathological features of AIA resemble RA, e.g. primarily affecting the peripheral joints and being symmetric polyarthritis. The onset of the clinical arthritis is evident after 10–14 days. The first histopathological signs of arthritis, an accumulation of mononuclear cells in synovial tissues, are already present 6 days after disease induction. The synovial infiltrate leads to pannus formation resulting in cartilage deformation and severe destruction of the joint. However, the disease is not joint-specific, and following AIA induction with CFA rats not only develop arthritis but also systemic features of inflammation such as uveitis and inflammation of the gastro-intestinal tract (Prakken et al., 2003). AIA can be passively transferred by T-cells derived from diseased rats to irradiated rats, showing that the disease is T-cell-mediated (Holoshitz et al., 1983; Whitehouse et al., 1969).

1.5.1.5 β- glucan-induced arthritis (GIA)

GIA is induced by the injection of β-glucan - a component of the cell wall of yeast. As an experimental arthritis model, GIA was well studied in SKG mice which are genetically prone to develop autoimmune arthritis (Sakaguchi et al., 2003). It has been shown that in a strictly controlled specific pathogen-free (SPF) environment, SKG mice failed to develop arthritis, while in a clean environment the administration of β-glucan triggered a chronic and severe arthritis. The disease severity correlated with the production of cytokine IL-6 levels. Blockade of Dectin-1, a major β-glucan receptor, prevented SKG arthritis triggered by β-glucan (Yoshitomi et al., 2005).

Furthermore, β-glucan can acts as an adjuvant for collagen-induced arthritis (Hida et al., 2006). In rats, β-glucan can also induce arthritis with early onset, and a severe and ankylosing disease course in DA and LEW.1AV1 rats (Lorentzen, 1999).

1.5.1.6 Collagen-induced arthritis (CIA)

CIA is induced through immunization with collagen type II (CII) emulsified with either IFA (for rats) or CFA (for mice), and it is the most commonly used arthritis model. In the rat CIA can be induced by both heterologous (human, bovine and chicken) and homologous (rat) collagen type II, where the clinical symptoms develop 10-14 days after the immunization. Similar to PIA, CIA is a chronic, joint-specific disease with a female preponderance, and characterized by T-cell infiltration in synovial tissues, presence of serum RF, pronounced bone and cartilage erosions (Trentham et al., 1977). In contrast to the previously described arthritis models induced by adjuvants alone, in the CIA model there is a specific immune response towards a defined antigen and the disease is critically not only dependent on T-cells but also on B-cells, since autoantibodies to CII are present in CIA and arthritis can be transferred by sera from arthritis mice to naïve recipients (Corthay et al., 1999; Stuart and Dixon, 1983; Svensson et al., 1998). In CIA a kinetic study of cytokine expression in rat DLNs revealed that DA rats expressed relatively high levels of TNF-α, IL-2 and IFNγ and little IL-4, whereas PVG.1AV1 rats expressed an opposite pattern with a marked induction of IL-4 mRNA and limited induction of TNF-α, IL-2 and IFN-γ (Mussener et al., 1997).

1.5.2 Animal models of infectious diseases

1.5.2.1 Staphylococcus aureus (S. aureus)-induced arthritis and sepsis

S. aureus-induced arthritis is a severe, rapidly progressing, erosive septic disease with high morbidity and mortality (Shirtliff and Mader, 2002). Staphylococci exhibit a high degree of selectivity for the joints (Verdrengh et al., 2006). Septic arthritis is induced by a single intravenous (i.v) injection of *S. aureus*. Within 24h both clinical and histological signs of arthritis occur which are characterized by granulocytes and activated macrophages infiltrate in the synovium, soon followed by T-cells. Within days, severe damage of cartilage and bone occurred (Bremell et al., 1992). Studies have shown that the severity of *S. aureus*-induced arthritis is dependent on T-cells (Abdelnour et al., 1994), macrophages (Verdrengh and Tarkowski, 2000) and

granulocytes (Verdrengh and Tarkowski, 1997). Macrophage depletion leads to less severe arthritis while granulocyte-depletion aggravates it (Verdrengh and Tarkowski, 2000). Furthermore, cytokines can influence the development of septic arthritis, in which the Th1 cytokine TNF aggravates the arthritis while the Th2 type cytokine IL-10 ameliorates arthritis (Gjertsson et al., 2002). In contrast, the B-cell compartment does not significantly influence survival or the outcome of arthritis (Gjertsson et al., 2000).

1.5.2.2 Herpes simplex virus-1(HSV-1)-induced encephalitis

Herpes simplex virus type 1 (*HSV-1*) is the most frequent cause of sporadic and fatal viral encephalitis in humans (Tyler, 2004). In rat *HSV*-1-encephalitis, the symptoms usually develop 5-to-7 days after intranasal viral challenge, often starting with repetitive, stereotypic movements and motor instability. Within 3 to 5 hours after the appearance of the first symptoms, the psychomotor behavior deteriorates with development of hind limb paralysis and thereafter death. In our study, animals were immediately sacrificed for ethical reasons when definitive symptoms of neurological dysfunction were assured.

In *HSV-1*-induced encephalitis mortality results from infection in central nervous system (CNS). Clinical and animal model studies have clearly demonstrated the importance of genetic makeup in regulation of *HSV-1* encephalitis (Bereczky-Veress et al., 2008; Ellison et al., 2000; Halford et al., 2004; Lopez, 1975; Lundberg et al., 2008; Nicholls et al., 1994)

The mechanism by which HSV-1 CNS infection causes death has not been defined. It appears to involve both virus- and immune- related processes. Recent studies have demonstrated that genetic variation of the host inflammatory response may play a major role in determining HSV fatality (Bereczky-Veress et al., 2008; Lundberg et al., 2008). *In vitro* and *in vivo* studies have shown that human and mouse microglia infected with HSV-1 express a variety of proinflammatory cytokines and chemokines, with TNFα and macrophage chemoattractant protein 1 being prominently expressed, consistent with their involvement in early innate responses against invading virus (Lokensgard et al., 2002; Lokensgard et al., 2001; Sergerie et al., 2007). Whether such innate inflammatory responses are protective or deleterious has not yet been clarified, since in a knock-out mouse model it was shown that both TNF-α and IL-1β have protective effects during HSV-1 encephalitis (Sergerie et al., 2007). Moreover, it has recently been shown that interferon responses are important for HSV-1 encephalitis in humans (Jouanguy et al., 2007).

1.6 C-TYPE LECTIN AND LECTIN-LIKE RECEPTORS

In the innate immune system, pattern-recognition receptors (PRRs) recognizing pathogen-associated molecular patterns (PAMPs) have critical roles in protection from invading pathogens. Toll-like receptors (TLRs), a large family of molecules which are the mammalian homologues of drosophila Toll, were identified as a part of PRRs and have been intensively investigated during the past decade (Takeda and Akira, 2004). Recently, an increasing number of non-TLR PRRs have been identified, in particular C-type lectin and lectin-like receptors (CLRs). The CLRs comprise a large family and are relevant to the pathogenesis of some infectious diseases such as *C. albicans* infection (Huysamen and Brown, 2009; Kimberg and Brown, 2008), and some inflammatory diseases such as RA (Eklow et al., 2008; Fujikado et al., 2008; Lorentzen et al., 2007).

1.6.1 Common structure of C-type lectins

CLRs are cell surface receptors characterized by sharing a highly conserved carbohydrate recognition domain (CRD) from invertebrates to higher mammalian species (Zelensky and Gready, 2005). These CRDs contain 18 highly conserved amino acid residues, and have calcium-binding pockets that are essential for carbohydrate recognition and binding. The receptors recognize carbohydrate structures which are often part of microbial pathogens in a Ca^{2+}-dependent manner via CRDs. Based on their molecular structures, the CLRs are divided into two large groups (Fig.3).

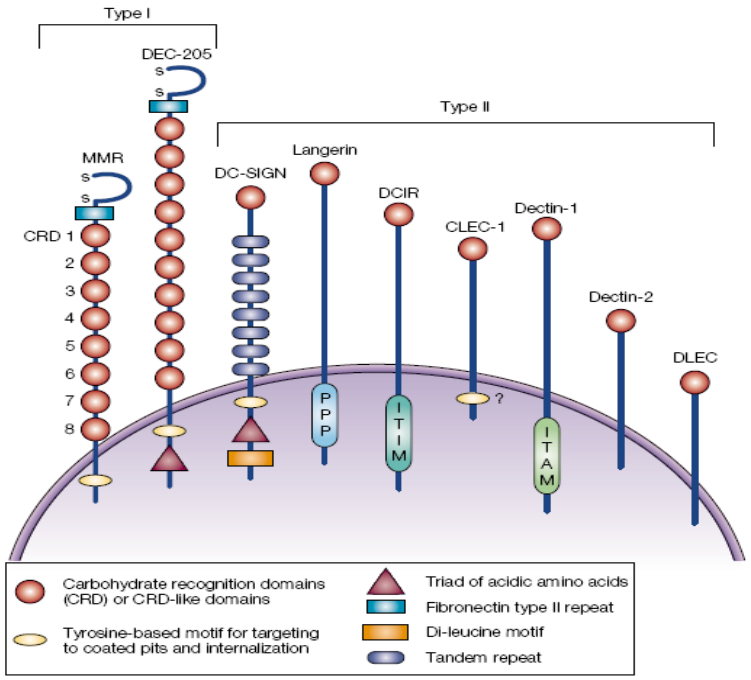

Figure 3. Two types of C-type lectins or lectin-like molecules (adapted from Carl G. Figdor, 2002). Type I C-type lectins (MMR and DEC-205) contain 8–10 carbohydrate recognition domains (CRDs), which contain multiple CRDs. Some of CRDs bind ligand in a Ca^{2+}-dependent manner. Type II C-type lectins contain only one CRD. The CRD is fully conserved and bind ligand in a Ca^{2+}-dependent manner.

One group, called the mannose receptor (MR) family, contains type I transmembrane proteins with multiple CRDs. This group includes the MR (CD206), DEC-205 (CD205), Endo 180 (CD280) and selectins (East and Isacke, 2002). They commonly contain 8 or 10 CRDs. Since not all of their multiple CRDs have been shown to bind Ca^{2+} or carbohydrates, the term C-type lectin-like domain (CTLD) is used to describe the domain without Ca^{2+}-dependent sugar-binding capacity. The amino acid sequence of CTLD is closely related to that of CRD, but conservation of the common 18 amino acid residues is incomplete (Drickamer, 1999; Kanazawa et al., 2004).

The other group, called the asialoglycoprotein receptor (ASGPR) family, comprises type II transmembrane (TM) proteins with a single CRD, such as macrophage galactose-type C-type lectin (MGL, CD301), DC-specific ICAM3-grabbing nonintegrin (DC-SIGN, CD209), and the members of the Dectin-1and the DCIR family of lectins. They contain fully conserved CRD and their carbohydrate binding has been confirmed in most cases (Drickamer, 1999).

The cytoplasmic domains of the C-type lectins are diverse but some CLRs contain important functional motifs in their intracellular part, e.g. immunoreceptor tyrosine-

based inhibitory motif (ITIM) (human *DCIR* and rodent *Dcir1* and *Dir2*) (Flornes et al., 2004; Kanazawa et al., 2003), predicting an inhibitory function; or immunoreceptor tyrosine-based activating motif (ITAM) (human and rodent *Dcar1*, *Mincle* and *Dectin-1*) (Kanazawa, 2007; Kanazawa et al., 2003; Yamasaki et al., 2008), suggesting an activating function. These structural differences reflect their putative different functions in the cells in which they are expressed.

1.6.2 Pleiotropic function of C-type lectin receptors

The large family of CLRs has been well described in their structural features, but how they influence immune responses is still poorly understood.

As mentioned above, some CLRs such as DC-SIGN and Dectin-1 can recognize PAMPs and function as PPRs. Evidence has also emerged that DC-SIGN and Dectin-1 not only play a role as PRRs, but they also might synergise or antagonise TLR signals (Gantner et al., 2003; Geijtenbeek and van Kooyk, 2003). The classical mannose receptors as well as DEC-205 and BDCA-2 have been suggested to be involved in the binding and uptake of pathogens (Cambi and Figdor, 2003; Dzionek et al., 2001; Mahnke et al., 2000). Other CLRs such as the selectins seem to have a major role in mediating cell adhesion and migration, rather than antigen internalization, or pathogen recognition (Cambi and Figdor, 2003). Furthermore, recent studies have shown that some of the type II CLRs, such as DC-SIGN, can function both as an adhesion receptor and as a PRR (Cambi and Figdor, 2003). In addition to acting as receptors for exogenous structures, recent evidence has suggested that some CLRs expressed by myeloid cells may also function as receptors for endogenous structures and are involved in the recognition of dead cells (Robinson et al., 2006), such as mannose-binding lectin (MBL) (Nauta et al., 2003; Ogden et al., 2001), MGL-1(Yuita et al., 2005) and Mincle (Yamasaki et al., 2008). Although some of these CLRs receptors may have phagocytic function to facilitate the clearance of dead cells, it is also possible that C-type lectins may recognize debris and transduce inflammatory signals to initiate an autoimmune response (Yamasaki et al., 2008).

1.6.3 Antigen presenting lectin-like receptor gene complex (*APLEC*)

In previous studies, our research group identified the arthritis-regulating QTL *Oia2* in a genome-wide scan in an F2 cross between DA and LEW.1AV1 (Lorentzen et al., 1998). By using a panel of congenic and sub-congenic strains, *Oia2* was further mapped and positioned to a gene cluster designated the antigen presenting lectin-like

receptor gene complex (*APLEC*) (Backdahl et al., 2003; Lorentzen et al., 2007; Ribbhammar et al., 2003). The gene cluster is located on RNO4 and it contains seven type II C-type lectin-like receptor genes (Table 2), i.e., macrophage inducible C-type lectin (*Mincle*), macrophage C-type lectin (*Mcl*), dendritic cell immunoactivating receptor-1 (*Dcar-1*) and dendritic cell immunoreceptor 1-4 (*Dcir1-Dcir4*), as well as the dendritic cell associated C-type lectin (*Dectin*) pseudogene *Dectin2p*, and the gene fragment named *Dcar2gf* (Flornes et al., 2004). The human and mouse orthologues of rat *APLEC* genes are *MINCLE* (Matsumoto et al., 1999), *MCL* (Balch et al., 1998), *DCAR* (Kanazawa et al., 2003), dendritic cell lectin (*DLEC*; also referred to as *BDCA2*) (Arce et al., 2001; Dzionek et al., 2001), *DCIR* (Bates et al., 1999) and *Dectin2* (Ariizumi et al., 2000) (Fig.4). The *APLEC* encoded receptors are preferentially expressed on neutrophils and antigen presenting cells, i.e. dendritic cells, macrophages, B-cells (Flornes et al., 2004). Rodent *APLEC* are summarized in Table 2, and specific gene information is described below.

Table2. Rodent antigen presenting lectin-like receptor gene complex (*APLEC*)

	Charged residue	Signaling motif	Ligand	Cellluar distribution
Dcir1-2	None	ITIM	unknown	Neutrophils, APCs
Dcir3-4	None	None	unknown	Neutrophils, APCs
Dcar1-2	Arginine	ITAM	unknown	Neutrophils, APCs
Mcl	None	None	unknown	Neutrophils, APCs, $CD4^+$ T cells
Mincle	Arginine	ITAM	nuclear protein	Neutrophils, APCs, $CD4^+$ and $CD8^+$ T cells

APCs - antigen presenting cells;

Reference: Flornes LM, et al 2004; Kanazawa et al., 2003; Yamasaki et al., 2008.

Dcir1-4 There are four different rat *Dcir* genes identified, denoted *Dcir1–4*. *Dcir1* and *Dcir2* contain a single ITIM inhibitory motif (Flornes et al., 2004), predicting inhibitory functions. In contrast, *Dcir3* and *Dcir4* exhibit neither ITIMs nor ITAMs. they may be involved in their signaling by forming heterodimeric receptors through association with other CLRs carrying the relevant amino-acid motifs, as analogous to some killer cell lectin-like receptors (KLRs) e.g. CD94 (Lazetic et al., 1996; Takei et al., 2001). Mice orthologues were identified for all four rat *Dcir* genes but there is only one *DCIR* gene in humans (Bates et al., 1999; Flornes et al., 2004). In recent studies it was reported that human *DCIR* associate with RA susceptibility (Lorentzen et al.,

2007), and could participate in DC-mediated capture and transmission of HIV-1(Lambert et al., 2008). It was also reported that Dcir-KO mice spontaneously develop autoimmune disease and are more susceptible to experimental arthritis (Fujikado et al., 2008).

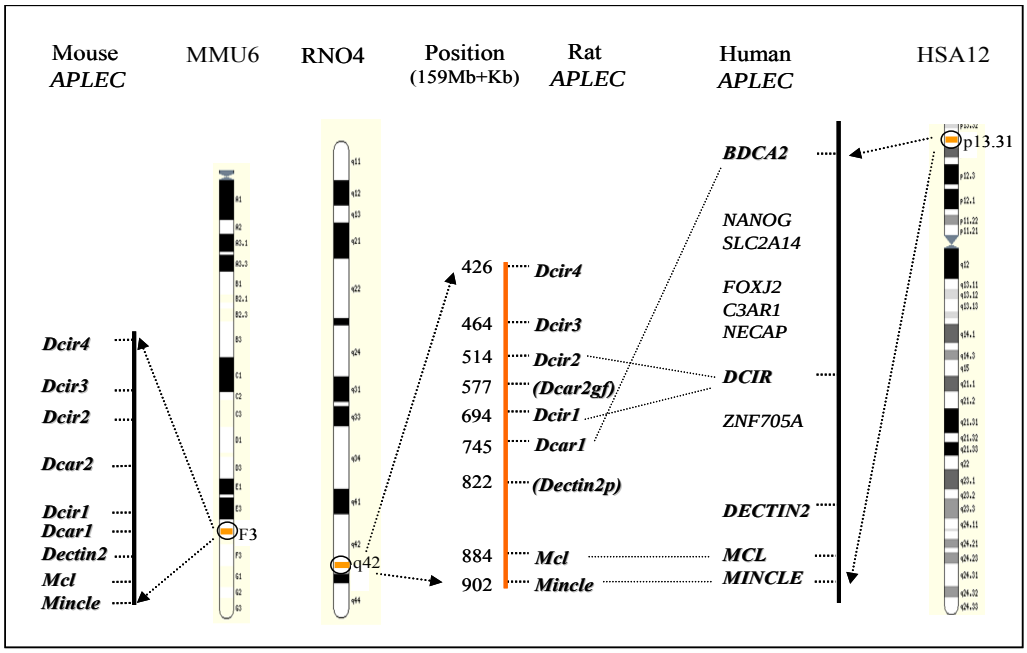

Figure 4. Physical map of the *APLEC* genes in the rat, the mouse and the human. Dotted lines between rat, mouse and human genes indicate *APLEC* orthologs as stated by the current National Center for Biotechnology Information database (NCBI, http://www.ncbi.nlm.nih.gov/entrez/gene). With the following adjustments following the recent updates of the NCBI (updated 3rd Sep., 2008) and Ensembl (http://www.ensembl.org/index.html, release 51, Nov. 2008) rat databases, mouse *APLEC* genes lie in tandem as described, whereas another novel rat pseudogene was annotated in the locus in Ensembl: RGD1559669 predicted (159.846-847), between *Dectin 2p* and *Mcl*; The interval between human *BDCA2* and *DCIR*, as will as between *DCIR* and DECTIN2 also contain several non-C-type lectin superfamily genes.

Dcar1-2 In the rat and mouse, two orthologues of *Dcar* genes were detected and named *Dcar1* and *Dcar2*, respectively (mouse *Dcar2* was reported under the name *Dcar*) (Kanazawa et al., 2003). In the rat *Dcar2* is predicted as a gene fragment (Flornes et al., 2004). In humans there are no DCAR genes predicted. But human *BDCA2* is considered to be the orthologue of rodent *Dcar* genes, since structurally *BDCA2* exhibits the same *Dcir*/*Dectin2* hybrid feature as *Dcar* genes do and thus may represent the equivalent to the rodent *Dcar* genes (Flornes et al., 2004). *Dcar* are characterized by the presence of a positive charged arginine residue in the TM region. This positive charged amino acid was suggested to interact with a negatively charged residue in the

TM region of an ITAM-containing adaptor molecule FcRγ, predicting activating function. The CRD of Dcar shows 91% amino acid sequence identity with that of Dcir genes (Kanazawa et al., 2003). Some studies suggest that DCIR and DCAR, which contain ITIM - an inhibitory motif or ITAM - an activating motif, respectively, possibly form paired immunoreceptors, functionally as well as structurally (Kanazawa et al., 2002; Kanazawa et al., 2003). For rat *Dcar1*, the DA allele carries a predicted early nonsense mutation. It leads to a stop codon and suggests *Dcar1* may be nonfunctional, which could be a natural knockout for *Dcar1* in the DA strain (Lorentzen et al., 2007).

Although ***Mincle*** and ***Mcl*** have high homology in their CRD region, their TM and cytoplasmic tails are quite different. *Mincle* contains a positively charged residue (arginine) in the TM region. This positively charged residue interacts with a negatively charged residue in the TM region of ITAM-bearing adaptor molecule FcRγ, predicting activating function (Flornes et al., 2004; Yamasaki et al., 2008). *Mcl* lacks both features (Arce et al., 2004; Flornes et al., 2004). It has recently been reported that *Mincle* is involved in the recognition of *Candida albicans* (Bugarcic et al., 2008; Wells et al., 2008). Also it has been demonstrated that *Mincle* can recognize a nuclear protein released by dead or dying cells (Yamasaki et al., 2008). It is therefore possible that *Mincle* is a dual sensor of non-self and abnormal self ligands. No function of *Mcl* has been suggested except for *MCL*-mediated endocytosis (Arce et al., 2004).

2 AIMS OF THE STUDY

The overall goal of this thesis was to identify and functionally characterize gene(s) that regulate arthritis as well as other immune-related diseases in the rat, and to gain functional insight to possibly obtain clues for disease prevention and treatment.

Specifically, I aimed:

- To fine map arthritis-regulating loci on rat chromosome 4 and 10, to determine if genetic interactions occur between the loci, and to examine potential association with rheumatoid arthritis of the corresponding human genomic loci (paper I)
- To determine whether the recently identified gene complex *APLEC*, which determines susceptibility to oil-induced arthritis in rats, can also influence other types of arthritis and other phenotypes than susceptibility. In addition, I aimed to determine whether sex effects occur, and to define mechanisms underlying impact of *APLEC* on arthritis (paper II).
- To determine over time the potential relationship between *APLEC* impact on oil-induced arthritis and the profiles in draining lymph nodes of transcript levels of APLEC members and cytokines (paper III), with special emphasis on IL-17 that is profoundly regulated by *APLEC* in autoimmune collagen-induced arthritis (paper III).
- To determine whether rat *APLEC* can influence infectious diseases, delayed type hypersensitivity, and macrophage responses to microbial activators of the innate immune system (paper IV).

3 RESULTS AND DISCUSSION

3.1 DEFINITION OF ARTHRITIS RISK GENES ON RNO 4 AND 10

Paper I

Our laboratory has a longstanding interest in the QTLs *Oia2* and *Oia3*, which were originally identified in a genome-wide scan of OIA in an F2 cross between DA and LEW.1AV1 rats (Lorentzen et al., 1998). The two QTLs are the first described in arthritis induced by a non-immunogenic adjuvant. Both QTLs overlap with arthritis-QTLs discovered in other models (Griffiths et al., 2000; Joe et al., 2000; Nordquist et al., 2000). Although *APLEC* was recently demonstrated to be the major determinant of susceptibility to OIA in the original QTL named *Oia2* (Lorentzen et al., 2007), it is still possible that other genes close to *APLEC* may regulate OIA, as well as other types of arthritis. It is also possible that genes in *Oia2* and *Oia3* interact, as our group previously reported limited penetrance of permissive DA alleles on resistant LEW.1AV1 background in single congenic strains, in contrast to full penetrance in the *Oia2*-*Oia3* double -congenic strain (Holmdahl et al., 2001).

To identify novel candidate risk genes in *Oia2* and *Oia3* and to detect possible gene-gene interactions between the two QTLs, a high-resolution mapping of PIA was performed in a seventh generation (G7) AIL. The AIL mapping approach allows the separation of closely situated QTLs as well as detection of gene-gene interactions using a pairscan analysis (Broman et al., 2003).

A pristane-exposed rat population was genotyped with genetic markers covering the targeted *Oia2* and *Oia3* QTLs located on RNO4 and 10, respectively. As a control for mapping precision and resolution, a small RNO12 region harboring the PIA severity gene *Ncf1* was also included in the study.

A high frequency of arthritis (57 %) was recorded in 422 rats injected with pristane. Maximum linkage to PIA occurred less than 130 kb from the known genetic arthritis determinants *Ncf1* (*Pia4*) and *APLEC* (here corresponding to *Pia27a*), demonstrating remarkable mapping precision. Five novel quantitative trait loci were mapped on RNO4 (*Pia29, Pia27b* and *Pia28*) and 10 (*Pia30* and *Pia31*), with narrow confidence intervals. Some exerted sex-biased effects and some were linked to chronic arthritis.

Genes in the five QTLs represent candidate genes. Since association data for RA susceptibility is available at genome-wide scale, we evaluated the human regions containing the candidate disease risk genes for potential impact on RA. Although no

association was significant following Bonferroni correction, human homologous genomic regions contain loci where multiple nearby SNPs associate nominally with RA, e.g. at the genes encoding protein kinase C alpha (*PRKCA*) and interleukin-17 receptor alpha (*IL17RA*).

This analysis is just a first glance. To confirm the possible associations, follow - up studies are needed, e. g. to perform association studies of *PRKCA* and *IL17RA* in EIRA and other materials.

Importantly, it may be that analysis in RA should take genetic interactions into account. Because in the rat, our analyses of the genotype and phenotype data with two QTL model test revealed that each QTL exerts additive effects with one or several other QTLs. The analysis also suggested two novel epistatic interacting QTLs on RNO10 that influence chronicity only in DA allele combinations. These two QTLs were undetectable in the one dimensional linkage analysis.

3.2 IMPACT OF *APLEC* ON EXPERIMENTAL ARTHRITIS

Paper II and Paper III

That *APLEC* determines OIA susceptibility was originally demonstrated in a single experiment by comparing DA rats with DA rats made heterozygous for DA and PVG alleles in *APLEC* (recombinant R17 heterozygotes) (Lorentzen et al., 2007). This small experiment did not enable analysis of sex effects or of other clinical parameters, such as severity and chronicity. It also remained uncertain whether genetic variation in *APLEC* could influence other types of arthritis, as the different models most likely represent different or partly different disease processes, all of which have clinical similarities to the RA syndrome.

One of the interesting models to investigate is CIA, which in contrast to adjuvant-induced models such as OIA, is clearly an autoimmune disease that allows functional analysis of autoimmunity in terms of serum autoantibody levels and isotypes, as well as cytokine mRNA profiles in draining lymph node.

On this basis, I continued and extended previous research in the lab aimed to determine whether *Oia2* regulates different types of arthritis, i.e. before *APLEC* was identified (Backdahl et al., 2003; Ribbhammar et al., 2003).

My first step was to make an *APLEC* congenic strain, by producing homozygous founders from the heterozygous R17 offspring (Lorentzen et al., 2007). After this, I aimed to establish, by comparing DA with R17, a firm basis for future mechanistic and

comparative genetic studies by delineating the impact of *APLEC* on arthritis in different contexts.

I found that congenic R17 rats generally deviated from DA by reduced arthritis susceptibility, delayed onset, decreased severity and reduced body weight loss in OIA, SIA, CIA and PIA (Fig.5). But paradoxical or opposite genetic effects were observed, e.g. a more severe disease course in congenic males in pristane-induced arthritis (shown in Paper II, Figure 3), and decreased clinical signs in collagen-induced arthritis despite increased auto-antibody levels (shown in Paper II, Figure 4). Compared to DA, the congenic rats had a skewed anti-CII IgG isotype profile and reduced lymph node mRNA levels for IL-17, IFNγ and IL-1β.

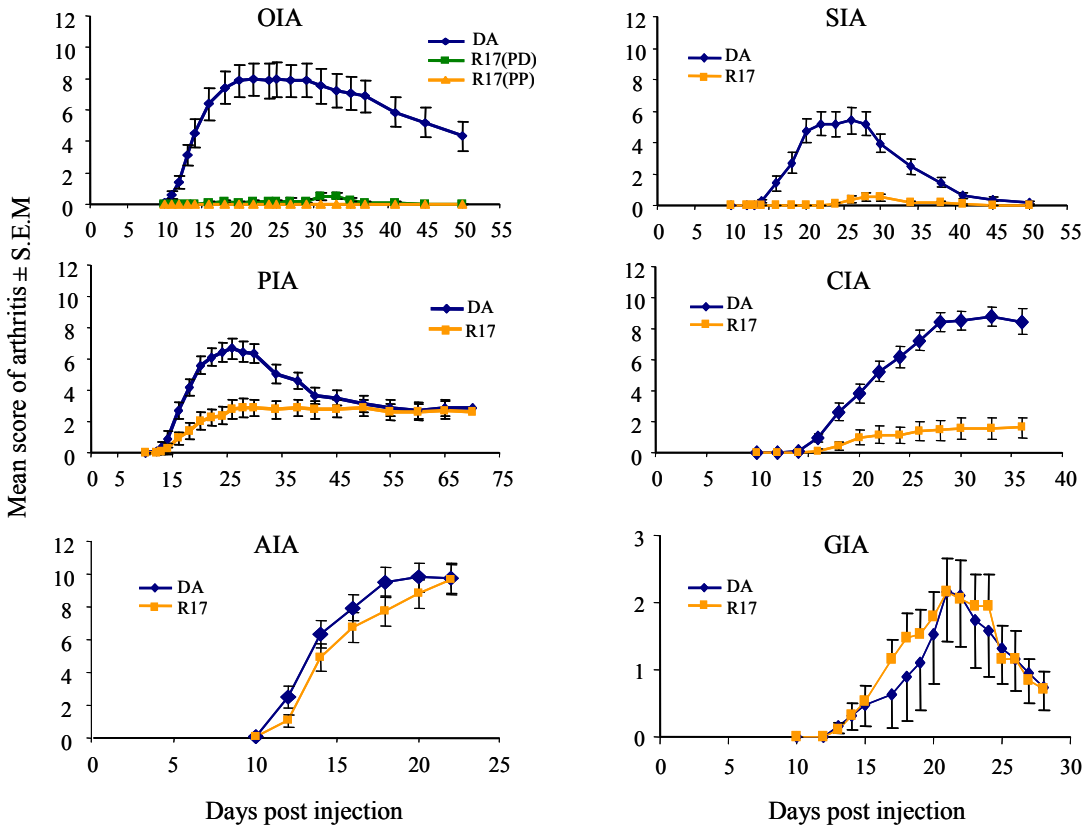

Figure 5. Arthritis development in congenic R17 rats and in parental DA rats in 6 models of rheumatoid arthritis. OIA, oil-induced arthritis; SIA, squalene-induced arthritis; PIA, pristane-induced arthritis; and CIA, collagen-induced arthritis; AIA, adjuvant-induced arthritis; GIA, β glucan-induced arthritis. Data are presented as mean score of arthritis in all experimental animals.

These results demonstrate that *APLEC* regulates autoimmunity, and they provide incitement for further genetic analysis of *APLEC* in humans, in continuation of the first study suggesting association of *APLEC/DCIR* with RA (Lorentzen et al., 2007).

Our data can be interpreted to suggest that association may be limited to certain subsets of RA, may be influenced by sex, and should be analysed for phenotypes besides susceptibility, e.g. severity and anti-collagen antibody levels and isotypes.

Concerning the rat arthritis data, it was notable that DA rats had high levels of IL-17 mRNA in DLN compared to DA. Increasing evidence suggest that the cytokine IL-17 plays a critical role in the pathogenesis of RA (Hwang et al., 2004; Kim et al., 2005; Miossec, 2003). Deficiency of IL-17 in mice results in resistance to autoimmune diseases such as collagen-induced arthritis and experimental autoimmune encephalomyelitis (Afzali et al., 2007; Nakae et al., 2003a; Nakae et al., 2003b). Our data in CIA demonstrate that DA alleles in the gene complex associates with a Th17-type profile in the autoimmune response against CII, whereas PVG alleles do not. Furthermore, this appeared to correlate with susceptibility or resistance in CIA, respectively. Thus, it was reasonable to suspect that this correlation might also occur in other arthritis models. We thus investigated whether skewing of T helper cell cytokine profiles may also explain the impact of *APLEC* on OIA, SIA, and PIA (Paper II, results described in the discussion). These adjuvant-induced RA models are induced by immunostimulating factors without added autoantigens. However, they are still T helper cell-dependent diseases characterized by inflammation restricted to joints (Holmdahl et al., 2001). Our results indicate that *APLEC* indeed influences IL-17 mRNA in both OIA and SIA, in the same way as in CIA (i.e., increased levels in DA rats on day 10 postinjection compared with congenic rats). Unexpectedly, there is no difference in IL-17 mRNA levels between two strains in PIA (Fig.6, showing data described in discussion of Paper II).

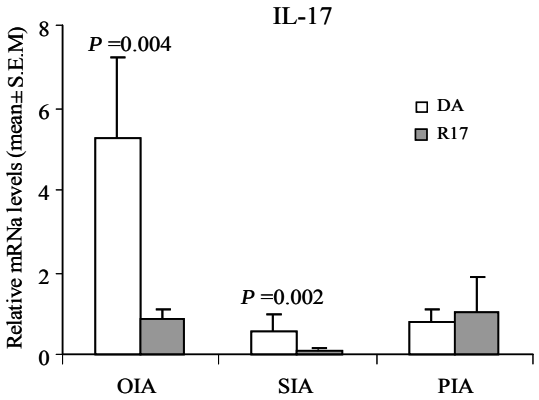

Figure 6. Impact of *APLEC* on cytokine IL-17 mRNA expression in adjuvant-induced RA models. mRNA was prepared from draining lymph node cells taken on day 10 postinjection. Expression levels are presented as the target gene quantity normalized to the GAPDH housekeeping gene. $P < 0.05$ were considered significant. The experiment was performed by Lorentzen JC and Guo JP.

One explanation for this unexpected result, may be that the impact of *APLEC* on PIA, is more or less independent of IL-17. It may also be that the dynamics of IL-17 mRNA differ between models. Nonetheless, the IL-17 levels in OIA differed dramatically between DA and R17, and this was also the model where the clinical picture differed most between the strains, i.e. DA rats developed arthritis whereas R17 did not.

In Paper III, we aimed to determine over time whether the expression levels of the *APLEC* receptors themselves are influenced by challenge with IFA, and whether this can be correlated with pro-inflammatory events, e.g. IL-17 mRNA transcript levels. Thus, age and sex matched DA and R17 rats were injected with IFA. The mRNA expression levels of *APLEC* genes and cytokines were analyzed at day 0, 2, 5, 10 and 15 postinjection. Concerning mRNA expression of *APLEC* genes, the strongest early response was seen for *Dcar1*, which peaked already by day 2, with similar magnitude in both strains. The most striking strain differences in transcription patterns were exhibited by *Mincle* and *Mcl*. Their expression levels were at all time points higher in DA than in R17 rats, with in particular the late high peaks in DA contrasting with low responses in R17. Concerning mRNA expression of cytokines, the most conspicuous strain difference was exhibited by IL-17. In DA rats, its expression rose from undetectable levels at day 0 and 2 to a marked peak by days 5 and 10. In R17 rats, the IL-17 transcriptional levels remained low. A similar pattern was observed also for the proinflammtroy cytokine IL-1β.

Considering the substantial evidence for a central pathogenic role of Th17 cells in autoimmunity, the IL-17 results from this study must be considered conclusive, taken

together with supporting evidence from CIA (Paper II). It may be an important finding that OIA is a suitable model for the study of how Th17 cells influence arthritis induced by non-immunogenic adjuvants.

The strong early upregulation of the *Dcar1* transcript is transcript is also noteworthy. Although the *Dcar1* transcription levels were similarly up-regulated in both strains, it should be borne in mind that this transcript is non-functional in DA rats, as we previously pointed to *Dcar1* as the most likely *Oia2* candidate (Lorentzen et al., 2007).

3.3 IMPACT OF *APLEC* ON INNATE IMMUNITY AND INFECTIONS

Paper IV

That *APLEC* associates with arthritis in rat and possibly in human warrants efforts to learn more about *APLEC* genes and gene products. Besides arthritis we also hypothesized that *APLEC* will be important in regulation of other immune mediated phenotypes, such as infectious diseases and innate immune response. This is based on the evidence that *APLEC* regulates clinical phenotypes in RA models induced by non-immunogenic structures that activate the innate immune system, e.g. incomplete Freund´s adjuvant-oil and yeast β-glucan (Guo et al., 2008). Moreover, the preferential expression of *APLEC* encoded receptors on APC and neutrophils, makes *APLEC* ideal candidates for study regulation of other immune-related traits such as infectious diseases and innate immune responses besides its impact on autoimmunity. Herein we tested the hypothesis by studying the impact of *APLEC* in experimental infectious diseases induced by *HSV-1* and *S. aureus*, in monocyte-dependent delayed type hypersensitivity (DTH) cellular responses *in vivo* and in functional responses to a variety of microbial agents in BMMΦ *in vitro.*

Following infection with *S. aureus* and *HSV* infections survival rates differed significantly between the two strains, but the effects were opposite in the two models. We also demonstrate that *APLEC* influences cellular responses, as evidenced by both reduced DTH response *in vivo*, and MΦ arginase activity *in vitro* in R17 compared to DA rats. In unstimulated BMMΦ some of *APLEC* genes appeared to already have polarized activation states between DA and R17, e.g. in mRNA expression levels of Dcir1, Dcir4, Mincle, Mcl as well as other innate immune receptors, e.g. CD163 and Dectin-1. Following stimulation with a panel of microbial agents, differences in induced mRNA levels were demonstrated for several cytokines, and differential protein levels were confirmed for IL-6 and IL-10 following stimulation with LPS, mannan and

β-glucan. Expression levels of *APLEC* gene mRNAs also differed, and both strains had a notably dichotomous expression of the genes, with general down-regulation of all four *Dcir* genes and up-regulation of *Mincle* and *Mcl*. Interestingly, during these experiments we discovered in both strains a rapid loss of *Dcar1* mRNA expression at already a few hours after cells were taken from bone marrow into culture.

Genetic variation(s) in *APLEC* may influence a wide range of infectious diseases, and our data suggest that the direction of impact will be difficult to predict in a particular disease, since survival rates of DA and R17 rats were opposite in viral-induced encephalitis compared to in bacterial-induced sepsis. We demonstrate that *APLEC* influences cellular responses both DTH *in vivo* and arginase activity *in vitro*. Similar data were previously reported between DA rats and PVG rats which is the donor strain for *APLEC* in R17 (Andersson et al., 2004). The gene complex also regulated transcript levels for a wide range of cytokines following stimulation with various microbial adjuvants. Although mRNA and protein levels do not always correlate, it is clear that *APLEC* regulates correlating mRNA and protein levels of IL-10, which is a suggested key regulator of immune response to viral, bacterial, and protozoal infections (Couper et al., 2008). Following stimulation the dichotomous transcript levels for *APLEC* genes may indicate a shift towards activating signaling, since rodent *Dcir1-2* contain ITIMs, predicting inhibitory function, whereas rodent *Dcar* and *Mincle* have a positively charged amino acid in the transmembrane region. This positively charged residue interacts with a negatively charged residue in the TM region of ITAM-containing adaptor molecule FcRγ, predicting activating function. The rapid loss of *Dcar1* mRNA expression *in vitro* may indicate that the strikingly differential *in vitro* responses of DA- and R17- derived BMMΦ are most likely not mediated by *Dcar1*, but rather by the other rat *APLEC* gene products.

Taken together, our data demonstrate that *APLEC* is a novel determinant not only for autoimmune diseases, but also for infectious diseases. We also demonstrated the striking genetic influence of *APLEC* in relating to innate immunity. Further study of the impact of *APLEC* in different infectious disease settings is warranted.

4 CONCLUDING REMARKS AND FUTURE PERSPECTIVES

In this thesis I have demonstrated that high resolution mapping in rat AIL populations defines limited sets of arthritis candidate risk genes, some of which appear to possibly associate with RA and thus may give clues to evolutionarily conserved pathways that lead to arthritis. The gene-gene interactions demonstrated between the arthritis QTLs in rats suggests that a similar interaction should be considered when analyzing the corresponding chromosomal regions in humans. I have also demonstrated that the gene complex *APLEC* exerts its effects not only on a collection of RA models but also on infectious diseases and innate immunity.

The impact of *APLEC* on arthritis was generally profound and interestingly, the impact was complex and sometimes paradoxical. It depended on factors such as sex and type of arthritis, i.e. how the disease was induced. Based on our results in the rat it is reasonable to suggest that genetic variation in *APLEC* may be important in human arthritis, but that solid genetic association with *APLEC* genes may be difficult to achieve without rational stratifications. This would include stratification for, e.g. sex and ACPA status, as well as for the way arthritis was precipitated, which is not yet possible duo to luck of such etiological information.

Furthermore, our present data in autoimmune CIA demonstrate that *APLEC* regulates T helper cell cytokine profiles, e.g. IL-17 and IFNγ mRNA expression levels, as well as anti-collagen IgG isotype profiles. Interestingly, *APLEC* also influences arthritis and IL-17 mRNA in OIA and in SIA, although no auto-antigens are used to induce arthritis in these adjuvant-oil induced RA models. Surprisingly, although *APLEC* also influences arthritis induced by the adjuvant oil pristine, there is no difference in IL-17 mRNA levels between two strains in this model. It may be that the dynamics of IL-17 mRNA differs between models and that we did not capture the critical time-point(s) in PIA, or the impact of *APLEC* on PIA is more or less independent of IL-17.

I also reported a strong genetic influence of *APLEC* on infectious diseases, but our data suggest that the direction of impact will be difficult to predict in a particular disease since the effects of *APLEC* were opposite in viral-induced encephalitis compared to in bacterial-induced sepsis. This paradox most likely reflects the complexity of the innate and adaptive immune responses involved in the two different types of provocations.

Moreover, I have demonstrated that *APLEC* influences general innate immune macrophage activities *in vitro*, i.e. following stimulation with a panel of microbial

adjuvants, *APLEC* regulates protein levels of IL-6 and IL-10. As several of the tested microbial adjuvants are pathogenic, and can e.g. induce arthritis, it will be interesting to investigate if *in vitro* phenotypes can be used to predict *in vivo* phenotypes, such as disease susceptibility and disease course.

Following stimulation the transcript levels for *APLEC* genes were notably dichotomous, with down-regulated mRNA levels for *Dcir* receptors but up-regulated levels for *Mincle* and *Mcl*. The relative up- or down-regulation of transcript levels were generally more pronounced in R17 compared to DA MΦ, which may reflect that the balance between opposing signals regulates MΦ effector functions.

The newly identified gene complex *APLEC* has here been proven to regulate both autoimmunity and innate immunity. In a future perspective, it will be important to further define the gene(s) underlying the impact of *APLEC* on immunity, and to perform more detailed functional studies of *APLEC*.

To define the critical genetic variation(s) in *APLEC*, several approaches can be applied: e.g. 1) Further positional mapping *APLEC* by creating intra-*APLEC* recombinant strains. 2) Knock-down of genes using small interfering (si) RNA, a powerful tool for genetic and gene functional studies; 3) Generation of antibodies targeting each of *APLEC* genes. In both 2) and 3), we expect that manipulated congenic rats will become susceptible when the PVG allele(s) that confer resistance is silenced or blocked; 4) Unfortunately it is unlikely to delineate the effect of each rat *APLEC* genes by application of knockout methodology, because homologous recombination is not applicable in the rat. Generation of knockout mice for each *APLEC* genes will be important tools for future studies; 5) Finally, it will clearly be important to define the nature of binding ligands, including the identification of agonists and antagonists for *APLEC* genes. Such information will elucidate the limited knowledge on functions of *APLEC* genes and gene products, and thus provide additional possibilities of pathway manipulations.

5 ACKNOWLEDGEMENTS

There are so many people that in different ways contributed to this thesis. I would like to express my gratitude to all of you. Especially I would like to thank:

Johnny C. Lorentzen, my main supervisor, thank you for directing me to the field of experimental genetics, for giving me the opportunity to work on such interesting projects, for your great supervision, for sharing your broad knowledge and the ways of thinking in science, for always keeping a critical eye at my work, and for all the help, support and inspiration during these years.

Lars Klareskog, my co-supervisor, thank you for your kindness and support, for always making time and giving me enthusiastic and constructive feedback on my study, and for providing us the best research environment with great people and superb laboratory facilities.

Bob Harris, my unofficial supervisor, thank you for being such an inspirational and enthusiastic supervisor, for your contribution with great knowledge in immunology, for always being supportive on my study, and for the highly appreciated linguistic advice.

My co-authors**, Liselotte Bäckdahl**, **Monica Marta**, **Linda Mathsson**, **Johan Rönnelid**, **Maja Jagodic**, **Kristina Becanovic**, **Bo Ding**, **Tomas Olsson**, **Margareta Verdrengh**, **Andrej Tarkowski**, **Stefan Lange**, **Eva Jennische**, **Robert A. Harris**, **Line M Flornes**, **Erik Dissen**, **Michael R. Daws**, **Sigbjørn Fossum** and **Johnny C. Lorentzen.**

All present colleagues at Rheumatology lab, **Vivi,** for your caring and encouragement, for introducing me to Johnny, and for your broad knowledge in immunology. **Leonid,** for providing your expertise in genetics and the help in analysis of survival rates, and for your concerns. **Ingrid**, for your enthusiasm and the wonderful pizza evening. **Anca**, for challenging me with for great knowledge on my half-time seminar, and for nice talks.
Marianne, Eva L and **Åse,** for being such kind and caring persons, let me feel at home, also thank **Marianne** for being such enthusiastic and skilled guide in immunohistochemistry, and for your great help. **Eva J,** for often having late lunch together without booking, and for nice chats. **Doina,** for taking care of my plants when I was away.
Therese, my fantastic roommate, for sharing good and bad things, for being so helpful at any time, and for guiding through important and detailed dissertation process **Heidi**, for being such wonderful person, for your always stretched hands whenever I needed, and for all your caring. **Erik S**, for all the nice talks about Sweden and China, for your enthusiastic recommendation of traveling in archipelagos, and for the wonderful time with your family. **Lasse**, **Omri** and **Marcus**, thank you computer guys so much, for rescuing me from my laptop crash and for other computer-related assistance, also thank **Omri** for your expertise in writing of RA autoantibodies, and for the nice talks. **Dimitrios**, for happy lab company at late evenings and weekends, and for all joyful talks. **Alex**, for your brilliant views in science and life in general. **Nannis,** for being so

sweet and enthusiastic. **Ingela**, for all talks regardless science, and for exciting moment when we met at Washington convention center. **Gustavo**, for warmly greeting "Ni Hao". **Hiba** for talks and smalltalks. **Shankar**, for being my first teacher in immunohistochemistry, and for all nice chats. **Mai** and **Karina,** for sharing clinical experience from different nations. **Maria**, for your important help in booking of Lecture Hall on 26th of March, thanks for your kind cancellation. **Marina K** and **Marie W,** for your caring. **Mei**, for feeling warm. **Marie Wahren**, **Helena**, **Sevim**, **Emeli**, **Gull-Britt, Patrick, Jayesh, Vilija**, **Aurélie**, **Vijole**, **Hulda**, **Karin P**, **Hanna**, **Lotta** and **Lena**.

All former colleagues in the lab, **Duojia**, for being true friend, for all your help and all the time we spent together. **Liselotte**, my another unofficial supervisor and colleague, for introducing me to practical work in the lab, for all shared work. **Sipra**, for being such good friend, for your understanding and encouragement. Long time no hear, I miss you. **Cecilia**, for your passion in science, it was pleasure to share your exciting moments with your amazing data. **Wei**, for having common language due to similar life experience. **Monika**, for interesting topic of life in England. **Sukanya**, **Carina**, **Andreas**, **Mona**, **Dzung** and **Ulrica**.

Our dear secretaries in Rheumatology Unit, **Gunnel** and **Susanne**, for being so patient and efficient, for always helping. Our dear secretary in Dept. of Medicine, **Camilla Berg**, I'm very grateful for all your help in big and small questions or mistakes.

The people at the animal department and especially **Elisabeth**, thank you for your great work and for your friendship. You contribute so much to this thesis.

Can't forget **All my rats**, wish all of you have happy life in your heaven.

All present and former colleagues in Neuroimmunology group, in particular, **Maja,** thank you for sharing your knowledge and brilliantness with us, and for all your help. **Monica**, for being such closed friend and wonderful co-worker. **Fredrik**, for great collaboration and supervision, and for your artistic dissection on spinal cord. **Maggan**, for being so warm and helpful, and for your great ideas in study of VRA model.
Rux, for your warm heart and genial smiles, for all the help and nice chats, and for sharing the struggling time at RNase free area. **Johan**, thank you for all your help during these years, especially for your critical reading in genetics part of my thesis frame. **Ame**, for your excellent teaching in sequencing. **Mikael** and **Rikard**, for your great enthusiasm in collaboration. **Rita**, for sharing wonderful time in Brazil, especially can't forget those lucky "Volkswagens" in Iguacu. **Cynthia**, for nice chats on lunch table. **Magnus**, for nice company on 5th floor. **Mohsen**, **Pernilla**, **Karin**, **Alan**, **Nada, Melanie** and **Jian Rong**.

My friends in Sweden, **Li Wang Sternäng**, for being such great friend in my life, for sharing happiness and sadness, for listening to my complaints, for your understanding and support I've always hoped for, and for all the funs we had. **Ola Sternäng**, for being such caring person, and for your wise advice. **Hailian Xie**, my dear friend and corridormate, thank you for the great company during these years. I felt empty when you were away. For your generosity and help, for all the time we spent together,

dinners, talks and travelings… **Jinfang Huang**, for being my first and special friend in Sweden, for your fantastic attitude to life, for caring me as your sister, and for constant supply of dumplings. **Hong Xu** and **Jie Lu**, for feeling as old friends at first glance. **Jinfeng Shen**, for being such happy and helpful girl. **Jikui Guan** and **Yanhong Li**, for friendship and nice company.

All my friends in China, in particular, **Liping Chen**, **Youzhi Bao** and **Xiaozhong Zhang**, for often spending hours to talk with me over the phone, this is so important to me in these years. **Liping**, my one day older friend and sister, for all you have done for me and my family. **Youzhi**, for your sidesplitting complaints when I felt distressed. **Xiaozhong**, for trying your best to boost me up when I was down. **Guiling Li**, for being best friend, and for all your help and support. **Zhengan Cao**, my dear friend and colleague, for your concerns and encouragement. Mr. **Baocheng Wang** for being my wonderful family friend, for all your wise talks. Mrs **Yulan Lai**, for being first teacher in my life, then being my mentor and friend forever. **Liu Yang** and **Peizhen Wang**, my ancient friends, seldom we meet, but we never separate.

My **parents,** and my **sister** and **brother**, for your endless love, for always believing in me. My lovely niece **Wenxian**, for your fresh and funny stories about your "diehard" friends, I enjoy it very much. The relatives from my husband side, my **brothers-in-law** and **sisters-in-law**, for all your caring and support.

Finally, if you agree with Chinese saying: "behind an accomplished man, there is always an accomplished woman supporting him", in my family I would like to say "behind an accomplished woman, there are two accomplished men supporting her".

Mingrui, my dear son, you are the real sense of our life. Your and dad's voice every second day is the breath of my life here. My energy is filled up with your each calling "A Ma". I'm so proud of you, for being such independent and excellent boy. You are my model in some aspects. From now on I'm able to enjoy your happy smiles everyday (may be not often in your deep sleep but on dinner table at least).

Zhigang, my dear husband, it would be impossible to make this thesis without your understanding and support. One instead of thousands: Thank you! for your endless love, for your sacrifice, for letting me being I am. The most romantic thing I could image is that from now on we are getting older together and never be apart any more. Let's collect every pieces of happiness on the way, and save them for chat while we are sitting on wheelchairs.

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