Genetic characterization to dissect the phenotypic complexity of autoimmunity

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Genetic characterization to dissect the phenotypic complexity of autoimmunity

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To my father

“Det är stoltare våga sitt tärningskast,
än att tyna med slocknande låge.

Det är skönare lyss till den sträng som brast,
än aldrig spänna en båge”

Verner von Heidenstam

Abstract

Autoimmune diseases are dependent on multiple genes and unknown environmental factors. Great efforts in the identification of genes conferring susceptibility to autoimmune diseases like Rheumatoid Arthritis (RA) and Multiple Sclerosis (MS) have recently been rewarding. A few genes have so far been associated to several autoimmune disorders and the understanding of the role of these genes in the pathogenesis and the identification of additional genes will be of great use in the designing of new and better therapies. The fine characterization of each disease is of importance in order to define sub-groups, which may respond differently to treatments.

The work in this thesis was done in mouse models for RA and MS and is based on four papers where the aim was to identify genes associated with these diseases. We have studied crosses between a susceptible and a resistant mouse strain to assess the genetic context for a disease-linked locus to appear. In an F2 intercross between the two strains, the *Eae2* locus on chromosome 15 was previously linked to disease in the MS model Experimental Autoimmune Encephalomyelitis (EAE) through interaction with *Eae3* on chromosome 3. The locus homologous to *Eae2* on human chromosome 5 was later associated with MS in a Finnish population. In order to identify additional loci, EAE was studied in an F2 intercross where the *Eae2* locus was neutralized (paper I) and in a N2 backcross (paper II). In paper III and IV a new strategy to study the interaction between *Eae2* and *Eae3* is described. Mice congenic for the *Eae2* and *Eae3* regions were bred in a Partial Advanced Intercross (PAI), which allows for the segregation of genes in the congenic intervals. More than 1000 PAI mice were investigated for Collagen Induced Arthritis (CIA). Different traits of disease were linked to seven sub-QTL within *Eae2* and *Eae3*. Furthermore, the importance of sub-phenotypes in order to identify disease-modifying genes was investigated and is discussed.

Original papers

This thesis is based on the following papers, which are referred to in the text by their roman numerals:

I. ***Identification of susceptibility genes for experimental autoimmune encephalomyelitis that overcome the effect of protective alleles at the eae2 locus.***
Jirholt J, Lindqvist AK, Karlsson J, Andersson A, Holmdahl R. Int Immunol. 2002 Jan;14(1):79-85.

II. ***Novel quantitative trait loci controlling development of experimental autoimmune encephalomyelitis and proportion of lymphocyte subpopulations.***
Karlsson J, Zhao X, Lonskaya I, Neptin M, Holmdahl R, Andersson A. J Immunol. 2003 Jan 15;170(2):1019-26

III. ***Identification of epistasis through a partial advanced intercross reveals three arthritis loci within the Cia5 QTL in mice.***
Johannesson M, Karlsson J, Wernhoff P, Nandakumar KS, Lindqvist AK, Olsson L, Cook AD, Andersson A, Holmdahl R. Genes Immun. 2005 May;6(3):175-85.

IV. ***Genetic interactions in Eae2 control collagen-induced arthritis and the $CD4^+/CD8^+$ T cell ratio.***
Karlsson J, Johannesson M, Lindvall T, Wernhoff P, Holmdahl R, Andersson A. J Immunol. 2005 Jan 1;174(1):533-41.

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Abbreviations

AEP	Asparagine endopeptidase
Aire	Autoimmune regulator
anti-CCP	Anti-Cyclic citrullinated peptide antibody
APC	Antigen Presenting Cells
AS	Ankylosing Spondylitis
Bbaa	Borrelia burgdorferi-associated arthritis
BcR	B cell Receptor
CAIA	Collagen-Antibody Induced Arthritis
CD	Cluster of Differentiation
CIA	Collagen Induced Arthritis
CFA	Complete Freunds Adjuvance
CNS	Central Nervous System
DC	Dendritic cells
EAE	Experimental Autoimmune Encephalomyelitis
HLA	Human Leukocyte Antigen
HS	Heterogenous Stock
IFA	Incomplete Freunds Adjuvance
IL	Interleukin
IFNγ	Interferon γ
IgG	Immunoglobulin G
Mbp	Megabasepair
MBP	Myelin Basic Protein
MHC	Major Histocompatibility Complex
MO	Macrophages
MOG	Myelin Oligodendrocyte Glycoprotein
MS	Multiple Sclerosis
NKT	Natural killer T cell
QTL	Quantitative Trait Loci
PAI	Partial Advanced Intercross
PADI	Peptidyl Arginine Deiminase I
PDCD1	Programmed Cell Death gene 1
PLP	Proteolipid Protein
RA	Rheumatoid Arthritis
RF	Rheumatic Factor
RUNX1	Runt-related transcription factor 1
SLE	Systemic Lupus Erythemathous
SNP	Single Nucleotide Polymorphism
Th	T helper
TcR	T cell Receptor
TEC	Thymic Epithelial Cells
TGFβ	Tumor Necrosis Factorβ
TNFα	Tumor Necrosis Factorα
Treg	Regulatory T cells
Trmq	T cell Ratio Modifier QTL

Introduction

Autoimmune diseases arise from an immune system triggered to attack the bodys own tissues. Rheumatoid Arthritis (RA) and Multiple Sclerosis (MS) are inflammatory autoimmune diseases that confer a lot of pain, malaise and disability. RA is estimated to affect approximately 1% of the worldwide population with a female predominance of 3 to 1(1). The prevalence of MS in northern Europeans is about 0,2% and also here, women are more frequently affected than men (2). One of the most characteristic traits of RA is the symmetrical swelling and inflammation in hand joints. MS affects the central nervous system and symptoms like numbness, weakness and difficulty to walk are early signs. Subsequently, disability and paralysis arise from electrical short-circuiting of the neurons and progressive loss of plasticity of the central nervous system (CNS). Finding satisfactory cures for these diseases have been a struggle for decades. Both RA and MS are rather syndromes than single diseases since the disease courses differ tremendously in between patients and could vary from mild to devastating disabling with severe destruction of the target organs. 85% of MS patients develop a relapsing-remitting disease course and 10-15 % suffer from primary progressive MS, a form, which so far lacks helpful treatments.

One way to develop better treatments for MS and RA is to identify disease-modifying genes. This would subsequently lead to a better understanding of the molecular basis of disease and enable the finding of new therapeutic targets. In order to identify disease-modifying genes it is important to have well defined diagnoses. So far, most linkage studies performed in humans have failed to identify disease-linked genes except for the Human Leukocyte Antigen (HLA), and a reason for this might be the heterogeneity of the diseases themselves but also the heterogeneity of the human genome. Since these disorders are genetically complex and dependent on multiple genes, some of which are working in interactive networks, we need tools to characterize the genetics to be able to get a better understanding of the disease mechanisms. Moreover, people live different lives and are affected by various environmental influences such as smoking, diet, social class et c. Taken together, this

makes it extremely hard to form homogenous test groups in humans. Although being different diseases, autoimmune disorders share some characteristics and possibly also some of the genetics. Data have emerged showing the involvement of shared genes in several autoimmune diseases in humans (3), (4), (5), (6), (7), (8).

Animal models have been developed to study the pathogenesis of RA and MS among other autoimmune diseases. By using these models the genetics is homogenous and the environment can be controlled. Another great advantage is that experiments made in animal models are faster and conclusions can be drawn quicker than is possible in human trials. Also in animal models, the involvement of shared autoimmunity genes has been proposed (9). By using crosses of inbred mouse strains, several quantitative trait loci (QTL) loci have been linked to the experimental model for MS, Experimental autoimmune encephalomyelitis (EAE) and for Collagen Induced Arthritis (CIA). Several of the QTL are overlapping and common genes might control both CIA and EAE.

In the present thesis, the *Eae2* is discussed, a QTL on mouse chromosome 15 (figure 1). *Eae2* controls both EAE and CIA supporting the hypothesis of the existence of shared autoimmunity genes. The emphasis is put on the importance of working with well-defined phenotypes and finding tools to comprehend the complex network of gene interactions contributing to the biological pathways underlying the various traits of autoimmune disease. The thesis is based on four papers, which follow the progression of gene identification methodology by investigating the *Eae2* locus in different genetic contexts.

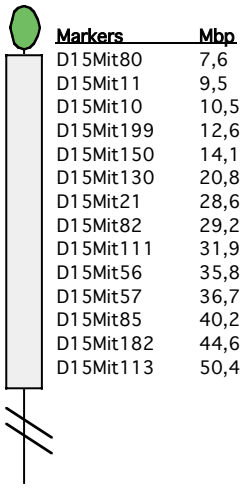

Figure 1. The *Eae2* region on mouse chromosome 15 discussed in this thesis.

Insight into the immune system

The immune system is designed to fight off invading microbes and pathogens and is divided into the innate and the acquired immune systems. One important feature of the immune system is its ability to discriminate self from non-self. Cells from the innate immune system ingest antigens derived from the own body as well as taking in foreign bacteria and viruses. The presentation of the digested antigens on the surface of antigen presenting cells (APC) to antigen specific T cells is then followed. The subsequent behavior of the T cells is an important event in inflammation and elimination of an infection. However, sometimes things go wrong along the way and certain tissue of the body may be subjected to an autoimmune attack and an autoimmune disease develops.

The innate immune system

The innate immune system is the first line of defense against infectious agents. It involves several soluble molecules including the complement system and interferons produced by virus-infected cells, along with cellular events such as phagocytosis of bacteria. The phagocytic cells are derived from myeloid progenitors during hematopoiesis in the bone marrow and include macrophages, monocytes and neutrophils. One of the events involved in phagocytosis is the activation of the NADPH complex, which generates microbicidal reactive oxygen species (ROS). It has been shown that decreased levels of ROS exacerbate arthritis in a rat model of RA. Linkage analysis and subsequent positional cloning identified the *Ncf1* gene. A single nucleotide polymorphism (SNP) in the gene was found to alter the ROS production and thus being responsible for this innate phenotype (10).

Antigen presenting cells

There are two kinds of APC. The professional APC include the dendritic cells (DC), macrophages (MO), B cells, and thymic epithelial cells (TEC), which constitutively express major histocompatibility complex (MHC) class II molecules. Non-professional APC can be induced to express MHC class II and include fibroblasts, mast cells and glial cells within the CNS. The primary role for the professional APC is to protect against pathogens by phagocytosis of invading bacteria/virus. The

pathogens are ingested in endosomes that fuse with lysosomes in the cytoplasma for degradation into peptides. These peptides are subsequently being presented on MHC class II molecules on the cell surface of the APC. Intracellular pathogens are presented on MHC class I molecules.

Activated macrophages express the co-stimulatory molecules B7-1 (CD80) and B7-2 (CD86) and in order to activate antigen specific T cells via the MHC-antigen-T cell receptor synapse, a co-stimulatory signal via the B7-ligand, CD28 is a prerequisite. It has been proposed that B7-1 and B7-2 engagement affect the differentiation of T cells into different effector functions (Th1 or Th2 cell lineage; see below), which have implications in autoimmune disease (11). Furthermore, the activation status of the APC is of great importance in autoimmune disease. In a transgenic system where the B7.2 molecule was constitutively expressed on resident microglia in the CNS, the mice developed a spontaneous demyelinating disease. The infiltrating cells were $CD8^+$ memory- and $CD4^+$ T cells implicating a role for the B7/ CD28 pathway in MS (12). Also, in mice deficient in the B7.2 molecule, the mice developed a milder disease upon EAE induction (13). Interestingly, as discussed in paper IV, a phenotype concerning the expression density of the B7.2, but not B7.1, on activated macrophages was linked to the *Eae2* region.

In the context of autoimmune disease, the APCs play a crucial role in the pathogenesis, by presenting peptides derived from the body's own target organs. The APCs connect the innate and the acquired immune system by presenting peptides to T and B cells via their respective receptors, BcR and TcR. Through this engagement, the T and B cells receive a first signal for activation. However, co-stimulation is required for full activation, and the APC gives a secondary signal to the T cell via the B7-CD28 interaction. For B cell activation, T cell help is needed through the CD40-CD40 ligand interaction in addition to stimulation by Th2 cytokines like IL-4 and IL-10, outlined in Figure 2.

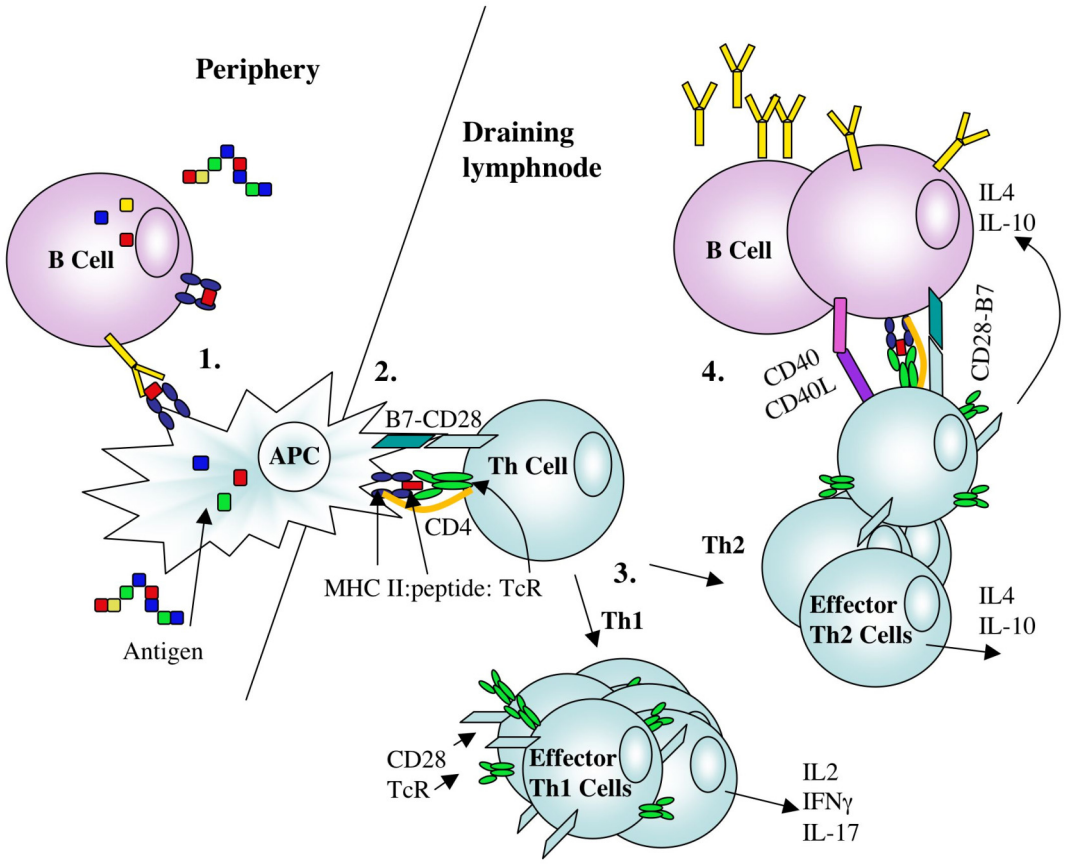

Figure 2. Schematic outline of antigen presentation. (**1.**) Antigen presenting cell (APC) engulf antigen in the periphery, degrade it and present peptides derived from the antigen on MHC class II molecules (blue). The APC can present the peptide (red) to antigen specific B cells via MHC and the B cell Receptor (BcR, yellow) in the periphery. B cells are also able to internalize antigen, and present peptides on MHC class II. (**2.**) Antigen-activated APCs migrate to the draining lymph node and up-regulate B7 (turquoise) and MHC class II (blue), and secrete cytokines (not shown). In the draining lymph node, the APC presents the antigen peptide (red) to $CD4^+$ T cells with epitope-specific T cell Receptors (TcR, green) along with a co-stimulatory signal via B7-CD28. (**3.**) Activated T cells submit into either Th1 or Th2 effector functions and produce different cytokines. (**4.**) Activated T cells may stimulate B cells through the TcR (green) and MHC class II, along with co-stimulation via CD40-CD40L, CD28-B7, and Th2 cytokines (e.g. IL-4 and IL-10), leading to clonal expansion of the B cells and production of IgG antibodies (yellow Y-shaped).

The acquired immune system

The acquired immune system comprises B and T lymphocytes derived from lymphoid progenitors during hematopoiesis. Lymphocytes express antigen-specific receptors that are generated to encompass an extreme variety of possible antigens evolved from

foreign intruders. The B cells mature in the bone marrow while the T cells are derived from the thymus. In autoimmune disorders, these cells recognize self derived antigens as foreign and mount an attack to the specific tissue of origin.

T lymphocytes

T cells express either CD4 or CD8 as a co-receptor to the T cell Receptor (TcR) depending on their affinity for MHC class II or MHC class I respectively. The $CD8^+$ T cells are called cytotoxic since they can kill tumor cells or cells infected with virus. The $CD4^+$ T cells are helper T cells and interact with the MHC class II bearing cells. The $CD4^+$ T cells could be categorized into T helper (Th) subclasses 1, 2, and 3 according the profile of the cytokines they secrete.

RA and MS and their respective animal models (CIA and EAE) are looked upon as Th1 driven diseases (14), (15). There is a vast infiltration of $CD4^+$ T cells into rheumatic synovial joints. It has been shown that these cells produce Interferon γ (IFNγ) and IL-17; pro-inflammatory cytokines produced by Th1 cells. IL-17 has osteoclast activating properties and may contribute to the destruction of cartilage in concert with the monocyte derived cytokines tumor necrosis factor α (TNFα) and IL-1 (16). The administration of anti-TNFα as a treatment in RA has been successful in reducing the degradation of cartilage (17).

Th2 cells typically produce the cytokines IL-4, IL-5, IL-9, IL-10 and IL-13. One of the criteria for RA is the Rheumatic Factor (RF), which is auto-antibodies towards IgG molecules in sera (18). In order for B cells to produce antibodies they are dependent on T cell help. This humoral response is triggered by activated Th2 cells by the production of cytokines in addition to cell-cell contact through the TcR-MHC. Moreover, a co-stimulatory signal is needed through the CD40-CD40 ligand in order for the B cells to commit to isotype switch and production of IgG antibodies (Figure 2). It has been shown that the Th2 cytokines IL-4 and IL-10 have a clear down regulatory effect on development of CIA (19).

The Th3 cells produce mainly Tumor Necrosis Factor β (TGFβ) and are implicated in the regulation of immune responses. An example where these cells play an important

role is in the gastrointestinal tract, which is exposed to myriads of dietary proteins. In order to avoid food allergies the regulatory Th3 cells play an important role by avoiding an immune response towards these antigens, a process called oral tolerance. By taking advantage of these regulatory T cells residing in the gut, much research has aimed at inducing oral tolerance towards auto-antigens and thereby suppressing an autoimmune attack towards these antigens in the tissue of origin (e.g. brain, joints, pancreas et c) (20), (21).

Positive and negative selection

The T cells mature in the thymus and the maturation process involves positive and negative selection. During development, T cells that express both CD4 and CD8 co-receptors are positively selected in the thymic cortex based on the ability of the TcR to interact with MHC expressed on thymic stromal cells. $CD4^+CD8^+$ double positive (DP) T cells survive (*positive selection*) if their TcR bind weakly to self-MHC expressed on thymic stromal cells whereas those DP cells with TcRs which do not recognize self-MHC or bind strongly to self antigens presented by self MHC undergo apoptosis (*negative selection*).

CD4 and CD8 T cell fate

Several theories exist on how the decision is made for a T cell to become a $CD4^+$ or $CD8^+$ T cell. Interestingly, the hematopoietic transcription factor runt-related transcription factor 1 (RUNX) proteins, recently identified to control autoimmune disease (see below) are implicated in lineage decision. The RUNX1 has been found to regulate the expression of CD4 during CD8 lineage commitment (22). It has been suggested that the strength of the TcR-MHC signal could be decisive in lineage fate, where a strong signal would promote a CD4 fate and a weaker signal would lead to the CD8 lineage (23). Also, the persistence of TcR signaling supports development of $CD4^+$ cells while interrupted signals result in cells going into the CD8 lineage (24). How and when thymocytes interact with the thymic antigen presenting cells (APC) during positive selection could affect cell fate. Thymic stromal cells, which are consisting of epithelial cells, dendritic cells, and macrophages play an important role in the maturation of DP T cells into single CD4 or CD8 positive T cells. CD83 is expressed on thymic epithelial and dendritic cells. It has been shown that the

engagement of CD83 in the interaction between maturing T cells and the APC is required for the generation of $CD4^+$ T cells (25). Thymic macrophages mainly play a role as scavengers of apoptotic T cells (26), but have also been suggested to present antigens and affect MHC restriction in close interplay with thymic nurse cells (27).

The $CD4^+$ and $CD8^+$ T cell ratio is under genetic control and a role for the MHC locus in the determination of the proportions of $CD4^+$ and $CD8^+$ cells has been reported in linkage studies in mice (28), and rats (29). Additionally, non-HLA genes are of importance and are illustrated by the difference in the ratio in the two MHC congenic mouse strains RIIIS/J and B10.RIII used in experiments discussed in this thesis. As shown in paper II, a N2 backcross of these strains revealed that the $CD4^+/CD8^+$ T cell ratio is controlled by three different QTL on chromosomes 2, 6, and 15 (*Eae2* region). Moreover, in paper IV we demonstrate that the $CD4^+/CD8^+$ T cell ratio is controlled by genetic interactions between sub-QTL in the *Eae2* (*Cia30*) and *Eae3* (*Cia5,* and *Cia22*). The sub-QTL controlling the $CD4^+/CD8^+$ T cell ratio and the proportion of $CD4^+$ and $CD8^+$ T cells in peripheral blood was named T cell ratio modifier QTL4-6 (*Trmq4, Trmq5, and Trmq6*).

Central Tolerance

The thymus is organized into cortical and medullary zones. Positive selection occurs in the cortical area whilst negative selection and tolerance induction happen in the medulla. It appears that in the thymic medulla, there are distinct tissue lobes organized in islets or organoids, which express antigens specific for various organs, e.g. lung and thyroid gland (30). These islets have descended from a few hundred epithelial stem cells (31). Thymic epithelial cells (TECs) in the medullary organoids are able to synthesize organ-specific proteins, process them, and display antigens on MHC molecules. The expression of the autoimmune regulator (*aire*) gene is involved in the transcription of these extra-thymic tissue specific genes. The TECs are able to induce tolerance and negative selection in $CD8^+$ T cells but not in $CD4^+$ T cells (32). The TECs may transfer antigens to the DCs, which are able to induce tolerance also in $CD4^+$ T cells.

Negative selection is affected by how strongly the T cells engage their TcR with MHC, but also by the concentration of MHC molecules in thymus. It has been shown that even extremely low levels of MHC class II on thymic DCs confer on them the ability to mediate clonal deletion of autoreactive T cells (33). In addition, the efficiency by which APCs process and present antigens is of importance. An endosomal-lysosomal protease, asparagine endopeptidase (AEP), found in thymic dendritic cells, has been shown to destroy the immunodominant epitope of the myelin basic protein (MBP85-99). Thereby T cells reacting towards this epitope could escape negative selection (34).

Peripheral tolerance

Whereas high avidity recognition of peptide-MHC complexes by developing T cells in the thymus results in deletion and promotes self tolerance (*central tolerance*), such strong interaction by mature T cells in the periphery results in activation and clonal expansion (35). We are continuously exposed to myriads of non-harmful antigens from the diet and environment, and by peripheral tolerance mechanisms a deleterious immune response is avoided (36). Even though many organ specific antigens are expressed in the thymus through the actions of the AIRE protein, not all are expressed in high enough concentrations or with the right posttranslational modifications to eliminate auto-reactive T cells. Indeed, some T cells specific to a self- antigen, but with low avidity TcR, escape central tolerance in thymus. When these T cells encounter self-antigens presented by professional APC in the periphery, they will get stimulated via the TcR, but without co-stimulation there will be no clonal expansion and after a few cell divisions they are deleted (37).

Ignorance is another mean of tolerability, simply meaning that T cells avoid recognition of their cognate antigen due to its expression in immunologically privileged locations e.g. the eye, or that the avidity of the TcR is too low. However, ignorance is a labile state and given the proper stimulatory milieu (e.g. viral infection), the T cells could get activated and start to proliferate, subsequently resulting in an autoimmune attack (38).

Potentially self-reactive T cells are kept in check by regulatory T cells (Treg). The suppression of autoreactive T cells is dependant on cell-cell contact and requires the engagement of B7 molecules on the target T cells (39). The CTLA-4, a ligand for the B7 molecules is constitutively expressed at high levels on Treg cells but not on normal T cells (40). The Treg are normally CD4 and CD25 (IL-2Ra-chain) positive cells but as a Treg marker CD25 is limited since all activated $CD4^+$ and $CD8^+$ T cells transiently upregulate CD25. A better Treg marker is Foxp3, which is expressed in Treg cells but not in other activated or resting T cells (41). Some thymocytes expressing self-reactive TcR with intermediate avidity, upregulate Foxp3 in response to increased strength or duration of a TcR signal, in combination with an unknown signal. Upon Foxp3 induction, thymocytes deviate towards Treg lineage (42).

The Autoimmune Diseases

Rheumatoid arthritis

Despite the tolerance mechanisms, autoreactive cells sometimes escape the control. Potentially harmful T- and B- cells could mount an attack towards "self" by recognizing self-antigen via their respective TcR or BcR. Rheumatoid arthritis is a systemic, chronic, inflammatory, autoimmune disease characterized primarily by destruction and inflammation of synovial joints. The three most abundant cell populations in RA synovium are synovial macrophages, synovial fibroblasts, and infiltrating T lymphocytes (43). Females are more affected than males in a ratio of 3:1. Depending on the variability of the disease, patients must fulfill the following four uppermost criteria for RA diagnosis:

1. Morning stiffness
2. Arthritis in at least three joints
3. Arthritis of hand joints
4. Symmetrical joint swelling
5. Rheumatoid nodules
6. Abnormal serum rheumatoid factor
7. Radiographic changes in hand joints

Morning stiffness and swelling of arthritic joints might be related to an altered hypothalamic-pituitary-adrenal axis and an imbalance of the production of cortisol and melatonin. The anti-inflammatory hormone cortisol is produced by the adrenal gland in circardian rhythm with the lowest plasma concentration during night and early morning. RA patients have lower ratios of serum cortisol to the pro-inflammatory cytokines IL-6 and TNF (44), which might contribute to the stiffness and swelling in the morning. Melatonin is produced by the pineal gland and the peak plasma concentration is reached at night and early morning. This hormone has opposite effects to those of glucocorticosteroids and could promote inflammation, especially through its stimulatory action on Th1 cells leading to cytokine production.

It has been shown that peripheral blood monocytes secrete IL-12 in response to melatonin (45), and melatonin has been reported to aggravate CIA in mice (46).

Although the involvement of symmetrical joint arthritis is a criterium for RA, not all patients have this diagnosis in the early phase of the disease. Some joints are more prone to be involved in symmetrical arthritis than others. The proximal interphalangeal (PIP) joints are more often involved in joint swelling symmetry than the metacarpophalangeal- (MCP), and wrist joints (47). The reason for symmetric inflammation and destruction remains obscure but might involve the nervous system (48).

The role of antibodies in RA

The three latter diagnostic criteria are only observed in subgroups of patients, like arthritic noduli (49), rheumatoid factor (RF), (50), (51), and antibodies towards cyclic citrullinated peptide antibodies. RF is found in sera of around 75% of RA patients and constitute of autoantibodies raised against the constant region of self IgG molecules. The antibodies are found in all Ig classes; IgA, IgG, IgE and IgM. However, the RF is not specific for RA, but it is a diagnostic and prognostic tool since all subclasses of RF predate the onset of RA (52), (53). RF is associated with a more severe disease course and could predict the need for a more aggressive treatment (54).

Another antibody, which has strong association to RA, is the Anti-Cyclic citrullinated peptide antibody (anti-CCP) (55). Citrullin is a “non standard” amino acid which is being generated by post-translational modification involving the enzyme peptidyl-arginine deiminase (PADI). The presence of anti-CCP antibodies in sera could predict the development of RA, indicating that citrullination of peptides and the production of anti-CCP and RF autoantibodies are going on in the early phase of RA (52).

RA and MHC class II

RA is thought to be a T cell driven autoimmune disorder and, in particular, $CD4^+$ T cells have been implicated due to the correlation with certain alleles of MHC class II. The haplotype HLA-DR4 has been associated with RA (56), and the inheritance of susceptibility to RA has been shown by twin studies to be around 60 % where MHC

class II is estimated to account for at least 30 % of the total genetic effect (57). The HLA is identified as having an impact on both RA and MS incidence and severity. Several alleles of the HLA-DRB1 gene (*0401, 0404, 0405, 1402 and 0101) associated with RA, share a conserved amino acid sequence within the DRB1 chain, which is part of one side of the antigen presenting binding site. It has been suggested that this "shared epitope" motif is involved in RA by presentation of an arthritogenic peptide and furthermore involved in the selection of autoreactive $CD4^+$ T cells during thymic maturation (58). The shared epitope has been associated with radiological severity in RA patients (59) and to development of rheumatoid nodules (60). Furthermore, it has been observed that smoking in combination with the shared epitope of HLA-DRB1 is a risk factor for a more aggressive disease development in patients seropositive for RF but not in seronegative RA (61). This is an example of how an environmental factor may interact with genes to increase the disease susceptibility. Several alleles of the HLA genes (DRB1*0401, DRB1*1001, DQB1*0302, and DQB1*0501) have, in addition been associated with the presence of anti-CCP antibodies. One or two HLA-DRB1 shared epitope alleles were significantly associated with production of anti-CCP antibodies along with a more severe disease progression (62).

Multiple Sclerosis

Multiple Sclerosis (MS) is a chronic autoimmune disease characterized by inflammation and destruction of the myelin sheath, as well as axonal injury and neuronal cell death leading to loss of neurological function. Women are more frequently affected than men. The frequency of MS varies over the globe and high frequency areas with a prevalence of more than 30 affected in 100 000 include most of Europe, Israel, Canada, northern US, eastern Russia, and southeast Australia and New Zealand. Africa and northern South America are low-frequency areas with prevalence less than 5 in 100 000. Migrants from high to low risk areas retain the MS risk of their birthplace only if they are at least 15 years old at the time of migration (63).

Infection and MS

One hypothesis is that MS is somehow acquired in early adolescence and that infectious agents could be the culprits in the disease onset (63). Viral infections may contribute to the initiation of autoimmune disease by molecular mimicry of the viral epitope and a self-epitope. Indeed, Theiler´s murine encephalomyelitis virus (TMEV) engineered to express a Haemophilus influenza epitope mimicking the immunodominant myelin proteolipid protein epitope (PLP139-151), induced EAE in susceptible SJL mice. Furthermore, infection could exacerbate an ongoing autoimmune disease (64). Also, T cells recognizing the myelin basic protein (MBP) peptide (93-105) have been shown to cross-react with, and be activated by a synthetic peptide corresponding to residues of human herpesvirus-6 (HHV-6) in MS patients (65), (66).

Inflammatory cells involved in MS

T cells are implicated in MS and have been shown to rapidly traffic the CNS. In a trial where the purpose was to neutralize auto-reactive T cells with antibodies, antibody-covered T cells were found in the cerebrospinal fluid after 72-96 hours post-injection of antibody (67). Up-regulation of CD4 and HLA-DRα mRNA has been found in MS brains indicating that antigen is being presented locally to activated CD4+ T cells (68). T cells, monocytes, and a few B cells characterize the inflammatory profile of active MS lesions. Macrophages are found in the center of the plaque and are filled with myelin debris. Oligodendrocyte counts are reduced in the lesions. The hallmark of the pathology of MS is the demyelinated plaque with reactive glial scar formation as visualized by magnetic resonance imaging (MRI). However, the pattern of demyelination and the cellular constitution of the inflammatory foci seen in MS brains are heterogeneous and indicate that different biological pathways may contribute to the same measurable end-point, the MS plaque (69). Thus, MS might be considered as a syndrome not only due to the different clinical sub groups but also because of the heterogeneous MS plaques. Therefore, fine-characterization of MS plaques may reveal different biological pathways in different MS patients and this could lead to the designing of new and more specific therapies.

MS and MHC class II

Epidemiological studies suggest that both environmental factors as well as heredity contribute to MS. The clearest evidence for genetic risk factors comes from twin studies showing that identical twins have 150-300 times greater risk than unrelated individuals in the population, and that siblings have a 20-40 times higher risk (70), (71). Association with HLA is suggested to explain 14-50 % of the genetic risk (72), and is primarily associated with onset and initiation of disease and not with the chronic phase (73). There is an increased prevalence of MS in isolated familial regions e.g. in Finland (74) and Sardinia (75). To date, the HLA is the only confirmed locus (76). The HLA-DR2 haplotype susceptibility allele has been associated to MS (77), and has been shown promote disease in a dose dependant manner, where two copies of the allele further increase the risk and render patients more likely to have a more severe outcome (78). These findings are in line with the more severe disease development in RA patients homozygous for HLA-DR4 alleles (79).

Animal models

Mouse and human genomes are homologous to approximately 90%, and many genes and biological pathways are conserved between the species. Animal models are crucial for developing new and better treatments for human diseases. The experiments can be done much faster: disease development is investigated for months instead of for years as is the case in humans. Studies of multi-factorial diseases like RA and MS are difficult to perform in man due to the heterogenous human genome, in combination with various interacting environmental factors. Homogenous test groups are not easily established taking into account e.g. age, sex, diet, social life, sleeping habits, everything that might have the slightest impact on disease. Animal models have been of great value in studying models of human autoimmune disorders e.g. MS, RA, diabetes, systemic lupus erythemathous (SLE), but the need for additional models for each of these diseases is increasing. Since we use inbred strains where all mice of one strain are genetically identical, we do not cover the whole picture of one disease but parts or subsets of the pathogenesis. Therefore, studying the disease in several strains of mice and rats will give a more complete picture, and will capture more features that are observed in different patient groups. Furthermore, different immunization protocols in the induction of disease might trigger different pathways and will, thus, contribute to the knowledge of disease.

Experimental arthritis models in the mouse

Collagen induced arthritis (CIA) is the most commonly used animal model for RA and is induced with collagen type II (80), type IX (81) or type XI (82). Other immunization protocols include the antibody transfer model (83) where collagen type II specific antibodies induce joint inflammation.

Glucose-6-phosphate (G6PI) is a secretable intracellular enzyme, which in the extra-cellular compartment can act as a cytokine. It induces B cell maturation and production of antibodies. In a T cell receptor (TcR) transgenic mouse model where the mice developed spontaneous arthritis, it was found that the TcR was specific for

G6PI (84). Furthermore, depending on the genetic background of the mouse strain, disease induction with G6PI in Complete Freunds Adjuvance (CFA) follows either an acute and severe disease course (DBA/1 mouse strain) or a chronic progressive arthritis (C3H.NB strain) (85). The model exemplifies the importance of controlling the genetic environment in order to study the disease phenotype.

The arthritis studies in this thesis are performed in an experimental setup using the susceptible B10.RIII mouse strain and the resistant RIIIS/J strain and the CIA model with collagen type II in Incomplete Freunds Adjuvance (CFA). B10.RIII male mice have an incidence of about 70-90 % while around 50 % of the females develop arthritis. Males are more prone to disease development than females and by castrating female mice they retain the same degree of susceptibility as males, indicating a role for sex hormones (86). It has been shown that the female sex hormone estrogen could mediate an increase in Th2 cytokines along with a decreased production of Th1 cytokines (IFNγ) and thereby modulating the immune reaction (87). Furthermore, estrogen may inhibit an inflammatory response by suppressing homing of inflammatory cells e.g. macrophages and granulocytes and their production of IFNγ and TNFα (88). In paper III and IV we show that specific gene regions in *Eae2* and *Eae3*/*Cia5* control an exacerbated development of arthritis in female mice only, thus implicating a role for sex hormones.

Experimental autoimmune encephalomyelitis

A model for MS was found through serendipity when the virologist Thomas Rivers in 1933 tried to explain a demyelinating disease that occurred in people after rabies vaccination. He injected brain tissue, in which the rabies virus was grown, into monkeys, which subsequently developed signs similar to MS; abnormal gait and eye movements as well as signs of inflammation and demyelination of the brain (89). The conclusion was that injected brain tissue into animals caused an allergic reaction with inflammation of the brain and spinal cord similar to MS, thereof the name experimental allergic (later autoimmune) encephalomyelitis (EAE).

Induction of a paralyzing MS-like disease in rodents is followed after injection of myelin specific proteins or peptides in susceptible strains. Induction of disease with

the myelin oligodendrocyte glycoprotein (MOG) or MOG peptides in B10.Q mice results in nearly 100% incidence with a chronic disease course (paper III). Moreover, SJL/J mice develop EAE when immunized with whole spinal cord homogenate (SCH), while B10.S/DvTe mice are resistant (90). The B10.RIII mouse strain develops a chronic disease course after induction with MBP peptide 89-101. The MHC class II haplotype is of importance for disease development as demonstrated by H2 congenic mice where H2-A^r, A^s and A^q were associated with EAE susceptibility (91). However, the MHC class II congenic strain RIIIS/J, which shares the H2-A^r, is resistant to EAE (and CIA), pointing towards the importance of other genes in addition to the MHC genes. This thesis deals with methods to find non-MHC genes in various crosses between the B10.RIII and the RIIIS/J mouse strains.

Identification of genes

In order to develop new drug targets and to increase the ability to predict the disease course it is important to identify disease modifying genes and which allelic variants are implicated in the pathogenesis. A main goal is to genetically identify clinical subgroups. A problem of the medications available today is that they only work for some patients whereas other patients are unresponsive or even become worse from the same treatment (92), (93). One reason for the unresponsiveness could be that these patients suffer from a variant of the disease. More custom-tailored drugs, aimed at different clinical sub-groups would be a positive outcome of the cloning of susceptibility genes.

The genome

Genes comprise only about 2% of the human genome. The remainder consists of noncoding regions, whose functions may include providing chromosomal structural integrity and regulating where, when, and in what quantity proteins are made. The human and mouse genomes are estimated to contain 20 000-25 000 genes (Human Genome Project). The genome is scattered with polymorphisms that can be used as markers in linkage and association studies of complex diseases. In the work presented in this thesis, we have used microsatellite markers to localize genetic regions linked to specific disease- and cellular phenotypes. Microsatellite markers are short tandem repeats of nucleotides and differ in length. Single nucleotide polymorphisms (SNPs) are the most abundant variations in the genome and the Human Genome Project, which has sequenced > 98% of the human genome, has yielded more than 3 million SNPs. SNPs are estimated to account for 90 % of the sequence variation in humans (94), (95).

Linkage analyses in humans

The first steps towards the identification of susceptibility genes have included linkage analyses, which link a certain allelic variant to a specific (disease) phenotype. In a

meta-analysis of all previously reported linkage genome screens in MS, involving a total of 719 families, six non-MHC regions have been identified (76). Many suggestive linkage peaks have been published and in a recent linkage analysis of RA, which took into account the heterogeneity of the disease, 19 non-HLA peaks were identified out of which 8 were estimated to be true-positives. Nine of the peaks overlapped with previously published regions (96). Other complex autoimmune disorders are dependent on MHC class I alleles. Behçet's disease is associated with MHC class I HLA-B51 alleles in addition to several other genes as suggested by a genome-wide linkage analysis (97). Ankylosing spondylitis (AS) mainly affects the spine and hip joints with inflammation and ossification. This disease is also strongly associated to MHC class I along with several non-MHC genes underlying linkage peaks (98). Interestingly, polymorphisms in the *ankh* gene have been associated with AS (99). The murine homologue, *Ank* is located within the *Eae2* region (100).

The efforts in trying to identify non-HLA susceptibility genes in human autoimmune diseases by linkage analysis approaches have proven difficult. The heterogeneity of the human genome and the variable disease courses decrease the power of detection of susceptibility alleles. Moreover, as discussed above, many of the autoimmune diseases are syndromes rather than one disease and may therefore be dependent on different genetic factors. Many clinically similar diseases are grouped together, irrespective of the underlying genetic contributions, possibly reducing the power of the studies. Genome scans and subsequent linkage studies usually locate the region of linkage, the QTL to a 10-30 cM region (1 cM ≈ 1 recombination in 100 individuals, which corresponds to ≈ 10 genes). To clone a gene in a QTL peak of this considerable size, the frequency of the predisposing allele needs to be very common in the population and confer a strong effect on the phenotype. In contrast, complex diseases like RA and MS, might depend on a large pool of alleles with low frequency in the population and with varying, often low effects on the disease risk (101). Linkage studies often show significance to a specific genomic interval only in specific ethnic groups (102) underscoring the vast impact of allelic and genetic heterogeneity on disease outcome.

Taken together, complex diseases result from a combination of several unfavorable sub-phenotypes many of which are under distinct genetic control. The set of phenotypes contributing to disease seem to differ in different ethnic populations (103), (104), (105). The importance to make the patient groups as homogenous as possible, in order to identify genes, is underscored. Furthermore, it is preferable to study the intermediate end-points, for example various sub-phenotypes, each of which contribute to the disease end-result. All these points argue for the use of animal models of autoimmune diseases, which could adjust for both genetic and phenotypic heterogeneity.

Linkage analyses in animal models

Complex trait mapping aim at detecting the effects of individual QTL (that is additive and dominant effects) on the phenotype. Linkage analyses in mouse models of EAE and CIA have so far identified more than 30 loci for each disease (www.informatics.jax.org). The basis of the method is to produce a gene segregating cross between a susceptible and a resistant mouse (or rat) strain, genotype the animals with microsatellite markers, induce and monitor the disease, and, subsequently, perform a genotype-phenotype statistical linkage analysis. Linkage studies have been performed in different strain combinations and with various disease induction protocols. Many of the *Eae* loci overlap with each other in different models of EAE. For example, the *Eae3*, in the central region on chromosome 3, has been linked to disease in a SJL/J-B10.S mouse cross immunized with the proteolipid protein (PLP) peptide (139-151) (106) and, in addition, was linked to disease in a RIIIS/J-B10.RIII cross immunized with the MBP peptide (89-101) (107). Furthermore, different disease models have co-localized QTL such as the *Eae39* and the proteoglycan induced arthritis (*Pgia16*) on chromosome 5 (108), and the QTL for CIA, *Cia5* to the same locus as *Eae3* (109).

Encouragingly, some linkages originally found in mouse models have subsequently been confirmed in human linkage studies. The QTL for SLE on human chromosome 2 (102), is syntenic to a QTL on mouse chromosome 1 (110), and two loci, found linked to disease in a mouse model for Ankylosing Spondylitis (AS), overlapped with a QTL controlling AS in humans (111). Moreover, the *Eae2* locus, which was originally

linked to EAE (107), is syntentic to a region on human chromosome 5p14-p12, which was later shown to be involved in MS (112).

Shared autoimmunity genes

The fact that several loci have been confirmed in various mouse crosses, which apart from having different genetic set-ups, also are immunized according to different protocols, points towards that the same genes are operating in these models. Furthermore, several QTL in mouse and rat disease models overlap with QTL in human diseases (113). In addition, co-localization of QTL for human autoimmune diseases (e.g. RA and diabetes) has been identified in linkage studies (114). As discussed above, *Eae3* is shared in several other autoimmune diseases; *Eae3*, *Tmevd2*, *Cia5*, *Idd10*, *Idd17*and *Idd18*, and this region is syntenic to human chromosome 1. Inevitably, these data support the existence of shared autoimmunity genes; genes or genetic pathways contributing to immune dysregulation and susceptibility to multiple autoimmune disorders.

At least 12 overlapping non-MHC autoimmune susceptibility QTL have been identified in syntenic regions of human and mouse in genome scans (113). Interestingly, many genes coding for proteins involved in the immune system are clustered in the genome. Therefore, it is possible that the co-localization of different autoimmune diseases reflects associations to different genes in the same cluster (coinciding in the same QTL peak) rather than to a common allele. Indeed, in paper IV, we conclude that in the *Eae3*/*Cia5* locus on mouse chromosome 3, an allele from the NOD strain, known to control diabetes, is not important for the control of neither CIA nor EAE. These results argue against a role for shared genes in the pathogeneses of diabetes, CIA and EAE in the *Eae3*/*Cia5* locus. These results emphasize the importance of a well-characterized genetic set-up, in order to closely pinpoint the genetic contribution to an autoimmune phenotype.

Epistasis- the importance of genetic interactions

Epistasis is classically defined as a genetic interaction in which the gene at one locus affects the phenotypic expression of a gene at another locus (113). The phenotype of a QTL can thus not be explained by simply summing the effects of individual loci. One

example of epistasis in a complex trait is the interaction between the C-allele of angiotensin II type1R (AGTR1) and the D-allele of it´s ligand, angiotensin II converting enzyme (ACE) leading to an increased risk of myocardial infarction (115). The phenotypic outcome, myocardial infarction, is dependent on both loci in a non-additive way.

Another important feature of epistasis is the suppression of autoimmune phenotypes by epistatic modifier genes, which was detected in a mouse model of SLE. The NZM2410 strain is moderately susceptible to SLE and females develop disease after 12 months of age (116). When the *Sle1*, *Sle2* and *Sle3* congenic fragments (originating from NZM2410), were introgressed onto the C57BL/6 background the mice developed fatal lupus nephritis in both males and females at a lower age (SLE). Thus, the effect of *Sle1*, *Sle2* and *Sle3* in the NZM2410 mouse strain is suppressed by modifying genes in the NZM2410 background. These modifier loci were subsequently detected (*Sles1-4*), and by congenic breeding of the *Sle1* and the modifier *Sles1* on the C57BL/6 background, the disease severity was reduced to the level of wildtype NZM2410 (117).

Some of the work in this thesis is built on the finding that 2 loci on mouse chromosome 3 and 15 were found to control development of EAE by genetic interactions. Surprisingly, it was shown that one allele from the resistant strain (RIIIS/J) in *Eae2* on chromosome 15 interacted with B10.RIII alleles in *Eae3* (chromosome 3) conferring increased susceptibility to EAE to a level far extending the level of the susceptible B10.RIII wildtype strain (107). In paper III and IV we discuss this epistatic effect further, and suggest a tool to unravel the genetic complexity of autoimmune phenotypes, namely a partial advanced intercross (PAI). By crossing the *Eae2* and *Eae3* congenic mouse strains for up to 8 generations, genetic recombinations were obtained and genetic control of the disease phenotypes were localized to seven sub-QTL within the *Eae2* and *Eae3* congenic fragments.

Association studies

Association studies of candidate genes located in linkage peaks in both humans and animal models have identified several genes shown to be involved in RA and other autoimmune diseases.

The PADI4 gene was associated to RA in a Japanese population (103). The PADI4 gene, located in a linkage peak on human chromosome 1, encodes for the peptidylarginine deiminase citrullinating enzyme 4. This enzyme is responsible for the conversion of the amino acid arginine into citrulline residues in various proteins. Citrullinated epitopes are associated with autoimmune diseases and antibodies recognizing anti-cyclic, citrullinated peptides are specific for RA. It has been shown that the myelin basic protein is more citrullinated in EAE brains and spinal cords and that severity of EAE coincides with increasing citrullination (118). Furthermore, an increased level of citrullinated myelin basic protein has been reported in the brains of MS patients (119).

By dense SNP mapping of a locus previously linked to several other autoimmune diseases (120) a risk allele of SLC22A4 was found to be associated with RA. The SLC22A4 is highly expressed in inflammatory joints in CIA mice. A SNP in an intron of the organic cation transporter gene SLC22A4 affects the transcriptional efficiency of the gene by altering the binding affinity of RUNX1. In addition, a SNP in the RUNX1 gene was contributing to the risk. SLC22A4 was identified to interact with RUNX1 in a way that homozygous alleles in both genes conferred increased susceptibility to disease. Furthermore, homozygosity for susceptibility alleles of SLC22A4 and heterozygous alleles in RUNX1 conferred moderately increased susceptibility to RA (8). This is a nice example of how genetic interactions could work in an additive fashion.

Another example is a SNP in the programmed cell death gene 1 (PDCD1, also called PD-1), which alters the binding site for the RUNX1. The mutation is located in an intronic regulatory enhancer of the PDCD1 gene and disrupts the binding site for a protein complex including RUNX1. The polymorphism in PDCD1 (called PD1.3) is

associated with development of systemic lupus erythematosus (SLE) in humans (121), type 1 diabetes (122), and RA (123).

By using SNPs with putative functional consequences, the hematopoietic specific protein tyrosine phosphatase, (PTPN22) was associated with RA. PTPN22 also called lymphoid-specific phosphatase (LYP) normally functions as a negative regulator of T cell activation (124). The risk allele changes the function of the protein, contributing to a lower threshold for T cell activation. Another allele of this SNP has been implicated in type 1 diabetes (125). Also, this genes has been associated to SLE (126). Altogether, this suggests that variants of the PTPN22 gene may increase overall reactivity of the immune system and susceptibility to autoimmune disease (127).

It has previously been suggested that an altered ability to maintain T cell homeostasis may play an important pathogenic role in MS (128). Cytokines are important in T cell homeostasis (129). Both naïve CD4 and CD8 T cells require engagements of both TcR and IL7 for survival. For memory T cell survival, on the other hand, IL7 is enough as well as crucial (130). Therefore, the maintenance of the T cell pool after an expansion triggered by antigen is dependent on memory T cell survival. This is mediated by both the expression of the IL7-R and IL-7 itself. Effector T cells loose their IL-7Rs whereas resting T cells have the highest level of expression (131). In an association analysis, 66 genes were genotyped with SNP markers chosen by chromosomal location and biological function. Here, SNPs in two genes, the IL-7R and the Lymphocyte Activation Gene-3 (LAG3) were associated with MS (132). The LAG3 (CD223) is an MHC class II ligand and is genetically related to CD4. It is embedded within the CD4 locus and may be controlled by CD4 regulatory elements. LAG3 is exclusively expressed on activated T cells and NKT cells. Through interaction of LAG3 with its receptor, activated T cells are down regulated (133). The location of the IL-7R on human chromosome 5p13, is syntenic to a region included within *Eae2*. Interestingly, as discussed in paper II and IV there is an increased $CD4^+/CD8^+$ T cell ratio in the *Eae2* congenic mice compared to wildtype mice.

Background to the Present Investigations

The present thesis is based on four papers focusing on the genetic characterization of the *Eae2* locus on mouse chromosome 15 and the dissection of linked autoimmune phenotypes. The *Eae2* locus was first identified in an F2 intercross between a susceptible (B10.RIII) and resistant (RIIIS/J) mouse strain. Importantly, heterozygous alleles in *Eae2* controlled an exacerbated development of EAE by interacting with homozygous B10.RIII alleles in the *Eae3* locus on mouse chromosome 3. Moreover, homozygous RIIIS/J genes in *Eae2* conferred complete resistance to disease induction (107).

The work in this thesis is based on studies in the same mouse model system and we have analyzed the *Eae2* region in different genetic set-ups, in addition to different disease models (EAE and CIA). In an effort to test murine candidate regions in a MS study, the human region syntenic to the *Eae2* locus on chromosome 5 was associated to susceptibility to MS in a Finnish population (112). These findings emphasize the significance of using animal models to detect susceptibility loci to complex trait diseases. With the QTL map in hand it has proven fruitful to perform high resolution mapping of the syntenic regions in humans (121), (104), (134). Also the animal models of complex diseases are extremely heterogeneous and depend on a large set of genes in combination with interactive components. Well-defined disease phenotypes allow for the identification of QTL that determine sub-groups of disease as discussed in paper II. This is more readily done in animal models where the impact of interactive environmental factors is decreased. Also, animal models may ease the detection of interactive loci as discussed in paper III and IV.

The *Eae3* locus co-localizes with the *Cia5*, which was linked to CIA in a F2 cross between the RIIIS/J and the B10.RIII strains (109). Since the susceptibility to EAE was dependent on interactive genes in the *Eae2* and *Eae3* loci, we tested the hypothesis that these loci also control development of CIA. In paper III and IV we

conclude that this hypothesis was correct and, furthermore, we suggest a method on how to characterize the genetics in order to pin down disease phenotypic QTL.

Paper I.

Identification of susceptibility genes for experimental autoimmune encephalomyelitis that overcome the effect of protective alleles at the Eae2 locus

In an effort to verify the protective effect from homozygous RIIIS/J alleles in *Eae2*, this locus was congenically bred onto the susceptible B10.RIII background. In generation 4, the intercrossed congenic mice were analyzed for development of EAE. In contrast to the hypothesis, the mice homozygous for RIIIS/J alleles in *Eae2* were not protected. However, mice heterozygous in the congenic fragment had a tendency to a higher incidence and a more severe disease course supporting the previous data. A reason for that the protective effect of the *Eae2* locus failed to depress EAE development could be that the 4th generation B10.RIII genetic background differs substantially from an F2 intercross and the number of RIIIS/J genes is reduced. Putative interactive loci in the background are not present in the context of a congenic strain. Furthermore, as discussed in paper III and IV the effect of nearby intra-chromosomal epistatic loci is neutralized in the congenic strain.

Moreover, by crossing an *Eae2* congenic mouse with RIIIS/J and subsequently produce F2, intercross mice the *Eae2* locus was fixed in the genome along with the MHC. Here, the impact of other loci on EAE development was analyzed. A QTL on chromosome 7 was significantly linked to acute disease and co-localizes with *Eae4* found in an EAE linkage analysis in a cross between SJL/J and B10.S (90). Additionally, linkages to SLE and CIA have also been associated to the same region (135), (136). Furthermore, the locus on chromosome 7 has been reproduced in CIA in mice congenic for a DBA/1 fragment introgressed onto the B10.Q background (Ahlqvist *et al*. manuscript in preparation). Suggestive linkage close to regions previously identified in other crosses and disease induction protocols (SLE, CIA EAE) were identified on chromosome 1, 6, 8, 10, 11, and 18.

This F2 intercross contained a limited number of animals, thus, reducing the power to detect significant linkages. Therefore, it would be worthwhile to analyze the suggestive linkages in additional crosses. Indeed, in paper II the QTL on chromosome 11 is reproduced to the same peak marker in a different genetic context and shows significant linkage to incidence of chronic EAE.

Paper II.

Novel quantitative trait loci controlling development of experimental autoimmune encephalomyelitis and proportion of lymphocyte subpopulations

To study the genetic contribution to EAE development in a backcross of the B10.RIII and RIIIS/J strains, we immunized more than 400 N2 backcross mice with the MBP peptide 89-101. In the context of a backcross, the genetic variance is reduced compared to an F2 intercross, and may neutralize or reduce the impact of previously identified QTL. We expected to detect the most penetrant loci and since the *Eae2* locus conferred susceptibility in heterozygous state interacting with B10.RIII homozygous alleles in *Eae3* in the previous F2 experiment, we expected to detect this interaction. Although, the importance of the *Eae2* locus in EAE was confirmed in this backcross experiment, the inheritance of susceptibility alleles was opposite to expectation. Intriguingly, mice heterozygous in a broad region close to *Eae2* were protected from disease development. This region extended down to the middle of the chromosome apparently indicating the involvement of genes other than those within *Eae2*. Furthermore, in line with our expectation, we did not observe an effect from the *Eae3* QTL since in the backcross there were no homozygous RIIIS/J alleles.

As expected, we detected some new QTL not previously detected in this strain combination. As observed in other linkage screens we detected some gender specific loci (137). Moreover, linkages on chromosomes 11, 16, and 18 might represent loci previously found in the SJL/J- B10.S strain combination; *Eae6b*, *Eae7* and *Eae23* (chromosome 11) *Eae11* (chromosome 16) and *Eae18* (chromosome 18). The three

peaks on chromosome 11 are homologous to a region on rat chromosome 10, which controls development of EAE (138).

By careful interpretation of clinical scores, we divided the sick mice into three clinical sub-groups; chronic, acute, or remitting-relapsing disease. We concluded that the different clinical subgroups were influenced by different QTL. The importance of categorization of disease development and the linkages to different QTL has previously been reported in a F2 linkage study. Furthermore, it has proven relevant to study clinical sub groups in genetic studies of MS. A previously identified QTL on human chromosome 6 just outside the HLA has been linked to MS (139). Analysis of allele frequencies in this particular locus revealed an increased frequency of a certain alleles in patients with early onset of relapsing-remitting MS (140).

Furthermore, we aimed at finding sub-phenotypes, which could reflect a biological pathway in disease. Studying sub-phenotypes of the disease instead of consider disease as one entity might enhance the power of QTL detection in linkage studies. Therefore, we investigated lymphocyte phenotypes possibly involved in the pathogenesis. The $CD4^+/CD8^+$ T cell ratio was linked to the *Eae2* and, the % $CD4^+$ and % $CD8^+$ T cells were linked to the *Cia22* (*Cia5*/*Eae3* see paper III and IV). We conclude that the study of sub phenotypes might detangle the complexity of autoimmune disease and ease the understanding of what pathways are of importance.

Paper III.

Identification of epistasis through a partial advanced intercross reveals three arthritis loci within the Cia5 QTL in mice

This study aims at identifying interacting genes in pathways determining disease outcome. The *Cia5*/*Eae3* locus has been reported to control several autoimmune disorders. The original QTL for diabetes susceptibility *Idd10,* has been mapped close to the *Eae3*/*Cia5* locus and was found to consist of three sub-QTL each with a subtle disease phenotype (141). Here, we demonstrate by using sub-congenic mice, that the *Cia5*/*Eae3* congenic fragment, referred to as R3 in the paper, contains several disease-modifying genes. In line with the heredity pattern of the diabetes loci, the sub-

congenic fragments contributed to disease in an additive fashion with the strongest disease phenotype seen in the R3 fragment. By excluding mycobacterial adjuvance (IFA) from the induction protocol, it was possible to enhance the CIA phenotype considerably in the R3 congenic mice. Furthermore, the R3 fragment conferred almost complete resistance to collagen-antibody induced arthritis (CAIA). It became clear that genes in the more centromeric sub-congenic fragment controlled the CIA-IFA disease phenotype. The more telomeric fragment did not affect CIA-IFA but showed a later onset of collagen-antibody induced arthritis (CAIA). This indicates that genes within the large R3 fragment work together to confer resistance to arthritis. We conclude that by modifying the disease induction protocol it is possible to enhance the phenotype considerably and enable the narrowing down of linked genetic distance.

To obtain a higher penetrance of the various subloci within the *Cia5*/*Eae3* congenic fragment, we needed to better define the genetic context in which they operate. Furthermore, we aimed at identifying the genes in *Eae2* and understanding the genetic interactions conferring the exacerbated disease development in mice heterozygous in the fragment. We took advantage of the formerly identified interaction between *Eae2* and *Eae3* in susceptibility to EAE, (107) and bred the *Eae2* and *Eae3* congenic mice together to produce bi-congenic mice. These mice were intercrossed in a partial advanced intercross (PAI) for 8 generations and thus, accumulating recombinations for every generation. The mice not chosen for breeding the next generation were immunized with CIA-IFA and altogether more than 1000 mice were studied for different disease phenotypes.

We identified three sub-QTL, *Cia5*, *Cia21*, and *Cia22* within the original *Cia5*/*Eae3* QTL and four loci in the *Eae2* congenic fragment; *Cia26*, *Cia30*, *Cia31*, and *Cia32*. The three subloci on chromosome 3 each interacted uniquely with loci on chromosome 15 and we demonstrate that by altering the genotype on the interacting locus on chromosome 15, the penetrance of the genes in each of the loci on chromosome 3 could be modulated. Interestingly, alleles descending from the protected RIIIS/J strain in a sublocus on chromosome 15 conferred exaggerated disease development. Mice heterozygous in this particular locus made the identification of the subloci on chromosome 3 possible. The importance of modifier

genes has been discussed before and the work by Morel et al. 1999 has showed how the *Sles1* modifier locus crucially affects the disease outcome inherited from the *Sle1* locus. Importantly, loci controlling complex traits seem to harbor multiple genes influencing subtle disease phenotypes (142). We therefore suggest using the new strategy, PAI to highly resolve the genetic map through the use of two congenic fragments harboring genes previously shown to interact. This method eliminates disturbing genes in the background, which might reduce the penetrance of the studied genes.

Paper IV.

Genetic interactions in Eae2 control collagen- induced arthritis and the $CD4^+/CD8^+$ T cell ratio

The centromeric region of mouse chromosome 15 have been shown to control several experimental models of inflammatory disease such as EAE (*Eae2*), proteoglycan-induced arthritis (*Pgia8*) (108), Borrelia burgdorferi-associated arthritis (*Bbaa14*) (143), Theiler´s murine encephalomyelitis virus-induced demyelination (*Tmevd8*) (144), and progression of autoimmune arthritis in MRL mice (*Paam1*) (145). As discussed earlier, QTL controlling autoimmune diseases in experimental models seem to cluster in the genome and it could be speculated whether the same genes or pathways operate in several autoimmune diseases. Another point of view is that genes potentially involved in the development of autoimmunity are clustered in the genome and this would explain the aggregations of QTL (113).

First, we investigated the *Eae2* locus for CIA even though this locus has not been linked to arthritis in any cross between the B10.RIII-RIIIS/J mouse strains. Interestingly, mice heterozygous for the fragment developed an exacerbated disease and to our surprise there was a sex influence. Moreover, mice homozygous for RIIIS/J alleles in the congenic fragment indeed reduced the disease severity in females and a tendency was seen for this also in males, thus supporting the previous statement of the inheritance pattern observed in development of EAE (107). To investigate the proposed interaction between the *Eae2* and the *Eae3* loci we performed a partially advanced intercross (PAI) (discussed in paper III). We investigated more than 1000

mice for different aspects of CIA and concluded that four sub-QTL within the *Eae2* locus (*Cia26*, *Cia30*, *Cia31*, and *Cia32*) are involved in CIA development. We pinned down the region conferring protection from CIA to the centromeric part of *Eae2*, *Cia30*. On the other hand, mice with homozygous RIIIS/J alleles in the *Cia26* sub-QTL (in the telomeric region of *Eae2*) and homozygous B10.RIII alleles in *Cia30* developed an exacerbated disease course, which in addition was female specific. Furthermore, heterozygous alleles in the *Cia32* interacted with two B10.RIII alleles in the *Cia22* locus on chromosome 3 also specifically for females. Males heterozygous in *Cia31* and homozygous B10.RIII in *Cia5* developed more severe disease before boost. These intra- and inter-chromosomal interactions visualize the extreme complexity of the disease taking into account that this method only considers two out of many QTL for CIA development.

As discussed earlier, not only definition of the genetic context is of importance to identify genes or pathways controlling complex diseases. We investigated the expression of the co-stimulatory marker B7.2 on stimulated macrophages from the *Eae2* congenic mice and B10.RIII wt and observed that one or two RIIIS/J alleles in the fragment altered the density of this marker on the cell-surfaces. Since co-stimulation is crucial for antigen-presentation function we consider this as a relevant phenotype. Furthermore, we analyzed the $CD4^+/CD8^+$ T cell ratio as a putative sub-phenotype of CIA. The $CD4^+/CD8^+$ T cell ratio was increased in the *Eae2* but not in *Eae3* congenic mice. In the backcross experiment (paper II) the ratio was linked to the *Eae2* region but not to *Eae3*. However, the $\%CD4^+$ and $\%CD8^+$ was linked to the *Eae3* region.

In the PAI mice, we showed how the $CD4^+/CD8^+$ T cell ratio is linked to the *Cia30* (T cell ratio modifier QTL; *Trmq4*) by interacting with *Cia5* (*Trmq5*) and *Cia22* (*Trmq6*) on chromosome 3. Furthermore, in line with the backcross data, the $\%CD4^+$ and $\%CD8^+$ is linked to the same marker within the novel sub-QTL *Cia22*. Moreover, the $\%CD8^+$ is also linked to the *Cia30* QTL. Taken together, by using the novel strategy of a PAI it is possible to increase the power to detect genetic interactions of complex phenotypes enabling the pinning down of the linked genetic regions.

Discussion and Conclusions

The work in this thesis provides a new strategy to characterize the genetics in order to unravel the phenotypic complexity of an autoimmune disease. By breeding bi-congenic mice in a partial advanced intercross (PAI), we have demonstrated a method to increase the penetrance of the genes in a QTL. Linkage analysis as such is built on genetic recombinations in the genome, and in the PAI strategy we collect mice with recombinations within a limited genetic interval thereby limiting the genetic complexity to two defined QTL. The analysis of interactions between two QTL and the understanding of the inheritance pattern for the phenotype, provide more statistical power to isolate the genes and further on to comprehend the genetic pathways. The data obtained from such fine-mapping strategies makes it possible to analyze data from studies in humans such that interacting loci could be taken into account. Furthermore, the importance of well-defined phenotypes is visualized through that different regions are linked to different disease sub-phenotypes.

How do we proceed to clone the gene in the respective sub-QTL? The first step is to produce sub-congenic lines for each sub-QTL containing small fragments that control the phenotype. The complex inheritance of the sub-QTL in *Eae2* is summarized in figure 3. The *Cia26* region conferred a strong disease phenotype in female mice. With the gender specificity in mind, this region is suitable for a candidate gene approach. By sequencing candidate genes, any polymorphisms between the two strains are detected. Furthermore, if they are located in the coding region they might confer an amino acid shift altering the function of the protein. However, SNPs in noncoding region are also of interest eg. regulatory functions. To ultimately conclude that a candidate gene is involved in the phenotype transgenic mice could be established. By transferring the gene polymorphism from the RIIIS/J to wildtype B10.RIII mice it is possible to investigate whether the transgenic mice behave similar to the congenic mice in disease development. Furthermore, it is possible to investigate existing gene knockouts for the phenotype or else producing such knockout as well as knockin mice.

It has been relatively easy to find recombinations in the *Cia30* and thus it is a suitable locus for making small overlapping congenic lines and subsequently positionally clone the gene. Furthermore, this region is not as dense in genes as many other regions. The bottleneck for this approach is markers; hence finding SNP markers in this region is a prioritized step. Heterozygous alleles in *Cia31* and *Cia32* were linked to exacerbated disease in males and females, respectively. There are few markers in *Cia31* and thus, finding the gene/s in this region would possibly not be achievable by regular positional cloning. Here, it would be useful to take advantage of the dense recombination frequency found in the outbred heterogenous stock (HS) mice. The HS mice are originally bred from eight mouse strains including the B10.RIII and RIIIS/J strains. By phenotyping a set of these mice and genotype the mice with a dense set of genetic markers it would be possible to decrease the genetic distance in the region considerably. The *Cia32* was strongly interacting with the *Cia22* on chromosome 3 conferring exacerbated disease in female mice. By narrowing down the two QTL and investigate the candidate genes in these regions it would be possible to speculate in putative interactions of candidate genes.

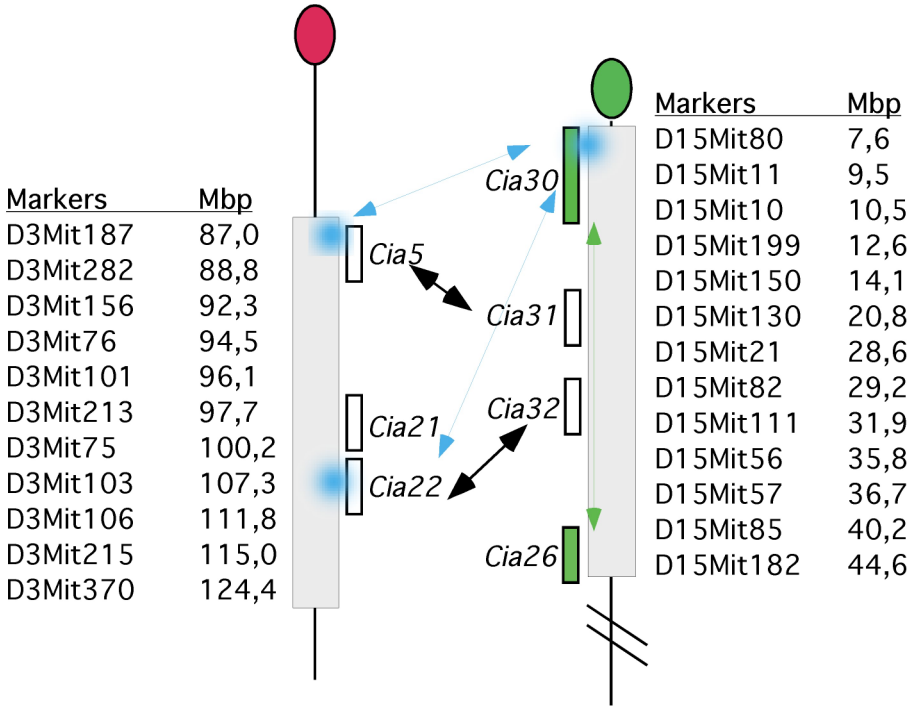

Figure 3. Sub-QTL in *Eae2* interacting within chromosome 15 (green arrow) and with chromosome 3 (black arrows). The interactive loci controlling the T cell ratio (*Trmq4*, *Trmq5*, and *Trmq6*) are indicated in blue.

Moreover, by investigating the sub-QTL for different disease phenotypes such as CIA and CAIA it would be possible to speculate whether the fragment controls the initiation phase or the effector phase of the disease. Investigation of sub-phenotypes concerning e.g. T cells, APCs or antibody-response etc, will further pin-point the action of the candidate gene.

The identification of genes involved in complex inflammatory diseases is in the initiation phase. Several linkage studies in both rodents and humans conclude that QTL for different autoimmune diseases co-localize in the genome and it is valid to speculate whether the same genes may operate in different autoimmune diseases. Indeed, as already discussed, different alleles of several polymorphic genes have been associated to different diseases. Both the PDCD1 and the PTPN22 are involved in T cell activation, and the involvement of these genes in several autoimmune diseases may mirror a common activation pathway. Also the Il7-R and the LAG3 gene, which recently have been associated to MS, are involved in T cell homeostasis and T cell activation, respectively (132). Moreover, in a gene expression analysis using a *Cia5*/*Eae3* QTL-chip to investigate differentially expressed genes in arthritis, several T cell specific genes were found (146). Accumulating data will piece by piece add to the picture of what pathways are implicated in the pathogeneses of RA and MS. The finding of biological pathways in sub phenotypes such as T cell activation may give an insight in how an autoimmune attack may occur and provide a target for drug development.

Populärvetenskaplig sammanfattning

Rheumatoid Artrit (RA) och Multipel Skleros (MS) är autoimmuna sjukdomar som drabbar ca 1 % respective 0.2 % av den nordeuropeiska befolkningen där kvinnor drabbas oftare än män. Dessa sjukdomar orsakas av att immunförsvaret attackerar kroppsegen vävnad. En fortgående inflammation förstör vävnaden och leder till nedsatt funktion. I RA angrips framför allt leder kan bli deformerade och förlora sin rörlighet. Även andra organ kan ibland vara inblandade och därför anses RA vara en systemisk sjukdom. MS är däremot en mer organ specifik sjukdom och drabbar det centrala nervsystemet (CNS). Det skyddande myelinet som omger nervfibrer bryts ned och beroende på var inflammationen är i hjärna och ryggmärg påverkas olika funktioner. Dubbelseende är ofta ett tidigt symptom i MS och orsakas av demyelinisering av den optiska nerven. Accumulering av sklerotiska plack i CNS leder så småningom till en successiv försämring som kan leda till svåra funktionshinder och förlamning.

Orsaken till dessa mycket komplexa sjukdomar är okänd men flera gener samt miljöfaktorer är inblandade. För att öka kunskapen om sjukdomarna och finna bättre mediciner är det viktigt att identifiera dessa gener. Den genetiska arvsmassan skiljer sig stort mellan individer och pga att olika kombinationer av många gener orsakar sjukdomarna. Detta gör det mycket svårt att finna nyckel generna i människa. Djurmodeller av de mänskliga sjukdomarna har fördelen att vi har kontroll över både miljöfaktorer och genetiken då vi använder inavlade stammar. I denna avhandling har vi utvecklat nya metoder för att förstå genetiken bakom RA och MS. Vi har visat att kollagen inducerad artrit, djurmodellen för RA samt djurmodellen för MS, experimentell autoimmun encefalomyelit (EAE) styrs av flera olika gener. Vi har visat att initeringsfasen och den kroniska fasen av artrit ej styrs av samma gener samt att interaktion av flera gener leder till olika förlopp. Vi har hittat genområden som styr vissa celltyper involverade i MS och RA. Vår forskningen hjäper oss att bättre förstå hur olika gener agerar tillsammans i utvecklingen av autoimmuna sjukdommar. I förlängningen kan detta leda till utveckling av nya och bättre läkemedel.

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