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# Genetic regulation of neuroinflammation: a translational approach

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Cover photo by Johan Öckinger. Sculpture at the Ootoyo Jinja shrine in Kyoto, Japan.

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*The capacity to blunder slightly is the real marvel of DNA. Without this special attribute, we would still be anaerobic bacteria and there would be no music.*

Lewis Thomas

# ABSTRACT

Multiple sclerosis (MS) is a chronic inflammatory disease of the central nervous system (CNS), causing demyelination and axonal damage. As other complex diseases with an inflammatory or autoimmune component, MHC is a strong regulator of the disease and acts in concert with multiple non-MHC genes and environmental factors. Recent studies have identified a number of risk alleles for MS, but the etiology and molecular mechanism underlying disease remains largely unknown. In order to identify new candidate genes and define mechanisms for neuroinflammation, myelin oligodendrocyte glycoprotein (MOG) induced experimental autoimmune encephalomyelitis (EAE) in rats has been used as a model for MS. MOG induced EAE share several disease characteristics with MS, including T- and B-cell involvement, demyelination and distribution of lesions in the CNS. High resolution linkage analysis in an advanced intercross line (AIL) was used for the definition and positioning of narrow EAE-regulating regions on rat chromosome 10; *Eae18a* and *Eae18b.*

*Eae18a* was further analyzed using synteny comparisons in humans, rats and mice within genetic regions that show linkage or association to MS and EAE, respectively. A 0.88 Mb consensus region, including 13 candidate genes for regulation of neuroinflammation, was identified.

*Eae18b* regulates severity of EAE and inflammation and demyelination in the CNS. The region contains a cluster of genes coding for chemokines involved in chemotaxis and activation of immune cells. In rats, *Ccl2*, *Ccl11* and *Ccl1* were shown to be *cis*-regulated and subsequent congenic experiments puts focus on genetically determined *Ccl11* expression in periphery and CNS as a key regulator of neuroinflammation. We also present the first evidence for a role of *Ccl12* in MOG-EAE regulation. Association studies including MS patient and controls from four Nordic populations revealed association to polymorphisms in *CCL2*, *CCL13* and *CCL1*, with a possible interaction from the HLA-DRB1*15 haplotype. In addition, CCL2 titers in the cerebrospinal fluid of patients are regulated by the genotype in the *CCL2* gene.

We also investigated the immune response in rat congenics including the *Mhc2ta* gene. We thereby confirmed that the *Vra4* region regulates MHC class II expression on microglia and other antigen presenting cells after nerve injury, and after *in vivo* and *in vitro* stimulations. The region also regulates susceptibility and severity of EAE, and we suggest the *Mhc2ta*-regulated MHC class II expression as a mechanism for EAE regulation.

In conclusion, we have identified 13 candidate genes for *Eae18a* and present evidence for the involvement of *CCL2, CCL13, CCL1, Ccl11* and *Mhc2ta*, in the regulation of MS and EAE.

# LIST OF PUBLICATIONS

I. Definition of a 1.06-Mb region linked to neuroinflammation in humans, rats and mice. JOHAN ÖCKINGER, Pablo Serrano-Fernández, Steffen Möller, Saleh M. Ibrahim, Tomas Olsson, Maja Jagodic. Genetics, 2006, vol. 173, 1539-1545

II. Variants of *CCL2*, *CCL13* and *CCL1* regulate neuroinflammation in rats and humans. JOHAN ÖCKINGER, Pernilla Stridh, Amennai Daniel Beyeen, Frida Lundmark, Maria Seddighzadeh, Annette Oturai, Per Soelberg Sørensen, Inger-Lise Mero, Elisabeth Gulowsen Celius, Virpi Leppä, Lars Alfredsson, Lars Klareskog, Leonid Padyukov, Jan Hillert, Ingrid Kockum, Maja Jagodic, Tomas Olsson. Manuscript

III. Genetically determined *Ccl11* expression regulates experimental autoimmune encephalomyelitis in rats. JOHAN ÖCKINGER, Amennai Beyeen, Alan Gillett, Melanie Thessen-Hedreul, Ruxandra Covacu, Milena Adzemovic, Hans Lassmann, Tomas Olsson, Maja Jagodic. Manuscript

IV. *Vra4* congenic rats with allelic differences in the class II transactivator gene display altered susceptibility to experimental autoimmune encephalomyelitis.Karin Harnesk, Maria Swanberg, JOHAN ÖCKINGER, Margarita Diez, Olle Lidman, Erik Wallström, Anna Lobell, Tomas Olsson, Fredrik Pieh. Journal of Immunology, 2008, 180, 3289-3296

# CONTENTS

# LIST OF ABBREVIATIONS

| | |
|---|---|
| AIL | advanced intercross line |
| ACI | AxC 9935 Irish |
| ACPA | anti-citrullinated peptide antibodies |
| APC | antigen presenting cell |
| BC | backcross |
| BN | Brown Norway |
| CFA | complete Freund's adjuvant |
| CNS | central nervous system |
| CSF | cerebrospinal fluid |
| DA | Dark Agouti |
| EAE | experimental autoimmune encephalomyelitis |
| EAN | experimental autoimmune neuritis |
| EBV | Epstein-Barr virus |
| EDSS | extended disability severity scale |
| ELISA | enzyme-linked immunosorbent assay |
| GWAS | genome wide association study |
| HHV-6 | human herpes virus 6 |
| HLA | human leukocyte antigen |
| HS | heterogeneous stock |
| IFA | incomplete Freund's adjuvant |
| IFN | interferon |
| LD | linkage disequilibrium |
| LEW | Lewis |
| Mb | megabase |
| mDC | myeloid dendritic cell |
| MHC | major histocompatibility complex |
| MOG | myelin oligodendrocyte glycoprotein |
| MS | multiple sclerosis |
| MSSS | multiple sclerosis severity score |
| pDC | plasmacytoid dendritic cell |
| PLP | proteolipid protein |
| PP | primary progressive |
| PVG | Piebald Virol Glaxo |
| QTL | quantitative trait locus |
| RA | rheumatoid arthritis |
| RIL | recombinant inbred line |
| RR | relapsing-remitting |
| SCH | spinal cord homogenate |
| SLE | systemic lupus erythematosus |
| SNP | single nucleotide polymorphism |
| SP | secondary progressive |
| T1D | type 1 diabetes |
| Treg | regulatory T-cell |
| VRA | ventral root avulsion |

# COMPLEX DISEASES

Most mammalian traits, including physiology, immune response, metabolism and behavior, are regulated by several genes in combination with environmental factors. Within this normal variation, there are also several genetic variants that can contribute to an increased risk of developing disease, although the majority of the population remains healthy. Combinations of certain risk alleles in one individual, in combination with environmental factors, can lead to the development of disease. Several of the common complex diseases involve chronic inflammation and dysregulation of the immune system. Examples of such disease are multiple sclerosis (MS), rheumatoid arthritis (RA), type 1 diabetes (T1D), systemic lupus erythematosus (SLE), Graves's disease and Crohn's disease. Together these diseases affect several percent of the western population and contribute to a significant reduction in life quality and represent a major burden on the health care systems.

## GENETICS OF COMPLEX DISEASES

In comparison with monogenetic diseases, where a single polymorphism can explain a major part of the inheritance, the genetics of complex diseases is controlled by multiple genetic variants, each with a small effect on the risk of developing the disease. There is also a significant genetic heterogeneity within the group of affected people; the combination of genetic risk factors can differ between patients, even though the clinical manifestations are similar. Many of the risk alleles will also be found in high frequency within the healthy population. There are a number of strategies used to identify these genetic risk factors contributing to disease susceptibility. Linkage in large families or trios comprised of two parents and affected offspring, has been successful for identification of genetic variants contributing to monogenic disorders, and has also been widely used for complex diseases. The principle of linkage is to identify genetic markers that co-segregate with a disease or other trait over generations, thereby identifying the location of presumable disease loci. This approach has limited success in most complex diseases, although familial variants with strong contribution from a single gene have been identified, for example in Alzheimer's disease, reviewed in (BERTRAM and TANZI 2008).

In recent years, association studies have been the method of choice for genetic studies, where the frequencies of alleles are compared between large groups of patients and

controls. The controls should ideally be population based, or matched to represent a comparable cohort to the group of cases with regard to ethnicity, known to affect allele frequencies, but also potential confounders such as sex, age and residential area. The most common method for association studies is the candidate gene approach, where a limited number of genes are investigated. The selection of candidate genes is limited by the current knowledge on gene function and molecular mechanisms. However, recent advances in high throughput genotyping technology make genome wide association studies (GWAS) possible. This approach has several advantages, for example that no *a priori* selection of candidate genes is needed, and information on a large number of polymorphisms makes interaction analyses possible. GWAS has thereby the potential of unmasking previously unsuspected disease pathways. Both candidate gene approaches and GWAS rely almost exclusively on linkage disequilibrium (LD), the correlated inheritance of alleles, between the unknown causative variants and the genotyped markers in markers.

## MULTIPLE SCLEROSIS

Multiple sclerosis (MS) is a chronic inflammatory demyelinating disease of the central nervous system (CNS). The disease onset is usually around 30 years of age and symptoms are characterized by sensory and motor disturbances, including numbness, muscular weakness, blurry vision, bladder dysfunction as well as pain, fatigue and cognitive impairment. This variability of symptoms between and within a patient probably reflects the location of inflammatory lesions within the CNS. There is no absolute test for the diagnosis of MS, but the current diagnostic criteria state that a patient should have at least two clinical bouts dispersed in time and location, or a single clinical bout but with additional evidence of lesions, using magnetic resonance imaging (MRI) (MCDONALD *et al.* 2001). The diagnostics can be further supported by identification of oligoclonal bands in the cerebrospinal fluid (CSF).

Disability in MS patients is usually graded using the extended disability severity scale (EDSS) (KURTZKE 1983). The EDSS scale itself does not measure the severity, since it does not take time into consideration, but can be used in combination with duration to calculate the multiple sclerosis severity score (MSSS) based on cross-sectional disability assessments in a large longitudinal database (ROXBURGH *et al.* 2005). Initially, the disease course is often characterized by relapses (disease bouts), followed by remissions (periods

of recovery). This relapsing-remitting (RR) form of MS is the most common variant affecting 80-95% of the patients, in contrast to the primary progressive (PP) MS, characterized by a steady deterioration and absence of remissions that affects 5-20% of MS patients. A majority of the RR patients do however develop gradual progression of disease and later enter a secondary progressive (SP) phase of MS, even though there is a big variation in type and severity of symptoms and the rate of disease progression, between patients (COMPSTON *et al.* 2006).

The available treatments for MS are disease modifying, rather than curing, and primarily have effect on the number and magnitude of relapses. Interferon-β and glatiramer acetate are the most commonly used therapies today and although they are effective in relapse reduction, they have only modest benefits on progression of disability (BUTTMANN and RIECKMANN 2007; WOLINSKY 2006). In addition, despite over 15 years of usage, the mechanisms for these treatments are still not fully understood. Anti-VLA4 monoclonal antibody (natalizumab) therapy is very efficient in suppressing relapses, but is used restrictively since cases of progressive multifocal leukoencephalopathy (PML), caused by the JC-virus, have occurred in patients on this therapy in combination with other immunosuppressive medications (KLEINSCHMIDT-DEMASTERS and TYLER 2005; LANGER-GOULD *et al.* 2005). New strategies for efficient MS therapies include a sphingosine-1P (S-1P) receptor agonist that increases the retention of lymphocytes in lymph nodes, a blocking anti-IL-2 receptor α chain (CD25) monoclonal antibody, as well as various strategies for induction of tolerance, reviewed in (GREENBERG and CALABRESI 2008).

## GENETICS

There is solid evidence for a genetic predisposition of MS, and that first, second and third degree relatives to MS patients have an increase risk of developing the disease, compared to the general population, reviewed in (DYMENT *et al.* 2004), whereas adoptees have the same risk as the general population (EBERS *et al.* 1995 ). The first genetic region linked to susceptibility of MS was the major histocompatibility complex (MHC) region (also called human leukocyte antigen (HLA) in humans) (JERSILD *et al.* 1973), and later the main effect has been mapped to a haplotype including HLA-DRB1*15, DRB5*0101, DQA1*0102 and DQB1*0602 (HILLERT and OLERUP 1993). Due to the high LD within the region, only the HLA-DRB1*15 allele is usually genotyped, and contain essentially the same information as the whole haplotype. Apart from being a risk factor for developing MS, the DRB1*15 allele is

associated with an earlier onset of MS (MASTERMAN *et al.* 2000). In recent years it has also been shown that the HLA-A*02 has a protective effect (BRYNEDAL *et al.* 2007), and the same has been suggested for HLA-C*05 (YEO *et al.* 2007) although it has not been proven that the HLA-C*05 effect is independent from the HLA-DR1*15 locus. While the MHC locus appears to have the main genetic influence on MS susceptibility, carrying the HLA-DR1*15 risk allele is not sufficient for the development of MS, as 30% of the non-affected population carry this allele, nor is it a prerequisite, as 50-40% of the MS patients do not carry this risk allele. This discordance clearly indicates that non-MHC genes are involved in the regulation and predisposition of MS. Numerous studies, including the studies presented within this thesis, have been designed for the identification of these genes and attributable variants.

Early genetic studies of MS, including linkage studies in extended pedigrees or trios, or association in case-control studies, identified candidate genes but the replication was notoriously poor or inconclusive. With a better understanding of the size of the genetic effects involved in MS and other complex disease, and the number of patients needed for conclusive identification of the underlying polymorphism (RISCH 2000; SAWCER 2008), larger studies including several populations where initiated. However, early meta-analyses and joint efforts failed to unambiguously identify polymorphisms or regions, apart from the MHC (GAMES and THE TRANSATLANTIC MULTIPLE SCLEROSIS GENETICS COOPERATIVE 2003; THE GAMES COLLABORATIVE *et al.* 2006). With the development of high-throughput genotyping methods new studies were initiated and in 2007, the interleukin 7 receptor (*IL7R*), earlier identified in a smaller study (ZHANG *et al.* 2005), was confirmed in parallel by two studies (GREGORY *et al.* 2007; LUNDMARK *et al.* 2007). A large GWAS conducted by the international multiple sclerosis genetics consortium (IMSGC) (INTERNATIONAL MULTIPLE SCLEROSIS GENETICS CONSORTIUM *et al.* 2007) also found an association to *IL7R* and identified a role for the alpha chain of the IL2-receptor (*IL2RA*), also known as CD25, in MS susceptibility. *IL2RA*, along with *CD58* (lymphocyte function-associated antigen 3, LFA-3), *RPL5* (ribosomal protein L5) and *CLEC16A* (C-type lectin domain family 16, member A), also identified in the original GWAS, have later been confirmed as associated to MS (DE JAGER *et al.* 2009; RUBIO *et al.* 2008; WEBER *et al.* 2008). Many of the recently identified risk genes for MS are involved in the immune system, for example IL2RA (CD25) is highly expressed on both activated Th1 cells and regulatory T-cells and CD58 is expressed on antigen presenting cells (APC) and is involved in the adhesion between T-cells and APCs. Another example is

IL7R, expressed on T-cells and involved in signaling that regulates T-cell survival and proliferation. There are however examples of genes that are not primarily involved in the immune system, such as *RPL5* and *KIF1B* (kinesin family member1B). The association to *KIF1B* was identified in an isolated Dutch population (AULCHENKO *et al.* 2008), and the corresponding protein is believed to have a function in axonal transport of mitochondria and synaptic vesicle precursors and may be related to neurodegeneration.

### COMMON REGULATION OF INFLAMMATORY DISEASES

Chronic inflammatory and autoimmune diseases such as MS, RA, SLE and T1D involve dysregulation of the immune system. These diseases share some characteristics including abnormal cytokine patterns, dysfunctional regulatory T-cells, and autoreactive T and B-cells. There is also evidence for increased risk of developing other autoimmune disease in relatives to MS patients (BARCELLOS *et al.* 2006; BROADLEY *et al.* 2000). All of these diseases have a strong risk influence from the MHC locus, and are associated with partly overlapping HLA haplotypes (FERNANDO *et al.* 2008) and share association to several sets of non-MHC genes. For example *SH2B3*, *CD226* and *CLEC16A* have been shown to affect susceptibility to both T1D and MS (INTERNATIONAL MULTIPLE SCLEROSIS GENETICS CONSORTIUM 2009) whereas *ILR2A* is associated with MS, T1D and RA (INTERNATIONAL MULTIPLE SCLEROSIS GENETICS CONSORTIUM *et al.* 2007; WELLCOME TRUST CASE CONTROL CONSORTIUM 2007). The interferon regulatory factor 5 (*IRF5*) is associated to several inflammatory diseases, including MS, RA, SLE and inflammatory bowel disease (DIDEBERG *et al.* 2007; KRISTJANSDOTTIR *et al.* 2008; SIGURDSSON *et al.* 2008; SIGURDSSON *et al.* 2005; SIGURDSSON *et al.* 2007), indicating the importance of the interferon-pathway in regulation of chronic inflammatory diseases. Taken together, there is an increasing genetic evidence for shared pathways involved in the development and regulation of chronic inflammatory diseases.

## EPIDEMIOLOGY

There is uneven geographical distribution of MS prevalence following a latitude gradient, with lower prevalence closer to the equator, and generally higher prevalence in northern Europe and America and in southern Australia (COMPSTON *et al.* 2006). The prevalence in Scandinavia is 0.1% (KOCH-HENRIKSEN 1995), with the exception of the Sami population that show a lower prevalence, as most indigenous populations (COMPSTON *et al.* 2006). Additionally, MS is more common in females than males, with an approximate ratio of 2:1 (COMPSTON *et al.* 2006; DUQUETTE *et al.* 1992), in most populations. One hypothesis for the explanation of the latitude gradient is sunlight exposure, causing decreased serum levels

of vitamin D, reviewed in (ASCHERIO and MUNGER 2007b; CANTORNA 2008). Cigarette smoking is another external factor implicated in both severity (PITTAS *et al.* 2009) and susceptibility to MS (ASCHERIO and MUNGER 2007b). Others suggest various infectious agents as risk factors for MS, where infections in the CNS could trigger the immune response and the immunopathology leading to demyelination. Many candidates have been investigated, but the most solid evidence is found for the gram-negative bacterium *Chlamydia pneumonia* and the herpes viruses Epstein-Barr virus (EBV) and human herpes virus 6 (HHV-6) (ASCHERIO and MUNGER 2007a; GILDEN 2005). Especially EBV is an interesting candidate since virtually all adult MS patients are seropositive for EBV, while the corresponding number is 86–95% for the general population. MS patients also have higher titers of anti-EBV antibodies than healthy virus carriers (GILDEN 2005; SALVETTI *et al.* 2009), and there is evidence for active EBV infection in MS lesions (SALVETTI *et al.* 2009; SERAFINI *et al.* 2007).

## HISTOPATHOLOGY

In the brain of MS affected individuals, the CNS white matter is characterized by multiple separate plaques characterized by inflammation and loss of myelin, with a preferential localization to the optic nerves, periventricular regions, brain stem and spinal cord. Histopathological examination of MS plaques reveals that virtually all lesions have infiltration of T-cells and macrophages, blood brain barrier damage and diffuse IgG-reactivity, but four different patterns have been suggested: I) active demyelination associated with T-cell and macrophage infiltration, II) as pattern I, but also including deposition of IgG and complement, III) diffuse plaques with infiltration of T-cells and macrophages, activated microglia, and apoptotic oligodendrocytes, IV) infiltrating T-cells and macrophages, non-apoptotic oligodendrocyte death and demyelination (LUCCHINETTI *et al.* 2000). However, others suggest that these lesion types represent different stages of disease progression and that lesions in established MS are more homogenous, characterized by macrophage associated deposition of complement, IgG and fibrinogen in demyelinating areas, and found little evidence for oligodendrocyte apoptosis (BREIJ *et al.* 2008).

## IMMUNOLOGY

Polyclonal $CD4^+$ and $CD8^+$ T-cells are found in MS plaques (WUCHERPFENNIG *et al.* 1992) and T-cells specific for autoantigens are believed to have a major role in the disease

development and plaque formation. There is evidence for T-cell clones in the periphery of MS patients, specific for the major myelin components myelin basic protein (MBP) (OTA *et al.* 1990) and proteolipid protein (PLP) (CORREALE *et al.* 1995), as well as for the minor component myelin oligodendrocyte glycoprotein (MOG) and other CNS proteins (KERLERO DE ROSBO *et al.* 1993; SUN *et al.* 1991; WALLSTROM *et al.* 1998). In addition, T-cell clones specific for MBP, MOG and PLP peptides have been identified in MS lesions, and many of these peptides can also be presented by the DRB1*1501 MHC class II molecule (FINN *et al.* 2004; MONTES *et al.* 2009; WUCHERPFENNIG *et al.* 1997). However, MOG protein or peptides have shown the highest incidence of reactivity, as well as higher magnitude of response compared to MBP or PLP, in several studies (KERLERO DE ROSBO *et al.* 1993; WALLSTROM *et al.* 1998).These proteins are also known to induce experimental autoimmune encephalomyelitis (EAE) in rodents and small primates, when immunized together with adjuvants. $CD4^+$ Th17 cells (ANNUNZIATO *et al.* 2007) transmigrate efficiently across the blood-brain barrier and are found in high numbers in MS lesions (KEBIR *et al.* 2007), and have been suggested as essential in MS development (MONTES *et al.* 2009). However, in the studies previously described, autoreactive T-cells specific for myelin components are also found in healthy controls. Thus, carrying autoreactive T-cells is not enough for the development of autoimmune disease. Antigen specific regulatory T-cells (Tregs), the main type characterized in humans by $CD4^+$ $CD25^{hi}$ $FoxP3^+$ expression, are important in the suppression of autoreactive T-cells and the induction of tolerance *in vivo* (SAKAGUCHI *et al.* 1995). Patients with MS have similar frequency of Tregs in the periphery, compared to healthy controls (VIGLIETTA *et al.* 2004), but the $CD4^+$ $CD25^+$ cells show reduced levels of FOXP3 mRNA and protein, and reduced suppressive function (HUAN *et al.* 2005). There is evidence for a reciprocal relationship between regulatory T-cells and Th17 cell development (BETTELLI *et al.* 2008), and dysregulation of this differentiation pathway might skew the ratio between the two cells types and thereby induce tissue inflammation rather that tolerance.

Even though MS often is regarded as a T-cell mediated disease, one of the hallmarks of MS is the intrathecal oligoclonal IgG production (KABAT *et al.* 1948), present in over 90% of MS patients (WALSH and TOURTELLOTTE 1986). In addition, antibodies are found in most demyelinating lesions (LUCCHINETTI *et al.* 2000) and myelin specific B-cells are found in both CSF and periphery (SUN *et al.* 1991). More recently, it has also been shown that a subset of secondary progressive MS patients form follicles containing B-cells, T-cells, plasma cells

and follicular DCs, close to inflamed blood vessels in the meninges (SERAFINI *et al.* 2004). Follicle formation is associated with earlier age at multiple sclerosis onset, irreversible disability and death, and by a more severe cortical pathology. The follicle formation is further associated with expression of EBV mRNA and proteins in B-cells and plasma cells infiltrating the brain, as well as EBV-specific oligoclonal bands and EBV DNA in CSF (SERAFINI *et al.* 2007). This could provide an antigen- and T-cell independent maturation and activation of B-cells, since expression of EBV proteins function as proliferation and survival signals to their host cells, and thereby suggests a mechanism for chronic inflammation in MS.

## NEURODEGENERATION IN MS

In CNS tissue from MS patients there is also evidence for axonal degeneration (FERGUSON *et al.* 1997) and axonal transections (TRAPP *et al.* 1998) associated with active inflammation and demyelination. Whole brain atrophy is also a correlated with physical disability in patents, and is often a better correlate than lesions-load measurements using MRI (BERMEL and BAKSHI 2006).The axonal injury is most prominent in the acute and active chronic lesions, but is also detected in the white matter of patients with active lesions (KORNEK *et al.* 2000). The neuronal degeneration in MS is often regarded secondary to inflammation, as a result of increased vulnerability of axons caused by proteolytic enzymes, cytokines, oxidative products and free radicals produced by activated immune and glia cells. However, the order of events and pathology in different types of lesions and disease courses is still under debate, and it has been suggested that neuronal degeneration could be a primary event is some cases, reviewed in (LASSMANN 2007; TRAPP and NAVE 2008).

## ANIMAL MODELS OF HUMAN DISEASE

Animal models of human diseases are valuable in studies of both pathological mechanisms and identification of genetic factors regulating disease. In the modern science, mice are the predominantly used laboratory animal but rats, guinea pigs, rabbits, dogs, cats, pigs, small primates and other species are also used. In genetic studies, animal models have advantages including the generation of genetically identical individuals (inbred and congenic strains), virtually unlimited samples size in experimental crosses and a controlled environment. The effect of environmental changes and interactions between environment and genetic factors can also be investigated by exposing animals with different genetic backgrounds to various environments, including potentially hazardous agents. Animal models also provide means for directed alterations of the genome, trough congenic breeding, mutagenesis and gene targeting in transgenic or knock-out models. Another big advantage is the availability of tissues for the study of pathogenesis and gene expression, especially CNS and other tissues usually not available from humans. In addition, the kinetics of pathogenesis can be studied in detail, including early events that appear before the diagnosis of the corresponding human disease. Animal models are also widely used for the screening, evaluation and mechanistic studies of new therapies and treatments for human disease.

## GENETIC ANALYSIS IN INBRED STRAINS

Inbred strains of rats and mice display different traits and phenotypes, from coat-color and size, to psychological phenotypes, immune response and metabolism to susceptibility to various models of human disease. By using strains with opposite phenotypes of the trait of interest, these differences can be used to identify the underlying genetic factors. One approach is to cross to inbred strains in a F2 intercross or backcross (BC) (Figure 1), and perform linkage analysis for the identification of genetic regions or markers that co-segregate with a specific trait. Since the F2 animals can carry any of three genotypes at a given location (11/12/22) this experimental population gives a better estimate of the number of quantitative trait loci (QTL) regulating the trait, and the effect of each identified QTL (i.e. additive, dominant or recessive models). In contrast, BC animals can have only two genetic variants at any given location (11/12), thus making this experimental population more powerful for identification of major effect QTLs with dominant mode of action (DARVASI 1998). Variance introduced by gene-gene interaction is

also reduced in the BC population, facilitating the identification of significantly contribution single QTLs, but hampering the identification of epistasis and thereby presenting a simpler genetic model.

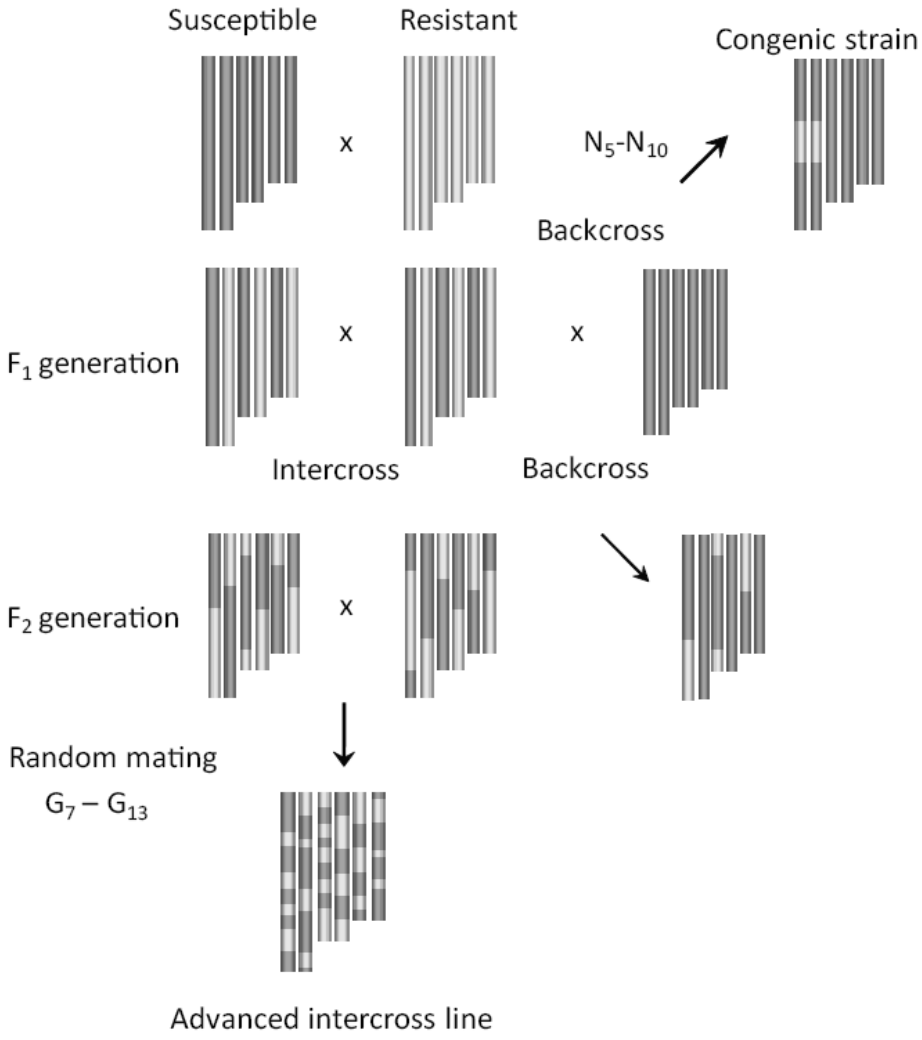


*Figure 1. Schematic outline of the breeding for F2 intercrosses, backcrosses, advanced intercrossed lines and congenic strains*

Recombinant inbred lines (RIL) (BAILEY 1971) are developed by crossing two inbred strains, followed by repeated sibling mating for the generation of new inbred lines whose genomes are a mosaic of the parental genomes. Panels of RILs can be used for the identification and localization of regions regulating a specific trait, but are less powerful compared to BC and F2 populations in most cases, if not a large set of RILs are included. However, once a panel of RILs is genotyped, it can be tested for a number of phenotypes

and in different environments, and enables confirmation of results in the same genetic setup (JIROUT *et al.* 2003). The concept can also be extended by using several parental strains, providing a higher phenotypic and genetic diversity and the possibility of high resolution mapping, given that many strains are tested (BROMAN 2005; COMPLEX TRAIT CONSORTIUM *et al.* 2004).

Advanced intercross lines (AIL) are created by continuous semi-randomized intercrossing, avoiding sibling-mating with at least 50 breeding couples in each generation (DARVASI and SOLLER 1995). In generation 6-13 an experimental cohort is produced, and can be used for high resolution linkage analysis. The accumulated recombinations in each generation enable a more accurate positioning of the QTL, with reduced confidence intervals, compared to BC, F2 and RIL (Figure 1). A higher number of tested animals as well as denser marker spacing are however needed, due to the number of accumulated recombinatory events in each generation.

Outbred heterogeneous stocks (HS) with known ancestry are developed in both rats (HANSEN and SPUHLER 1984; JOHANNESSON *et al.* 2009) and mice (HUANG *et al.* 2009; TALBOT *et al.* 1999; VALDAR *et al.* 2006), and represent another powerful approach for identification and fine mapping of QTLs in animals. As AILs, the HS gain resolution in genetic studies from the accumulation of recombinatory events but HS has the advantage of multiple founder strains, and additional generations in breeding before the experimental cohort are bred. The currently used heterogenous stocks in mice and rats have eight inbred founder strains, and are bred for more than 50 generations. The resolution in genetic studies can reach below the 1 Mb level and potentially identify single genes, but have the disadvantage of requiring high number of animals and high density genotyping, and more complex haplotype analysis.

Congenic strains carry a genetic fragment of interest, on the background of another strain (Figure 1), for example a genetic region conferring relative resistance to inflammation, on a susceptible background strain. This allows for analysis of the effect from the specific region. In order to develop a congenic strain, animals carrying a region of interest are bred from F1 animals or selected from an experimental cross (F2, BC, AIL, RIL) and are then backcrossed for several generations while selecting for the region of interest. Theoretically, 10 generations of backcrossing give <0.1% donor contamination outside the

desired fragment, but through marker assisted selection, also known as speed congenics, the same purity can be obtained by 5 backcrosses (WAKELAND *et al.* 1997). In our experience however, at least 7 generations are generally needed to obtain a pure congenic strain even using the speed congenic approach as the reduction of donor regions in the background is lower than the proposed 70% in each generation. But even with this limitation, the speed congenic approach is preferable, since it gives an actual measurement of the background contamination for each line, not only a theoretical estimate.

Congenic strains are seen as the standard method for the confirmation of novel QTLs and are useful in further characterization of the phenotype attributed by the genetic variants in the region. In EAE for example, the congenics are used for both confirmation of a certain QTL linked to clinical score, and for a more extensive mapping of immune response, cytokine profile and kinetics as seen in Papers III and IV. The same congenic strain can also be investigated in various animal models to test if the same genetic variant contributes to different traits (Paper IV). To test interaction between genetic regions, bi-congenic animals with two separate fragments introduced on the same background, can easily be created through crossing of established congenic strains. Through further backcrossing, interval-specific recombinants or sub-congenic strains can be developed. Congenic strains containing small genetic regions or single genes are seen as the ultimate evidence for the involvement of a gene in a certain trait.

Panels of congenic (IAKOUBOVA *et al.* 2001) or chromosome substitution strains (NADEAU *et al.* 2000) can also be used for the identification of novel QTLs (SINGER *et al.* 2004) and have the benefits of simple statistics for QTL identification and possibility of reproduction and testing identical animals for multiple phenotypes. However, the number of animals needed for testing is equal or higher compared to F2, BC and RIL, (BELKNAP 2003) but the resolution of linkage is lower, especially in chromosome substitution strains, and the analysis also do not generally allow for analysis of interactions, except in specific cases (NADEAU *et al.* 2000).

## EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS

Experimental autoimmune encephalomyelitis (EAE) is a widely used model for MS and neuroinflammation, where animals are immunized with CNS antigens in combination with

adjuvants, causing an autoimmune reaction with demyelinating lesions in the CNS. The inflammation causes motor symptoms including gradual paralysis of tail, hind limbs and forelimbs in a monophasic, relapsing-remitting or chronic manner, often preceded by weight loss. EAE can be induced in a number of species, including mice, rats, guinea pigs, rabbits and primates. The most commonly used antigens in rodents are spinal cord homogenate (SCH), purified myelin, myelin protein such as MBP, PLP and MOG, or peptides of these proteins, all resulting in distinct models with different disease characteristics regarding both immunology and pathology (MANNIE *et al.* 2009; MILLER and KARPUS 2007). The antigen is distributed in an emulsion with Freund's adjuvant, in some models including heat inactivated *mycobacterium tuberculosis* (complete Freund's adjuvant, CFA) and additional injections of pertussis toxin. In rats, one of the most widely used protocols is MBP in CFA, induced in LEW rats, resulting in an acute monophasic disease with inflammation but almost complete lack of demyelination in the CNS (SWANBORG 2001). In contrast, when using MOG or SHC in DA or LEW$^{av1}$ strains, animals develop a chronic or relapsing-remitting disease with both inflammation and demyelination in the CNS (LORENTZEN *et al.* 1995; STORCH *et al.* 1998; WEISSERT *et al.* 1998), and thus show a closer resemblance with the human disease. EAE can also be induced in naïve animals trough the adoptive transfer of T-cells specific for myelin antigens (BEN-NUN and COHEN 1982; BEN-NUN *et al.* 1981; PATERSON 1960; PETTINELLI and MCFARLIN 1981), sometimes in combination with transfer of myelin specific autoantibodies (MEESON *et al.* 1994). Mice transgenic for both MOG specific T-cell receptors and antibodies with MOG-specific IgG, spontaneously develop a opticospinal EAE that share some similarities with MOG induced EAE, although the lesions are predominantly located in optical nerves (KRISHNAMOORTHY *et al.* 2006).

## EAE GENETICS

Inbred rat and mouse strain differ in susceptibility to EAE, demonstrating a genetic regulation of EAE. The course and severity of disease is however to some extent relative to induction protocol, antigen, dose and adjuvants used in the specific environment. Early studies recognized a genetic difference in EAE susceptibility in rats (GASSER *et al.* 1973) and mice (BERNARD 1976), and linked the major effect to the MHC region (GUNTHER *et al.* 1978; WILLIAMS and MOORE 1973). It was however also shown that additional non-MHC genetic variants influenced susceptibility (GUNTHER *et al.* 1978; MOORE *et al.* 1980).

The first whole genome scan for identification of non-MHC loci regulating EAE in rodents was performed in an F2 intercross between the B10.RIII and RIIIS/J mice strains, using $MBP_{89-101}$ peptide for induction. This study identified two QTLs on mouse chromosome 15 and 3, respectively, thereby confirming the non-MHC regulation of the model (SUNDVALL *et al.* 1995). This study was followed by a number of backcross studies using different induction protocols, that all identified novel suggestive QTLs. (BAKER *et al.* 1995; ENCINAS *et al.* 1996, Croxford, 1997 #954). Significant loci on a whole genome level, to some extent overlapping with the previously suggested loci, was first identified in a large (681 genotyped mice) F2 intercross between the B10.S/DvTe and SJL/J mouse strains using spinal cord homogenate for induction of EAE (BUTTERFIELD *et al.* 1998). The first whole genome scans for QTLs regulating EAE in rats were presented in 1999 (DAHLMAN *et al.* 1999a; DAHLMAN *et al.* 1999b; ROTH *et al.* 1999), identifying four separate significant and 19 suggestive QTLs, some of these overlapping. These studies were followed by additional studies that identified new QTLs (BECANOVIC *et al.* 2003; BERGSTEINSDOTTIR *et al.* 2000).

All whole genome scans for EAE-regulating loci in rats and mice have identifying approximately 50 separate significant QTLs (OLSSON *et al.* 2006). The number of identified QTLs confirms the polygeneity of the disease and clearly shows that there is no single major contributor, apart from the MHC region. Many of the studies identifies sex specific QTLs or modifying effects, depending on sex. There is also evidence for significant influence from age as well as seasonal effects on susceptibility (TEUSCHER *et al.* 2004) and QTLs specific for season at immunization (TEUSCHER *et al.* 2006). The identification of overlapping QTLs using different strain combinations and induction protocols argues in favor of common disease mechanisms, although QTLs specific to species, strain-combination, induction protocol or disease type also exist. This is further supported by a combined analysis of four genome scans in rats, all including different strain combinations and using three different induction protocols (MBP, SCH and MOG), where both common and cross specific QTLs were recognized (JAGODIC and OLSSON 2006). QTLs regulating EAE are also known to regulate other animal models involving autoimmunity and chronic inflammation including experimental arthritis (BERGSTEINSDOTTIR *et al.* 2000; DAHLMAN *et al.* 1998) and experimental autoimmune neuritis (EAN), a rat model of Guillian-Barré syndrome (HUBERLE *et al.* 2009). The overlap of genetic candidate regions suggests common pathways for several complex diseases, and indicates that dysregulation of the immune response is one of the main mechanisms for disease development.

## GENETIC REGULATION OF MOG-EAE IN RATS

In this thesis MOG induced EAE in rat has been used as a model for identification and evaluation of candidate gene regions for neuroinflammation, and for characterization of the immune response. Inbred rat strains differ in susceptibility to MOG induced EAE, and the of susceptibility follow largely the pattern that is seen in other induction protocols, although BN rats are susceptible to MOG induced EAE, but resistant to MBP and spinal cord homogenate inductions (STEFFERL *et al.* 1999). In general, the DA, LEW, BN strains are susceptible to different degree, whereas ACI and PVG strains are resistant (SAKUMA *et al.* 2004; STORCH *et al.* 1998; WEISSERT *et al.* 1998, Stefferl, 1999 #456). The susceptibility is to some degree dependent on the dose and adjuvants used, and there is a strong influence from the MHC region (RT1 region in rats). For example the $RT1^n$ haplotype generally segregates with early onset, fulminant disease and high antibody response (STEFFERL *et al.* 1999; WEISSERT *et al.* 1998). The $RT1^a$ and $RT1^{av1}$ haplotypes are often associated with a chronic or relapsing-remitting disease course, whereas $RT1^u$ and $RT1^l$ are associated with mild or no disease (SAKUMA *et al.* 2004; STORCH *et al.* 1998; WEISSERT *et al.* 1998). The use of intra MHC recombinant strains indicates that MHC class II harbours the major effects, but is modified by the MHC class I region (WEISSERT *et al.* 1998).

Two whole genome scans has been performed for identification of MOG-EAE regulating QTLs in rats. In the first study, a (DAxACI) F2 intercross identified suggestive loci on chromosome 10, 12 and 13 for the regulation of inflammation and severity of disease, and a significant QTL for the regulation of demyelination in CNS on rat chromosome 18 (DAHLMAN *et al.* 1999b). The second study included a ($LEW^{av1}$ x $PVG^{av1}$) F2 intercross and identified significant QTLs on chromosome 8, regulating severity, and 13, regulating both severity and incidence of disease, as well as suggestive QTLs for regulation of disease severity, incidence demyelination and anti-MOG IgG production (BECANOVIC *et al.* 2003). A limited screen in a small F2 (DAx$PVG^{av1}$) also identified a QTL on chromosome 4, regulating severity of MOG-EAE and anti-MOG IgG2c production (DAHLMAN *et al.* 1998).

Fine mapping of the identified QTLs is essential for a more accurate location and unbiased identification of candidate genes, and can be performed in AIL (DARVASI and SOLLER 1995). In MOG-EAE, an AIL originating from the MHC identical DA and $PVG^{av1}$ strains has successfully been used in a number of studies, for example to subdivide *Eae18* on chromosome 10 into two narrow separate loci (JAGODIC *et al.* 2004), and for the identification of interacting QTLs on rat chromosome 4 (*Eae20-22*) in a region previously

linked to experimental arthritis and EAE (JAGODIC *et al.* 2005). Another example is the fine mapping of *Eae5* on chromosome 12 that could be redefined to a 1.3 Mb region including the candidate genes *Ncf1* and *Cldn4* (BECANOVIC *et al.* 2006). The AIL has also been used in combination with congenic mapping, for the confirmation of a previously identified suggestive locus (*Eae19*) on chromosome 15 (SHENG *et al.* 2005). In Paper I and II, a later generation (G10) of the AIL has been used, thereby taking advantage of additional accumulated recombinations in the cross, that enables higher resolution in the linkage analysis.

## MOG INDUCED EAE IN RATS

MOG was first described as an autoantigen associated with demyelinating antibody response in guinea pig EAE, immunized with spinal cord homogenate (LEBAR *et al.* 1986), and later it was demonstrated that MOG induce EAE in a number of species and inbred strains (VON BUDINGEN *et al.* 2001). In rats, MOG immunization result in a chronic, relapsing-remitting, severe acute or monophasic disease, depending on the strain and induction protocol (ADELMANN *et al.* 1995; SAKUMA *et al.* 2004; STEFFERL *et al.* 1999; STORCH *et al.* 1998; WEISSERT *et al.* 1998). In the DA and LEW$^{av1}$ rats, MOG in incomplete Freund's adjuvant (IFA) induces EAE without the need for additional adjuvants (STORCH *et al.* 1998). The benefits of this immunization protocol is that the immune system is not influenced by the *m. tuberculosis* superantigens (OHMEN *et al.* 1994) or other adjuvants, known to have a distinct genetic regulation (BLANKENHORN *et al.* 2000). The model shares some of the pathological characteristics with MS, including perivascular inflammation with infiltrating T-cells, macrophage, microglia and eosinophils in the lesions. Demyelinating plaque formation with depositions of IgG and complement, and glial scar formation in the CNS are also apparent in both MS and EAE (BREIJ *et al.* 2008; LUCCHINETTI *et al.* 2000; STORCH *et al.* 1998; WEISSERT *et al.* 1998).

The inflammation in EAE is dependent on MOG specific T-cells (LININGTON *et al.* 1993), primarily of the Th1 and Th17 subtypes (LANGRISH *et al.* 2005; STROMNES *et al.* 2008; THESSEN HEDREUL *et al.* 2009) but macrophages are also essential for antigen presentation and disease development (DITTEL *et al.* 1999; HUITINGA *et al.* 1990). Regulatory T-cells are known to influence the susceptibility to EAE in transfer models (ELLERMAN *et al.* 1988) and in actively induced EAE (KOHM *et al.* 2002), but fails to suppress effector T-cells in the CNS, in affected animals (KORN *et al.* 2007). The demyelination in EAE dependent on anti-MOG

IgG (ADELMANN *et al.* 1995; LININGTON *et al.* 1992; MEESON *et al.* 1994), as well as the complement system (LININGTON *et al.* 1989).

Although the immune response towards the autoantigen in EAE is the primary event in pathogenesis, there is evidence for neurodegenration in MOG induced EAE, especially in animals with a chronic or progressive disease. In DA rats with a progressive MOG-EAE, there is progressive axonal loss already 10 days after disease onset. The extent of axonal loss is associated with macrophages/microglia infiltration and demyelination, and correlates with disease severity (PAPADOPOULOS *et al.* 2006). The axonal injuries in chronic active MOG-EAE lesions show qualitative and quantitative similarities to active MS plaques (KORNEK *et al.* 2000).

## VRA

As a rat model for neurodegeneration we have used ventral root avulsion (VRA), where anesthetized animals are subjected to unilateral avulsion of lumbar ventral roots. This is a highly reproducible proximal axotomy in the border between central and peripheral nerve systems, causing retrograde degeneration and death of spinal motor neurons, in combination with activation of microglia, infiltration of macrophages, $CD4^+$ and $CD8^+$ T-cells and upregulation of proinflammatory cytokines (KOLIATSOS *et al.* 1994; PIEHL 1999). Rat strains differ in response to the VRA injury, both in the local immune response and in the extent of neuronal death, primarily linked to non-MHC genes (LUNDBERG *et al.* 2001). For example, the DA strain show high microgial activation and MHC class II expression and extensive neuronal death, whereas both ACI and $PVG^{av1}$ show lower microglia activation and reduced neuronal death. The susceptibility to immune activation and neuronal death in inbred rat strains follows the pattern of MOG-EAE susceptibility, reviewed in (OLSSON *et al.* 2005). A few QTLs regulating neurodegeneration (*Vra1* and *Vra2*, on rat chromosome 8 and 5, respectively), T-cell infiltration (*Vra2* and *Vra3*, both on rat chromosome 5), and MHC class II expression (*Vra4*, on rat chromosome 10), have been identified (LIDMAN *et al.* 2003). A further fine mapping using an AIL and haplotype mapping identified *Mhc2ta* as the regulator of MHC class II expression, and a SNP in the homologous human *MHC2TA* promoter was associated with MHC class II expression in humans and risk of developing MS, RA and MCI (SWANBERG *et al.* 2005).

# THE CHEMOKINE SYSTEM

Chemokines are a family of sequentially related soluble proteins, with chemoattractant and activating properties. They mainly act on neutrophils, monocytes, lymphocytes, and eosinophils during inflammation but also play fundamental roles in the development and homeostasis of the immune system and angiogenesis. The classification of chemokines is based on conserved cysteine residues, and the number of amino acids between these. The CC, CXC and CX3C chemokines have 0, 1 and 3 amino acids located between the cysteines, respectively, whereas the XC chemokines lacks cysteines in the typical chemokine motif. Chemokines signal trough G-coupled receptors with distinct but overlapping specificity (JANEWAY *et al.* 2005). A summary of identified chemokines, receptors and cellular targets in humans and rodents is presented in Table 1, but because of their close sequence similarity, it is not always possible to unambiguously determine which rodent ligand corresponds to which human ligand. In addition to the receptors in Table 1, the high affinity receptors DARC (CHAUDHURI *et al.* 1993; LEE *et al.* 2003), D6 (NIBBS *et al.* 1997a; NIBBS *et al.* 1997b) and CCX-CKR (TOWNSON and NIBBS 2002) with no known signaling function are identified. These silent receptors are proposed to function as scavenger receptors that may neutralize extracellular chemokines or act as transporters or reservoirs for chemokines (COMERFORD and NIBBS 2005), and thereby modulate inflammatory responses.

The roles of chemokines and chemokine receptors in MS pathogenesis have been widely investigated in blood, brain sections and CSF, for their role in immune regulation and migration of cell into the CNS. The results are diverse and sometimes contradicting, indicating that there is no single chemokine regulating the leukocyte trafficking into the CNS in MS. However, some of the more robust findings show an increase of CCR5 and CXCR3 expression on T-cells in both periphery and CSF of MS patients compared to controls. (LUQUE and JAFFE 2007; SAVARIN-VUAILLAT and RANSOHOFF 2007). In the CSF, there is also elevated concentration of CXCL9, CXCL10, CCL3 and CCL5, in MS patients compared to controls, whereas MS patients show reduced levels of CCL2 in many studies (CARTIER *et al.* 2005; LUQUE and JAFFE 2007). Studies of active and chronic lesions have demonstated the expression of *CCL2, 3, 4, 5, 7*, and *8* in MS lesions (MCMANUS *et al.* 1998; SORENSEN *et al.* 1999) together with expression of the receptors *CCR1, 2, 3, 5* and *CXCR3* (LUQUE and JAFFE 2007).

Many of the chemokines and chemokine receptors are also studied in different models of EAE in rats and mice. Studies using chemokine receptor knockout-mice show contradicting results but indicate a role for CCR1, 2, and 8 expression by macrophages and monocytes in the development of clinical disease (CARTIER *et al.* 2005). Both in lesions and before onset of EAE, there is a strong upregulation of chemokines in the CNS, including CCL1, 2, 3, 4, 5 and CXCL1 and 10 (KARPUS and RANSOHOFF 1998; UBOGU *et al.* 2006). Upregulation of *CXCL1* and *CXCL10* together with *CCR2* occurs in acute EAE, whereas relapses are associated with *CCL2* and *CCL20* expression (UBOGU *et al.* 2006). There is also evidence for the requirement of CCR6-CCL20 interaction for the entry of Th17 cells into the CNS, during the initial immune response in EAE (REBOLDI *et al.* 2009). Together, the data from studies in both humans and animal models indicates a complex regulation of the temporal and spatial expression of chemokines and chemokine receptors in neuroinflammation that is not yet fully understood.

| Name | Alias | Target cell | Receptors |
|---|---|---|---|
| CXCL1 | GROa | Neutrophil | CXCR2, 1 |
| CXCL2 | GROß | Neutrophil | CXCR2 |
| CXCL3 | | Neutrophil | CXCR2 |
| CXCL4 | PF4 | Fibroblast | CXCR3B |
| CXCL5 | ENA-78 | Neutrophil | CXCR2 |
| CXCL6 | GCP-2 | Neutrophil | CXCR1, 2 |
| CXCL7 | LDGF-PBP | Fibroblast, neutrophil | CXCR2 |
| CXCL8 [b] | IL-8 | Neutrophil, basophil, T-cell | CXCR1, 2 |
| CXCL9 | Mig | Activated T-cell | CXCR3, 3B |
| CXCL10 | IP-10 | Activated Th1 cell | CXCR3, 3B |
| CXCL11 | I-TAC | Activated T-cell | CXCR3, 3B, 7 |
| CXCL12 | SDF-1a/ß | CD34+ bone marrow cell, T-cell, DC, B cell | CXCR4, 7 |
| CXCL13 | BLC/BCA-1 | Naive B cells, activated CD4 T-cells | CXCR5 |
| CXCL16 | BUNZO | T-cell, NK T-cell | CXCR6 |
| CCL1 | I-309 | Neutrophil, T-cell | CCR8 |
| CCL2 | MCP-1 | T-cell, monocyte, basophil | CCR2 |
| CCL3 | MIP-1a | Monocyte/macrophage, Th1 cell, NK cell, basophil, immature DC | CCR1, 5 |
| CCL4 | MIP-1ß | Monocyte/macrophage, Th1 cell, NK cell, basophil, immature DC | CCR1, 5 |
| CCL5 | RANTES | Monocyte/macrophage, T-cell, NK cell, basophil, eosinophil, DC | CCR1, 3, 5 |
| CCL7 | MCP-3 | T-cell, monocyte, eosinophil, basophil, DC | CCR1, 2, 3 |
| CCL8 | MCP-2 | T-cell, monocyte, eosinophil, basophil | CCR1, 2, 3, 5 |
| CCL11 | Eotaxin | Eosinophil | CCR3 |
| CCL12 [a] | MCP-5 | Eosinophil, monocyte, T-cell | CCR2 |
| CCL13 [b] | MCP-4 | T-cell, monocyte, eosinophil, basophil, DC | CCR2, 3 |
| CCL14 [b] | HCC-1 | Monocyte | CCR1 |
| CCL15 | MIP-5 | T-cell, monocyte, neutrophil, DC | CCR1, 3 |
| CCL16 | HCC-4 | Monocyte | CCR1 |
| CCL17 | TARC | Th2 cell, immature DC, NK cell | CCR4 |
| CCL18 | DC-CK1 | Naive T-cell | Unknown |
| CCL19 | MIP-3ß | Naive T-cell, mature DC, B cell | CCR7 |
| CCL20 | MIP-3a | Memory T-cell, DC | CCR6 |
| CCL21 | 6Ckine | Naive T-cell, B cell, mesangial cells | CCR7 |
| CCL22 | MDC | Immature DC, IA NK cell, Th2 cell, thymocyte | CCR4 |
| CCL23 | MPIF-1 | Monocyte, T-cell, neutrophil | Unknown |
| CCL24 | | T-cell, eosinophil, basophil | CCR3 |
| CCL25 | TECK | Macrophage, thymocytes, DC | CCR9 |
| CCL27 | CTACK | T-cell | CCR10 |
| CCL28 | MEC | T-cell, eosinophil | CCR10, 3 |
| XCL 1 | Lymphotactin | T-cell, NK cell | XCR1 |
| XCL2 | SCM-1β | T-cell, NK cell | XCR1 |
| CX3CL 1 | Fractalkine | T-cell, monocyte, neutrophil | CX3CR1 |

*Table 1. Selected chemokines found in humans, rats and mice, with specific receptors and targets cells. [a] found in rodents only, [b] found in humans only. Adopted from (JANEWAY et al. 2005) and (ZLOTNIK et al. 2006)*

# AIMS OF THE STUDY

The aim of this thesis was to use a translational approach to identify and evaluate candidate genes and gene regions that regulate neuroinflammation in rats and humans, in order to better understand the underlying disease mechanisms. More specifically, the aims for each study were:

**Paper I**

- Investigate if the 10$^{th}$ generation of a rat AIL could provide an increase in resolution in linkage analyses compared to earlier generations, and redefine *Eae18a*, previously linked to EAE.
- Use bioinformatics in parallel for the identification of the corresponding syntenic regions in humans and mice, also linked or associated to neuroinflammation.

**Paper II**

- Perform high resolution linkage analysis in a rat AIL in *Eae18b*, and identify candidate genes for the regulation of EAE.
- Investigate the identified candidate genes for association to MS, in case-control cohorts.

**Paper III**

- Confirm the influence from *Eae18b* on EAE severity, using a congenic strain.
- Study the immune response in the congenic rats, with particular focus on the chemokines genes, located in the region.

**Paper IV**

- Study the *Vra4* region including *Mhc2ta*, in congenic rat strains, with focus on MHC class II expression *in vivo* and *in vitro*, and investigate if the region regulates MOG induced EAE.

# METHODOLOGICAL CONSIDERATIONS

## ASSOCIATION STUDY OF CANDIDATE GENES IN MS

### POPULATION

The MS patients from all Nordic countries included in the study were diagnosed according to the McDonald criteria (MCDONALD *et al.* 2001) and collected from various centers and hospitals in each country. The majority of the controls are blood donors of Nordic origin collected from the corresponding countries, except controls from Sweden 2, Finland, and part of the controls from Denmark. The controls in the second Swedish cohort (Sweden 2) are collected as a part of a larger epidemiological survey and therefore matched to MS patients with regards to age, sex and area of residence. Finnish controls are population based, and a subset of controls from Denmark are healthy hospital staff members.

### GENOTYPING

The case-control cohort from Sweden, Denmark and Norway were genotyped using the Taqman technology (LIVAK 1999; LIVAK *et al.* 1995) for SNPs in the chemokine cluster. *HLA-A* and *-DRBI* loci were genotyped using sequence specific primers for the different alleles (OLERUP and ZETTERQUIST 1991), providing a 2-digit resolution of HLA alleles. The Finnish cohort was genotyped using the Sequenom MASSarray platform for SNPs in the chemokine cluster, and rs3135388 as a tagging SNP for HLA-DRB1*15 status.

### PHENOTYPES

The main outcome variable in the association analyses in Paper II is presence of MS, according to McDonald criteria (MCDONALD *et al.* 2001). The majority of included patients display a relapsing-remitting disease, but minor groups with primary or secondary progressive MS are also included. None of the controls were showing signs of MS at the time of sampling. We have also used MSSS (ROXBURGH *et al.* 2005) as an outcome variable in a linear regression analysis in an attempt to capture severity of MS and find its associated genotypes in the chemokine cluster.

### LABORATORY PHENOTYPES IN MS

Enzyme-linked immunosorbent assay (ELISA) was used for the detection of chemokines in the CSF of MS patients and controls. Since lumbar punctures are rarely performed on

healthy individuals, the controls group consisted of patents diagnosed for other neurological diseases, where lumbar puncture is performed as a part of the diagnosis procedure. Patients with inflammatory neurological diseases where were not included in the control group. The expression of chemokine mRNA in CSF was also measured using quantitative real-time PCR, but most patients and controls had undetectable expression levels.

## STATISTICS

The simplest analysis method for association studies is to compare the frequency of alleles in cases and controls; the allele is associated with the trait if any of the groups significantly deviate from the expected frequency. This can be done in a $\chi^2$ or Fischer exact test and is the method of choice in whole genome scans, due to the easy computation and the intuitive interpretation of results. The analysis of allele frequencies do not take in to account the actual genotypes in each individual (11/12/22), and can thus possibly have lower power for analysis of association under certain genetic models. Association can also be tested using logistic regression model that allow for inclusion of covariates, such as sex, disease subtype or genotypes at additional loci, for analysis and adjustment of confounders, interaction or epistasis. We used the SNPassoc package (GONZALEZ *et al.* 2007) for R (R DEVELOPMENT CORE TEAM 2009) in Paper II for the simulations analysis of multiple genetic models, for each SNP. The analyses include testing of codominant (11 vs. 12 vs. 22), dominant (11 vs. 12+22), recessive (11+12 vs. 22) overdominant (11+22 vs 12) and multiplicative ("allele-dose") models using logistic regression. For each model, the Akaike's information criterion (AIC) (AKAIKE 1974) is given, and can be used for the selection of the best model for each SNP, given the data. AIC provides means for ranking the models, but does not give estimates on how much of the outcome variable is explained by the genotype or evidence for rejection of a model. SNPassoc was also used for the analysis of MSSS, analyzed as a continuous variable in a linear regression analysis.

Association to haplotypes within the chemokine genes was also investigated. When the actual causative polymorphism is not known, as it rarely is, analysis of haplotypes can be more efficient, since any new mutation occurs on a specific haplotype, and will be in LD with this ancestral haplotype. In addition the number of haplotypes found in a population is lower than the number of possible combinations in a given set of SNPs (MYERS *et al.* 2005). It has also been speculated that a combination of several polymorphisms together

affect a functional unit of the gene product, so that the haplotype is the actual risk allele, whereas individual polymorphism are neutral (HOLLOX *et al.* 2001). Haplotypes were constructed within each gene analyzed in Paper II. Since the actual phase of genotypes (genotype on each chromosome in a diploid genome) is not given by standard genotyping methods, the haplotypes were estimated using the expectation-maximization algorithm (NIU 2004) in Haploview (BARRETT *et al.* 2005).

Meta analyses were performed using the r-meta package (LUMLEY 2009) in R (R DEVELOPMENT CORE TEAM 2009), as previously described (HAKULINEN 1981) for markers and haplotypes showing association in any of the cohort. Polymorphisms and haplotypes with a significant heterogeneity in the allele frequency between (WOOLF 1955) the cohorts were not included in the meta-analysis

## GENETIC ANALYSIS IN RATS

For the identification of well defined candidate gene regions for the regulation of neuroinflammation, linkage analysis in rats were performed.

### EXPERIMENTAL POPULATIONS

In this thesis the focus has been to perform high resolution linkage analyses in the $10^{th}$ generation of a rat AIL (DARVASI and SOLLER 1995), originating from the inbred MHC-identical DA and $PVG^{av1}$ rat strains. Other generations of the same AIL has previously been used for linkage analysis in EAE (BECANOVIC *et al.* 2006; JAGODIC *et al.* 2004; JAGODIC *et al.* 2005; SHENG *et al.* 2005), VRA (SWANBERG *et al.* 2005), and EAN (HUBERLE *et al.* 2009).

### ANIMAL MODELS

#### MOG INDUCED EAE

In all papers included in this thesis, myelin oligodendrocyte glycoprotein (MOG) was used for the induction of EAE. Recombinant MOG (aa 1-125 from the N-terminus) was produced in *E.coli* as and purified to homogeneity by chelate chromatography as previously described (AMOR *et al.* 1994). MOG is emulsified with PBS and Freund's adjuvant in a 1:1 ratio, and 200µl of the emulsion is injected subcutaneously in anesthetized rats. The exact dose varies depending on the efficacy of the batch used. Each

batch is titrated in a small number of DA rats for identification of a dose that give approximately 80% EAE incidence (data not shown). No additional adjuvants are needed for EAE induction in the susceptible DA rats or congenic strains with DA background. The rats were weighed and monitored daily for clinical signs of EAE from day 8 post immunization until the end of each experiment. For the analysis of immune response and expression of chemokines in Paper II and III, animals were euthanized at different time points during the early phase of disease development.

### VENTRAL ROOT AVULSION

In Paper IV rats were subjected to unilateral avulsion of the left L3-L5 ventral roots under standardized conditions and in deep isoflurane anesthesia. 21 day after surgery, animals were euthanized with $CO_2$ and perfused with cold PBS. The meninges of the spinal cord were removed and the L3 segment dissected out, and the ipsilateral ventral quadrant was isolated for expression analysis. The L4-L5 segments were kept intact at -80°C, for histological analysis.

### IFN INJECTIONS

In Paper IV, IFN-γ, a regulator of MHC calls II expression on microglia (PANEK and BENVENISTE 1995), was administered via intraparenchymal injections in anesthetized rats, for the investigation of genetically regulated MHC class II expression *in vivo*. Three days after administration, animals were euthanized and brains removed, and stored at -80°C. Histopathological analysis for MHC class II expression was performed on brain sections.

## MARKERS AND GENOTYPING METHODS

Microsatellite markers have been used for genotyping experimental crosses and congenic strains, in all studies included in this thesis. Using the DA and PVG$^{av1}$ strains permits dense genotyping, since these strains display a high rate of polymorphic microsatellite markers (http://www.broad.mit.edu/rat/public/). A standard PCR protocol has been used for amplification of microsatellites, including [γ-33P] ATP end-labeled forward primers. The PCR products were size fractioned on 6% polyacrylamide gels and visualized by autoradiography. A safer more high-throughput method is the use of fluorescently labeled primers and capillary electrophoresis for size separation. This approach also enables multiplex PCR reactions and detection, and was used in Paper III. A comprehensive study

characterizing over 20.000 SNPs in 167 inbred rat strains, including the strains used in this thesis, have recently been published (SAAR *et al.* 2008). This will facilitates the use of SNPs rather than microsatellites in future genetic studies in rats.

## LINKAGE ANALYSIS

Linkage analysis in the AIL was performed in current versions of the R/qtl package (BROMAN *et al.* 2003). R/qtl implements a number of different methods and models for the mapping of binary and quantitative traits in animal crosses. It is however not adjusted for analysis in generations of an intercross later than a F2, a problem shared with all other available software packages for linkage analysis in experimental crosses. The AIL will thus be analyzed as a F2, which does not consider the more complex pedigree structure present in later generations. This also results in the length of the chromosomes being inflated if the physical positions are not used.

### MODELS

The genetic regulation of a trait can be investigated using t-tests or non-parametric statistics, or Fisher exact test for binary traits, where stratification on each marker can reveal the phenotypic influence from a specific marker. Single marker test can also be performed using linear or logistic regression that allow for the introduction and evaluation of covariates potentially affecting the traits. This is however not a powerful method for the positioning of QTLs, and has not been used as the main method for analyzing the AIL. A more powerful approach is interval mapping (LANDER and BOTSTEIN 1989), where any given location in the genome can be regarded as the position for a QTL, even between markers. The evidence for linkage between a position in the genome and the studied trait is given in LOD (log10 of the odds), which is the likelihood of the presence of a QTL at a specific position, compared to the likelihood of no QTL at that position, given the data. The Haley-Knott regression model (HALEY and KNOTT 1992) is an approximation of the interval mapping algorithm, with the advantage of being less computational intense, and can be used for more complex models including multiple QTLs and covariates. In the linkage analysis, sex has been used as covariate, since the incidence rate was different in males and females. It is however unclear if there is an effect that actually is related to sex and not weight or antigen dose corrected for body weight, as males in general are bigger at the age of 8-16 weeks when experiments are started. In the congenic experiment, sexes are usually analyzed separately. This is also possible for the linkage analysis, although power is usually higher when analysis is performed in the whole set. In Paper II, we have

also included breeding couple as a covariate in order to correct for the family effect i.e. relatedness, since siblings are genetically more similar to each other, than more distantly related animals. Other covariates that could be included in the analysis include age, experimental set, weight etc, but these does not influence the analysis or results significantly in this particular experiment (data not shown).

### THRESHOLD OF SIGNIFICANCE

In F2 crosses and BCs, the thresholds level can be determined either by using a theoretical value according to a given set of parameters (LANDER and KRUGLYAK 1995). Alternatively, permutation tests can be used to estimate an empirical threshold, given the actual data (CHURCHILL and DOERGE 1994). In an AIL, there is a complex relationship between all individuals, since the selection of breeding pairs only is controlled for sibling mating in each generation, not for relationship in earlier generations. This could lead to a reduced heterogeneity and fixation of alleles within the offspring from some breeding couples, and potentially coinheritance of multiple regions within a pedigree. Since permutation approach is only valid if the complete structure is known, and theoretical values are also difficult to estimate, for the same reason, the exact thresholds for linkage in the AIL are unknown. However, in the studies presented in this thesis, we have not aimed for discovery of novel QTLs, but rather fine mapping of known QTLs. We have also estimated how much of the phenotypic variance is explained by the family factor alone, and used this as a minimum threshold for linkage.

### CONFIDENCE INTERVALS

The confidence interval for the QTL locations were by the markers outside a 1.5 reduction in LOD score approximately equivalent to a 95% confidence interval (MANICHAIKUL *et al.* 2006). We have also calculated the the probability for the positioning of QTLs using a bootstrap approach. Simulated pedigrees were sampled with replacement from the AIL to create a new data set with the same number of samples (794), which was mapped using a single-QTL model in R/qtl. The maximum LOD and the location of that maximum were recorded and the re-sampling was repeated 1000 times to obtain an estimate of the probability of a QTL being present at a given location in the region.

## CONGENIC STRAINS

Congenic strains are used for confirmation of the influence from QTLs and further studies of EAE related traits, as well as mapping of kinetics of the immune response, (Paper III and IV). All congenic strains are originally selected from the AIL, and thereafter backcrossed while selecting for the desired fragment. When starting the breeding of a congenic strain from an AIL, one can take advantage of the recombinations in previous generations, and select animals with a relatively small fragment in region of interest, thereby avoiding the tedious breeding of recombinants from the established congenic strain. The DA.PVG-*Eae18b* strain used in Paper III was backcrossed for 8 generations, while the reciprocal DA.PVG-*Vra4* and PVG.DA-*Vra4* strains used in Paper IV were backcrossed for 10 generations, before being bred to homozygosity and used in experiments.

## PHENOTYPES IN EAE

The linkage analysis was done using phenotypes directly reflecting the clinical manifestations of EAE, including EAE score and weight loss during disease. In the subsequent evaluation of candidate genes and congenic experiment, a more careful investigation of the immune response was also implemented. The clinical manifestations of autoimmune inflammation, demyelination and neuronal degradation during EAE can be estimated in the living animal using a simple scoring scale. Although an exact translation cannot be done, higher scores usually correlate with a more prominent demyelination and neuronal damage during disease (PAPADOPOULOS *et al.* 2006). The clinical score was graded as follows: 0, no clinical signs of EAE; 1, tail weakness or tail paralysis; 2, hind leg paraparesis or hemiparesis; 3, hind leg paralysis or hemiparalysis; 4, tetraplegia or moribund; 5, death. The following clinical parameters were assessed for each animal: EAE incidence (clinical signs for >1 day), onset of EAE (the first day that clinical signs were observed), maximum EAE score (the highest clinical score observed during EAE), cumulative EAE score (the sum of daily clinical scores), duration of EAE (the number of days with EAE) and weight loss ([weight at day 8 p.i. - minimum weight during the experiment] / weight at day 8 p.i.)

## ENZYME-LINKED IMMUNOSORBENT ASSAY

Anti-MOG antibodies in sera collected day 12-14 post immunization were measured using ELISA, with IgG isotype specific detection antibodies. Relative quantification was

calculated using serial dilutions of a pool of sera from immunized DA rats. Ccl2 was also measured in sera, using commercial ELISA kits, with a standard of recombinant Ccl2.

## PRIMARY CELL CULTURES

Primary cell cultures from parental and congenic strains are used for the evaluation of the impact from genetics variant on the immune systems. Cells from lymph nodes, spleens, bone marrow and brains have been used to study different aspect of the immune response.

### IN VITRO STIMULATIONS

In Paper II, lymphocyte cultures from immunized animals where used to study the effect on chemokine transcription when restimulated *in vitro* with MOG protein. This mimics the process where activated autoreactive T-cells encounter the autoantigen in the CNS, during the initiation of EAE. In parallel cultures, the proliferative response of lymphocytes was also tested, after restimulation with MOG or stimulation with the superantigen Concavalin A (ConA). The proliferation is measured trough the incorporation of radioactive $H^3$ thymidine, into the dividing cells.

In Paper III, astrocyte cultures from DA and the DA-PVG-Eae18 congenics were established. The axons of newborn rats are not myelinated until a few days after birth, and the establishment of cell cultures from the CNS is preferably done from these brains, since the myelinated brains need extensive washing when establishing cell cultures. After the separation of microglia from the cultures, astrocytes were stimulated with the proinflammatory cytokine TNF (tumor necrosis factor), in order to study if the parental and congenic animals show different patterns of chemokine production, in a proinflammatory environment. In Paper IV, bone marrow cells were cultured under different conditions for the generation of plasmacytoid and myeloid dendritic cells (pDC and mDC, respectively). The pDCs, mDCs and splenocytes from naïve animals were stimulated *in vitro* with IFN-γ to investigate the effect on MCH II expression.

### EXPRESSION ANALYSIS

Quantitative real time PCR with SYBR green dye has been used for quantification of transcription of specific targets in Paper II, III and IV. Using target-specific primers and

standardized PCR programs, this method provides a fast and reliable method for studying the differential regulation of transcription in cell cultures and tissues, in animals with different genetic background. All expression analysis in this thesis has used relative expression, where the target gene expression is related to a reference gene. The expression of the reference gene should remain stable in all cells and not be affected by external factors, such as proinflammatory cytokine in vivo, or ongoing inflammation in animals. The reference gene is sometimes referred to as "housekeeping" gene, since genes coding for proteins involved in basic cellular function may have a more stable expression. In Paper II *Gapdh* was used as reference gene, whereas *Hprt* was used for most targets in Paper IV. Multiple reference genes have been used, in Paper III (*Gapdh* and *β-actin* or *Gapdh*, *β-actin* and *Hprt)* and for some targets in Paper IV *(Gapdh* and *Hprt*). The geometrical mean of 2 or more reference genes gives a more stable value, and thereby provides a better normalization. The relative expression can be expressed as a ratio between the target and the reference, or as the fold change compared to a calibrator sample. When calculating the ratio (Paper II and IV), a standard curve created by serial dilutions is used for determining the relative expression of target and reference. In this thesis, the standard curve was created from a pool of undiluted cDNA samples, but stimulated cells or amplified PCR products can also be used. The relative expression is directly correlated to the threshold cycle ($C_t$), the PCR cycle were the signal from the amplified product can be detected in a specific sample. The fold change is calculated as the $2^{-((Ct_{calibrator} - Ct_{reference}) - (Ct_{target} - Ct_{reference}))}$ known as the $2^{-\Delta\Delta Ct}$ -method. The calculation is also easily adjusted for the efficiency of the PCR reaction, calculated from the standard curve. Efficiency adjusted fold changes are presented in Paper III, and the mean expression within the experiment is used as calibrator.

## FLOW CYTOMETRY

The expression of cell surface markers that are specific to differenT-cell types enables the analysis of number of cells within a cell type as well as quantification of specific markers. Within this thesis flow cytometry has been used to investigate if genetic variants contribute to quantitative differences of immune cells or surface marker expression. In Paper III, flow cytometry was used for the analysis of cell populations in the lymph nodes, after MOG-EAE induction. CD3 was used for identification of T-cells, and CD4 for identification of T-helper cells. CD25, expressed on activated $CD4^+$ T-cells and Tregs was also included. CD45RA was used for identification of B-cells, whereas CD11b was used for

macrophages/monocytes. In Paper IV, the MHC class II (OX-6) expression was determined in different types of antigen presenting cells (APC), using flow cytometry. Splenocytes were stained with CD11b (not CD11c, as erroneously written in the paper) for identification of macrophages and B220 for B-cells. Bone marrow derived plasmacytoid and myeloid dendritic (pDC and mDC, respectively) cells were distinguished based on the expression of CD11c and B220. $CD11c^{+}B220^{-}$ cells were identified as mDCs whereas $CD11c^{low}B220^{+}$ cells were characterized as pDCs.

### IMMUNOHISTOCHEMISTRY

In Paper IV, cryosections (14 µm) were cut from the L4-L5 segment in the spinal cord, after VRA, or the area surrounding the injection site in the brain after intraparenchymal injections of IFN-γ. Sections were stained with antibodies specific for CD3 for T-cell infiltration, Cd11b and ED-1 for identification of microglia and macrophages, and OX-6 for quantification of MHC class II expression, and visualized with corresponding conjugated secondary antibodies. In Paper III, histological evaluation was performed on paraformaldehyde-fixed, paraffin embedded sections from brain and spinal cord, collected 15 days post immunization. Sections (0.4 mm) were stained with hematoxylin-eosin and luxol fast blue to assess inflammation and demyelination respectively. Specific antibodies against ED1 and Iba-1, for identification of microglia and macrophages, and Ccl2, for protein quantification, were also used.

## BIOINFORMATICS AND HOMOLOGY ANALYSIS

The concept of using animal models for the studies of human disease is based on the idea that mammals, or eukaryotes, have fundamental cellular mechanisms in common, and that homologous pathways are involved in pathological processes in several species. Accordingly, if animal models are driven by the same genetic mechanisms as those for human traits, we should expect to find conserved genetic sequences shared by QTLs of the animal models and susceptibility regions of the human trait. The identification of regions with shared synteny linked to the human disease and a corresponding animal models can partly be used to estimate the common genetic risk factors across species,, and hence an estimate of the validity of the animal model, but can also be used to prioritize candidate regions (KUOKKANEN *et al.* 1996); (BARTON *et al.* 2001). A more comprehensive analysis of the synteny within regions linked or associated to MS and EAE in rats and mice was presented by Serrano-Fernández et al (SERRANO-FERNÁNDEZ *et al.*

2004), including all published QTLs and susceptibility regions, to that date. From this dataset 14 consensus regions with genetic evidence for linkage or association to neuroinflammation and high homology in all three species (90% sequence similarity between rats and mice, and 60% sequence similarity between rodents and humans) where identified. The same approach applying more stringent criteria was used in Paper I, for a focused analysis of regions in mice and humans sharing synteny with rat chromosome 10.

# RESULTS AND DISCUSSION

## LINKAGE ANALYSIS ON RAT CHROMOSOME 10

In order to fine map previously defined EAE QTLs in rats, the 10$^{th}$ generation of an AIL, originating from the MHC identical DA and PVG$^{av1}$ strain was used. In total 794 rats were immunized with MOG, and 29% (224 rats) developed signs of EAE, whereof 152 females. Among the affected animals, several distinct disease courses could be distinguished (Figure 2). The most common disease course was the monophasic disease (39.7%) but animals also displayed chronic/progressive (29.5%) and relapsing-remitting disease courses (25.5%). This indicates a diverse regulation of the EAE phenotype within the population, but no QTLs regulating a specific disease course have been identified in the AIL. However, the power of detecting differences within the affected subset of the experimental population is very low, and would require a larger experimental population, or preferably a higher incidence.

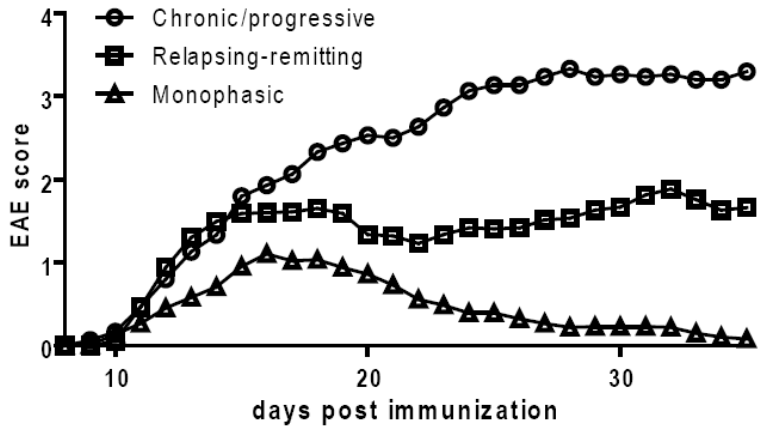


*Figure 2. AIL animals display different disease courses in response to MOG immunization.*

High resolution linkage analysis focused on rat chromosome 10 successfully confirmed the previously identified QTLs *Eae18a* and *Eae18b* and redefined the positions and confidence intervals for both loci. *Eae18a* (Paper I) was defined as a 5Mb region, with peak marker OT90.48 (60.86 Mb), and is linked to both severity and susceptibility of disease. In later studies, the *Eae18a* locus has also been linked to the titers of anti-MOG IgG2b titers in sera during the early disease phase (data not shown). *Eae18b* was defined as a 0.88 Mb region, linked primarily to severity of EAE, with the peak marker MJ310 located at 70.27

Mb (Paper II). The influence of the region on severity of disease was also confirmed using a congenic strain, with a PVG insert in the Eae18b region on a DA background (Paper III). We could thereby confirm that the linkage analysis in the 10th generation of the AIL allow a more accurate positioning and definition of narrower confidence intervals, compared to both traditional F2 intercrosses (DAHLMAN *et al.* 1999b) and previous generations in the same AIL (JAGODIC *et al.* 2004)

## HOMOLOGY MAPPING

In Paper I, rat chromosome 10 was also investigated using *in silico* mapping, for the identification of genetic regions linked or associated to neuroinflammation in humans, rats and mice that also have a shared synteny (SERRANO-FERNÁNDEZ *et al.* 2004). Through the use of more stringent criteria, compared to previous synteny mappings, the identified intergenomic consensus region was subdivided into four separate regions, whereof one overlap *Eae18a.* This analysis provided additional support for the localization and importance of *Eae18a* as an EAE-regulating QTL, and identified 13 candidate genes (Table 2) for regulation of EAE and MS.

| Location (bp) | RGD Symbol | Description |
|---|---|---|
| 58616509 | *Wscd1* | WSC domain-containing protein 1 |
| 58879846 | *Aipl1* | Aryl hydrocarbon receptor-interacting protein-like 1 |
| 58895247 | *Fam64a* | Family with sequence similarity 64, member A |
| 58903762 | *Pitpnm3* | PITPNM family member 3 |
| 59052750 | *RGD1304728* | KIAA0753 |
| 59100053 | *Txndc17* | Thioredoxin domain containing 17 |
| 59104238 | *Med31* | Mediator complex subunit 31 |
| 59107906 | *LOC687778* | Hypothetical protein |
| 59133558 | *Slc13a5* | Solute carrier family 13, member 5 |
| 59185136 | *LOC687784* | Similar to XIAP associated factor-1 isoform 2 |
| 59198308 | *Fbxo39* | F-box protein 39 |
| 59219924 | *Tekt1* | Tektin-1 |

*Table 2. Updated list of genes within the consensus region on rat chromosome 10, identified by both linkage analysis and synteny mapping in Paper I. Location (start position) retrieved from Ensembl v52 (www.ensembl.org), and gene symbols and descriptions from Rat Genome Database (http://rgd.mcw.edu)*

Given the development in both animal and human genetics during the recent years, it would be interesting to perform a new analysis similar to the original study in the whole genome (SERRANO-FERNÁNDEZ *et al.* 2004). A comprehensive study including all the confirmed and suggestive risk alleles form the recent whole genome scan in MS (INTERNATIONAL MULTIPLE SCLEROSIS GENETICS CONSORTIUM *et al.* 2007) and other association studies, together with data from recent linkage and congenic studies in mice and rats would enable identification of narrow intergenomic consensus regions for the regulation of neuroinflammation. This would also provide a more accurate estimate of the shared genetic regulation of neuroinflammation, in multiple species.

## CHEMOKINES AS CANDIDATES FOR EAE REGULATION

*Eae18b* includes a cluster of chemokine genes *(Ccl2, 7, 11, 12, 1)*, known to direct the chemotaxis of monocytes, eosinophils, neutrophils and other immunecells. No sequence differences between DA and PVG were found in the exons of the chemokine genes. This puts the focus on gene expression, and we analyzed if genotype in the chemokine cluster influence the expression of the chemokine genes during the early phase of the disease, using the 13$^{th}$ generation of the DAxPVG$^{av1}$ AIL. Animals were stratified based on genotype (DA or PVG homozygous) in the chemokine cluster, thus allowing for identification of *cis*-regulation of transcription on a heterogonous background. Differential expression of chemokine genes in the lymph nodes, regulated by genotype in the locus, was identified for *Ccl2*, *Ccl11* and *Ccl1* (Paper II). In Paper III, the genetically regulated chemokine expression during the induction and early disease phase was analyzed more in detail, using a congenic strain, and the regulation of *Ccl11* in lymph nodes was confirmed. *Ccl11* was also upregulated in the spinal cord during early EAE, as well as in the spleen and spinal cord during the late stage of the disease.

Ccl11 signals trough the Ccr3 receptor on eosinophils (DAUGHERTY *et al.* 1996; GAO *et al.* 1996; KITAURA *et al.* 1996; PONATH *et al.* 1996), basophils (BEAULIEU *et al.* 2002) and Th2 cells (SALLUSTO *et al.* 1998), but binds also to receptors Ccr2 and Ccr5 (BLANPAIN *et al.* 1999) and can function as an antagonist for Ccr2 (OGILVIE *et al.* 2001). Even though additional studies are needed for determining how the *Ccl11* expression regulates EAE, we suggest that upregulation of *Ccl11* expression in the resistant strains would lead to a preferential recruitment of Th2 cells to lymph nodes and CNS, and thereby suppress the immune reaction. Alternatively, increased *Ccl11* expression could reduce the chemotaxis of

pathogentic T-cells, macrophages and other Ccr2 expressing cells to the CNS, trough Ccr2 antagonism.

When comparing chemokine expression in the parental strains, there is also evidence for differential expression of *Ccl2* in the lymph nodes of naïve animals and *Ccl12* in both lymph nodes and spinal cords of immunized animals. These differences are however not primarily regulated by genotype in the chemokine locus, as demonstrated by the expression analysis in congenic animals, but rather a consequence of inflammation. The involvement and temporal upregulation of *Ccl2* in EAE is well documented but this is, to my knowledge, the first evidence for the involvement of *Ccl12* in EAE. Together, the data in Paper III clearly show that the *Eae18b* locus influences the severity of disease, together with macrophage infiltration and demyelination in the CNS during disease, and suggest a considerable role for *Ccl11* in this regulation. Linkage analysis for the identification of the loci regulating the expression of *Ccl2* and *12* during EAE will be performed in two reciprocal (DAxPVG$^{av1}$) backcrosses each including approximately 450 rats, recently completed in our laboratory

## ASSOCIATION TO HUMAN CHEMOKINE CLUSTER

The genetic association between SNPs in the chemokine cluster and MS was first investigated in a large Swedish case-control cohort, followed by an additional investigation of selected polymorphisms in additional cohorts from Sweden, Denmark, Norway and Finland. In total, 3831 patients and 4046 controls were included in the association study. In a meta-analysis where all cohorts were combined, association was identified to haplotypes in *CCL2* (GCC: rs2857656, rs2857657, rs4586) and *CCL13* (TC: rs159313, rs2072070), as well as a single SNP (rs3136682) in *CCL1.* The associated haplotype in *CCL2* and the SNP in *CCL1* are protective (OR: 0.86 and 0.71, respectively) whereas the haplotype in *CCL13* is predisposing for disease (OR: 1.07). In addition, a rare haplotype in *CCL13* (CC: rs159313, rs2072070) was associated with a strong protective effect in Swedish and Danish cohorts, but was not included in the meta-analysis, due to significant heterogeneity in allele frequency between the cohorts.

The interaction between genotypes in the chemokine locus and HLA-A*02, HLA-DRB1*15 and sex, previously shown to influence the risk of developing MS, was investigated. No significant statistical interaction was identified, but including HLA-DRB1*15 resulted in

novel associations between SNPs in the chemokine cluster and MS. In the analysis of the HLA-DRB1*15 positive subgroup in the original Swedish cohort, SNPs in *CCL2*, *CCL1* and *CCL13* showed association. A meta-analysis of all HLA-DRB1*15 positive patients and controls established association to disease predisposing haplotypes in *CCL2* (GCT: rs2857656, rs2857657, rs4586), and *CCL13* (TC: rs159313, rs2072070), with OR 1.22 and 1.14, respectively. Interestingly, the variant of the *CCL2* haplotype includes the same SNPs as the haplotype identified in the whole cohort, but with a different genotype in rs4586, and have an opposite effect. Thus, HLA-DRB1*15 may have a modifying effect on the association between the chemokine cluster and risk of developing MS, or the HLA-DRB1*15 positive patients may represent a genetically distinct subgroup among the MS patients. Further, we could also demonstrate MS patients have higher concentrations of CCL2 protein in the CSF, compared to controls, and that the genotype in *CCL2* is associated with the CCL2 levels in MS patients. Here, the T-allele of rs4586 is associated with lower CCL2 concentrations compared to the other alleles. However, the protein levels in CSF may not reflect the levels of CCL2 in the brain tissue, or in the lesions.

Selected SNPs were also investigated in a relation to rheumatoid arthritis, since several chemokines have been suggested to regulate inflammatory processes in the joints of RA patients, including *CCL2* and *CCL13* (HARINGMAN *et al.* 2006; IWAMOTO *et al.* 2006; IWAMOTO *et al.* 2007). In addition, the chemokine cluster region showed suggestive association in a whole genome study of RA patients positive for anti-citrullinated peptide antibodies (ACPA) (PLENGE *et al.* 2007). We could demonstrate that SNPs in *CCL2* (rs4586, OR=1.34), *CCL8* (rs3138038, OR=2.37) and *CCL13* (rs159313, OR=1.12) showed association to an increased risk of RA, in the ACPA positive set. In the whole cohort of RA patients, including also the ACPA negative patients, only SNPs in *CCL2* and *CCL8* were associated with RA. None of the haplotypes identified in the association study in MS were found to be associated to RA. In conclusion, we demonstrate that variants of *CCL2, CCL13* are associated with both MS and RA, providing further support to the hypothesis of shared pathways and risk factors in common inflammatory diseases. Additional genes are associated with the each of the two diseases, which could indicate organ or disease specific pathways.

There is a slight discrepancy between the identified genes in EAE, where the strongest evidence is found for *Ccl11*, but also *Ccl1, 2* and *12* are indicated, and MS, where *CCL1, 2*

and *13* are associated. However, even if the chemokine systems in rats and humans share many similarities, there are also differences and species specific chemokines. The close sequence similarity between chemokines also makes it difficult to unambiguously define the orthologous genes in each species. For example rat *Ccl11* and human *CCL11* share 77% sequence identity, whereas rat *Ccl11* share 70% of the sequence with human *CCL13* (www.ensembl.org). In addition, CCL13 can interact with the same receptors as CCL11, including CCR2, 3 and 5 (BLANPAIN *et al.* 1999; DAUGHERTY *et al.* 1996; GAO *et al.* 1996; GARCIA-ZEPEDA *et al.* 1996; OGILVIE *et al.* 2001). Hence, *CCL13* may represent a human specific functional homolog to *CCL11*.

## VRA4 CONGENICS

The *Mhc2ta* (MHC class II transactivator) gene, located in the *Vra4* region on rat chromosome 10 (LIDMAN *et al.* 2003; SWANBERG *et al.* 2005), regulates the expression of MHC class II, and related proteins such as CD74 (MHC class II- associated invariant chain) (LEIBUNDGUT-LANDMANN *et al.* 2004). It has been demonstrated that the MHC class II expression segregates with a haplotype in the promoter region of *Mhc2ta,* in several rat strains (SWANBERG *et al.* 2005). For the confirmation of the genetic regulation of *Mhc2ta*, MHC class II and related molecules, two reciprocal congenic strains, DA.PVG-*Vra4,* and PVG$^{av1}$.DA-*Vra4,* were used in VRA. Analyses in the spinal cord 21 days post operation show that *Mhc2ta* and *CD74* mRNA expression and protein levels of MHC class II are regulated by the *Vra4* region after VRA.

The regulation of MHC class II expression was further studied using intraparenchymal injections of IFN-γ. The expression of MHC class II is known to be differentially regulated in the DA and PVG$^{av1}$ rat strains after intraparenchymal IFN-γ injections (SWANBERG *et al.* 2005), and here we can show that the expression in dependent on genotype in the *Vra4,* with a relative upregulation in the DA strain, compared to the congenic DA.PVG-*Vra4* rats. The MHC class II expression of the cell surface was also studied in other antigen presenting cells (APC; B-cells, macrophages, mDC and pDCs), and the DA strain showed upregulation compared to the congenics, in untreated cells or after *in vitro* stimulations with IFN-γ. The congenics were also tested in MOG induced EAE, and DA.PVG-*Vra4* congenic animals show a significantly lower incidence, lower degree of weight loss, and less severe disease compared to parental DA animals. This indicates a connection between quantitative differences in MHC class II expression, and susceptibility to autoimmune

disease. The reduced expression of MHC class II in the congenic strains could potentially result in a reduced activation of T-cells in the periphery, during the induction of EAE.

*Clec16a,* a homolog to the human *CLEC16A*, associated to an increased risk for development of MS (DE JAGER *et al.* 2009; INTERNATIONAL MULTIPLE SCLEROSIS GENETICS CONSORTIUM *et al.* 2007; RUBIO *et al.* 2008; WEBER *et al.* 2008) is also located within the defined confidence interval for *Vra4* and in the congenic fragment, and is a potential candidate for EAE regulation. However, expression analysis in DA and PVG$^{av1}$ strains show no difference in expression of *Clec16a* during early EAE (THESSEN HEDREUL *et al.* 2009) and preliminary data show no expression difference between DA and PVG$^{av1}$ or DA.PVG-*Vra4* rats, 5 days after VRA (Harnesk et al., unpublished data). Further studies, including resequencing of *Clec16a* in several rat strains and description the molecular functions, are needed to better understand the role of this gene in neuroinflammation.

## FUTURE DIRECTIONS

The modern technologies for high throughput genotyping, improved statistical methods and large international efforts have enabled the identification of several genetic risk factors for common complex diseases (INTERNATIONAL MULTIPLE SCLEROSIS GENETICS CONSORTIUM 2009; INTERNATIONAL MULTIPLE SCLEROSIS GENETICS CONSORTIUM *et al.* 2007; WELLCOME TRUST CASE CONTROL CONSORTIUM 2007; WELLCOME TRUST CASE CONTROL CONSORTIUM and CONSORTIUM 2007). Within the following years we will see a continuous development of human genetics, with deep sequencing of individual genomes on a large scale (KUEHN 2008), and even larger GWAS studies identifying new genes and risk alleles.

The development in the field of human genetics will also change the way we use the animal models of human diseases. When a large number of associated genes and risk alleles for human disease are known, the focus cannot longer be to identify new broad candidate regions. Genetic studies in animal models should instead take advantage of the genetic homogeneity in inbred strains, in combination with the new technologies in genotyping and expression analysis, for a systematic assessment of genetic interactions and epistasis in animal models of human diseases, in order to present better models for the echanisms underlying specific traits. Experimental populations such as heterogeneous stocks will also enable high resolution mapping and direct identification of genes, from an experimental cross. In an ongoing study (JOHANNESSON *et al.* 2009) using a HS in rats,

multiple traits and phenotypes are recorded for each animal, thus enabling not only identification of genes regulating individual or multiple phenotypes, but also systematic studies of the interrelation between phenotypes.

Another important challenge for the research community is to understand the mechanisms of risk alleles for complex disease. Congenic animals, with disease modifying polymorphisms in selected genes and pathways will be a valuable resource for these studies, in combination with methods for silencing targeted genes.

## FROM RATS TO HUMANS AND BACK AGAIN

In conclusion, within this thesis we have used a translational approach for the combination of genetic information from multiple species in order to identify and evaluate candidate genes and genetic regions, for regulation of neuroinflammation.

This was done in several steps, including:

- Definition of narrow QTLs (*Eae18a* and *Eae18b*) for the regulation of EAE in rats
- Identification of a candidate gene cluster in *Eae18b,* with particular focus on *Ccl1, Ccl2* and *Ccl11*
- Association of the human chemokine genes *CCL1, CCL2* and *CCL13* to MS
- Evaluation of genes associated to MS using congenic rat strains, providing solid evidence for *Mhc2ta* and *Ccl11* in the regulation of neuroinflammation.

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