From the Neuroimmunology Unit
Department of Clinical Neuroscience
Karolinska Institutet, Stockholm, Sweden

Genetic and Immunological Regulation of Neuroinflammation

Mélanie Thessén Hedreul

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To my beloved family and friends

"Le génie n'est qu'une plus grande aptitude à la patience"
"Genius is only a greater aptitude for patience"

- Georges Louis Leclerc, Comte de Buffon
(Montbard 1707 - Paris 1788)

ABSTRACT

Multiple Sclerosis (MS) affects young adults and is characterized by chronic inflammation and demyelination in the central nervous system that leads to progressive worsening of disease. The cause of MS is incompletely understood and there is a need for more specific and effective treatments.

This thesis aimed to characterize genetically controlled pathogenic mechanisms in the model of MS, experimental autoimmune encephalomyelitis (EAE), and to translate findings from experimental models to human disease.

We demonstrated that genetic risk factors and pathogenic mechanisms in EAE are similar to those of MS. The EAE-susceptible strain had increased expression of MS candidate genes *Il2ra* and *Il7r* among others and up-regulation of MS-associated immunological pathways such as T_H1 and T_H17. Expression of *Il18r1* was increased in both the susceptible strain and in periphery and cerebrospinal fluid of MS patients. This might contribute pathogenically to disease through T cell differentiation and activation. Clinically isolated syndrome (CIS) patients had elevated *IL18R1* expression, thus it could potentially serve as an early disease biomarker.

Using an expression quantitative trait loci (eQTL) approach we detected numerous *cis*-regulated positional candidate genes for EAE and defined several disease correlated gene networks enriched for pathways involved in cell-mediated immune mechanisms of relevance for both EAE and MS. *Mfsd4* was identified as a candidate gene for *Eae34* which conferred a functional effect on T cell proliferation and activation. The importance of autophagy related genes in the pathogenesis of neuroinflammation was investigated. *Atg7* expression was higher in the EAE-resistant strain and in MS patients it associated with remission and less severe symptoms.

Results presented in this thesis collectively demonstrate genetic regulation of known and novel mechanisms in EAE and MS and point to causal pathogenic pathways. Combining various research fields in both human cohorts and experimental models is a promising approach to increase our ability to define MS susceptibility genes and pathways to target for currently unfulfilled therapeutic needs.

LIST OF PUBLICATIONS

This thesis is based on the following studies, which are referred to in the text by their Roman numerals.

I. **Melanie Thessen Hedreul***, Alan Gillett*, Tomas Olsson, Maja Jagodic and Robert A. Harris.
Characterization of Multiple Sclerosis candidate gene expression kinetics in rat experimental autoimmune encephalomyelitis.
Journal of Neuroimmunology. 2009;210(1-2):30-9.

II. Alan Gillett*, **Melanie Thessen Hedreul***, Mohsen Khademi, Alexander Espinosa, Amennai Daniel Beyeen, Maja Jagodic, Ingrid Kockum, Robert A. Harris and Tomas Olsson.
Interleukin 18 Receptor 1 expression distinguishes patients with multiple sclerosis.
Multiple Sclerosis. 2010;16(9):1056-65.

III. **Melanie Thessen Hedreul**, Steffen Möller, Pernilla Stridh, Rasmus Berglund, Andre Ortlieb Guerreiro-Cacais, Ann-Kristin Grimm, Yask Gupta, Johan Öckinger, Alan Gillett, Amennai Daniel Beyeen, Margarita Diez, Tomas Olsson* and Maja Jagodic*.
Genome-wide expression profiling in experimental autoimmune encephalomyelitis highlights a gene network enriched for T cell functions and *Mfsd4* as a candidate gene regulating autoimmunity.
Submitted Manuscript.

IV. **Melanie Thessen Hedreul**, Rasmus Berglund, Jenny Link, Roham Parsa, Juliane Becher, Petra Bergman, Mohsen Khademi, Francesco Cecconi, Jan Hillert, Ingrid Kockum, Maja Jagodic* and Tomas Olsson*.
Expression of the autophagy related gene *Atg7* is genetically regulated in experimental autoimmune encephalomyelitis and altered in patients with multiple sclerosis.
Manuscript.

* These authors contributed equally to the study

ADDITONAL PUBLICATIONS

Related publications and manuscripts not included in the thesis.

Amennai Daniel Beyeen, **Melanie Thessen Hedreul**, Alan Gillett, Marie N'Diaye, Andre Ortlieb Guerreiro-Cacais, Steffen Möller, Tomas Olsson and Maja Jagodic. **Epistasis between genetic variants of natural killer cell ligands and receptors determines NK cell regulation of experimental neuroinflammation.** *Submitted Manuscript.*

Pernilla Stridh, **Melanie Thessen Hedreul**, Amennai Daniel Beyeen, Milena Z. Adzemovic, Hannes Laaksonen, Alan Gillett, Johan Öckinger, Monica Marta, Hans Lassmann, Kristina Becanovic, Maja Jagodic and Tomas Olsson. **Fine-mapping resolves *Eae23* into two QTLs and implicates *ZEB1* as a candidate gene regulating experimental neuroinflammation in rat.** PLoS One. 2010;5(9):e12716.

Pernilla Stridh, Petra Bergman, Sabrina Ruhrmann, **Melanie Thessen Hedreul**, Sevasti Flytzani, Amennai Daniel Beyeen, Alan Gillett, Nina Krivosija, Johan Öckinger and Maja Jagodic. **Missing heritability resides in parent-of-origin effects and implicates significant epigenetic regulation of inflammation.** *Submitted Manuscript.*

Steffen Möller, René Schönfelder, Hajo Krabbenhöft, Benedikt Bauer, Yask Gupta, Robert Hoffmann, Ann-Kristin Grimm, Jan Kolbaum, Pjotr Prins, Morris Swertz, Danny Arends, Patrik Wernhoff, Chris Sander, Hans-Jürgen Thiesen, Dirk Koczan, **Melanie Thessen Hedreul**, Maja Jagodic and Saleh M. Ibrahim. **TiQS: an interactive web environment for expression QTL analysis.** *Manuscript.*

Sevasti Flytzani, Pernilla Stridh, **Melanie Thessen Hedreul**, Monica Marta, Andre Ortlieb Guerreiro-Cacais, Maja Jagodic and Tomas Olsson. **Genetic regulation of antibodies against myelin oligodendrocyte glycoprotein (MOG) in rat experimental autoimmune encephalomyelitis.** *Manuscript.*

Rita Nohra, Amennai Daniel Beyeen, Jian Ping Guo, Mohsen Khademi, Emilie Sundqvist, **Melanie Thessen Hedreul**, Finn Sellebjerg, Cathrine Smestad, Annette B. Oturai, Hanne F. Harbo, Erik Wallström, Jan Hillert, Lars Alfredsson, Ingrid Kockum, Maja Jagodic, Johnny Lorentzen and Tomas Olsson. ***RGMA* and *IL21R* show association with experimental inflammation and multiple sclerosis.** Genes and Immunity. 2010;11(4):279-93.

Milena Z. Adzemovic*, Johan Öckinger*, Manuel Zeitelhofer, Sonja Hochmeister, Amennai Daniel Beyeen, Atul Paulson, Alan Gillett, **Melanie Thessen Hedreul**, Ruxandra Covacu, Hans Lassmann, Tomas Olsson and Maja Jagodic. **Expression of *Ccl11* associates with immune response modulation and protection against neuroinflammation in rats.** *Submitted Manuscript.*

Mikael Ström, Faiez Al Nimer, Rasmus Eurén, **Melanie Thessen Hedreul**, Mohsen Khademi, Ingrid Kockum, Tomas Olsson and Fredrik Piehl. **Variability in *Gsta4* is associated with intrathecal antibody responses in experimental autoimmune encephalomyelitis and suggested clinical and immune phenotypes in multiple sclerosis.** *Manuscript.*

* These authors contributed equally to the study

CONTENTS

LIST OF ABBREVIATIONS

AIL	Advanced Intercross Line
APC	Antigen-Presenting Cell
Atg	Autophagy-Related Protein
BBB	Blood Brain Barrier
BC	Backcross
cAMP	Cyclic Adenosine Monophosphate
CIITA	MHC Class II Transactivator
CIS	Clinically Isolated Syndrome
CNS	Central Nervous System
Crtam	Cytotoxic and Regulatory T Cell Molecule
CSF	Cerebrospinal Fluid
CXCL13	Chemokine (C-X-C motif) Ligand 13
DA	Dark Agouti
DC	Dendritic Cell
DMD	Disease-Modifying Drug
DNA	Deoxyribonucleic Acid
EAE	Experimental Autoimmune Encephalomyelitis
EBV	Epstein-Barr Virus
EDSS	Expanded Disability Status Scale
eQTL	Expression Quantitative Trait Locus
ER	Endoplasmic Reticulum
F_2	Intercross Generation 2
FoxP3	Forkhead Box P3
G_{10}	Generation 10
GM-CSF	Granulocyte-Macrophage Colony Stimulating Factor
GWAS	Genome-Wide Association Study
HHV6	Human Herpes Virus 6
HLA	Human Leukocyte Antigen
IBD	Inflammatory Bowel Disease
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IL18R	Interleukin 18 Receptor
Itk	IL2-Inducible T-Cell Kinase
KO	Knockout
Lck	Lymphocyte-Specific Protein Tyrosine Kinase
LD	Linkage Disequilibrium
Lef1	Lymphoid Enhancer-Binding Factor 1

LOD	Logarithm of Odds
MBP	Myelin Basic Protein
MFS	Major Facilitator Superfamily
Mfsd4	The Major Facilitator Superfamily Domain Containing Protein 4
MHC	Major Histocompatibility Complex
MOA	Mechanism of Action
MOG	Myelin Oligodendrocyte Glycoprotein
MRI	Magnetic Resonance Image
MS	Multiple Sclerosis
NK	Natural Killer
OND	Other Neurological Disorders
OR	Odds Ratio
PBMC	Peripheral Blood Mononuclear Cell
Pde3b	Phosphodiesterase 3B
p.i.	Post Immunization
PLP	Proteolipid Protein
PP	Primary Progressive
PVG	Piebald Virol Glaxo
qPCR	Quantitative Polymerase Chain Reaction
QTL	Quantitative Trait Locus
RA	Rheumatoid Arthritis
Rgma	Repulsive Guidance Molecule A
Rgs	Regulator of G-Protein Signaling
rMOG	Recombinant MOG (amino acids 1-125)
RNA	Ribonucleic Acid
RNO	Rat Chromosome
RR	Relapsing Remitting
SLE	Systemic Lupus Erythematosus
SNP	Single Nucleotid Polymorphism
SP	Secondary Progressive
T1D	Type 1 Diabetes
TCR	T Cell Receptor
TGF	Transforming Growth Factor
T_H	T Helper Cell
TIR	Toll/IL1 Receptor
TLDA	TaqMan Low Density Array
TNF	Tumor Necrosis Factor
Treg	T Regulatory Cell
VitD	Vitamin D
VLA4	Very Late Antigen 4

THESIS AIMS

The work presented in this thesis aimed to characterize the genetic regulation of experimental neuroinflammation in order to better understand underlying pathogenic mechanisms and to translate findings from experimental models to human disease.

Specific scientific goals:

Study I   To determine the development of autoimmune responses and the kinetic expression of MS candidate genes in the EAE model.

Study II   To determine whether expression of *IL18R1* varies between MS patients and controls and to test genetic association of *IL18R1* with MS.

Study III   To identify candidate genes and molecular pathways that are dysregulated during EAE by using an eQTL approach and network analysis.

Study IV   To investigate the involvement of the autophagy-related gene *Atg7* in the pathogenesis of MS and its experimental model EAE.

1 INTRODUCTION

1.1 COMPLEX DISEASE

Complex diseases result from the interplay of genetic, epigenetic, environmental and additional as yet undefined risk factors (Figure 1A) [1-4]. Such diseases are regulated by multiple genes, each exerting small effects. To add to the complexity there is genetic heterogeneity [5], meaning that patients with similar clinical phenotypes can carry a different combination of risk alleles. In addition, genetic risk alleles can also be present in unaffected individuals. Apart from risk alleles, epigenetic influences, changes in gene expression without a change in the genomic sequence (e.g. DNA methylation and noncoding RNAs) also likely contribute to disease susceptibility [6,7].

There is an undefined contributing genetic effect named the *missing heritability* [8]. This missing heritability can be explained by gene variants contributing very small effects, rare gene variants or parent-of-origin effects. Epistatic effects are also considered an important factor in complex diseases, but are largely unexplored to date. These gene-gene interactions could explain a major part of the missing heritability. In the end, a unique combination of genetic and environmental risk factors will influence the susceptibility to disease and accumulated risk factors can eventually make an individual pass a threshold with ensuing development of clinical symptoms (Figure 1B) [9-11]. The interplay of genes and environment may also define disease severity, clinical course and response to treatment.

Examples of complex diseases involving chronic inflammation are multiple sclerosis (MS), rheumatoid arthritis (RA), Crohn's inflammatory bowel disease (IBD), systemic lupus erythematosus (SLE) and type 1 diabetes (T1D). Many gene polymorphisms influencing complex diseases have been identified. However, more research is needed to gain an understanding of how these risk alleles functionally influence disease. In this thesis the regulation of MS, for which disease etiology is largely unknown, was explored. This was mainly achieved by studying pathogenic mechanisms and by identifying risk genes in its experimental model.
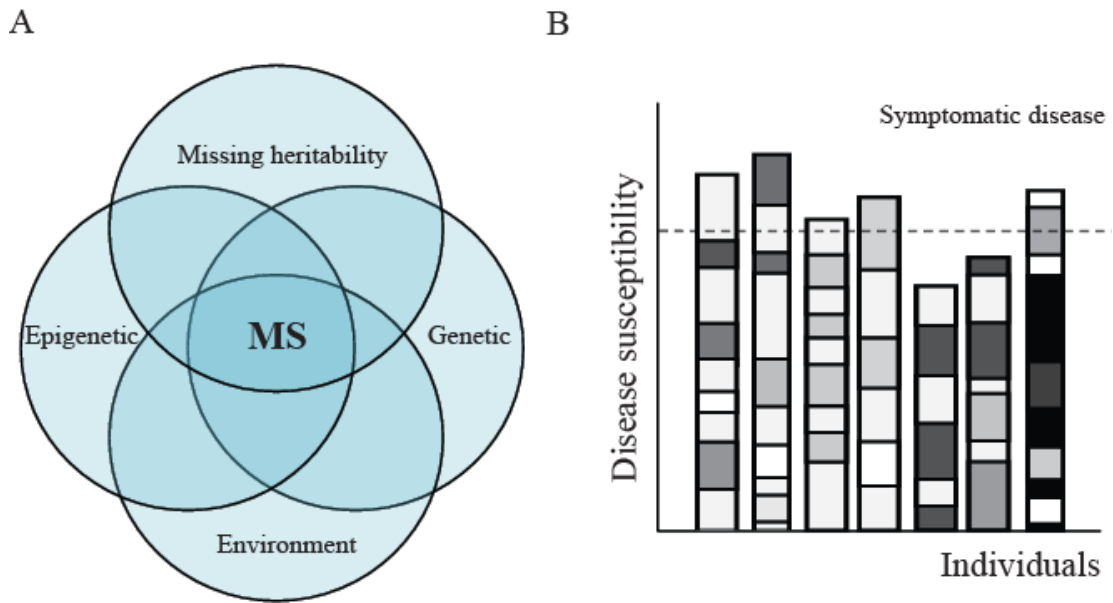
Figure 1. A) The proposed model of inheritance for MS, a complex disease in which genetic, epigenetic and environmental factors all interact to influence susceptibility. B) The threshold model for susceptibility to complex diseases. The accumulation of risk alleles and environmental factors, and the interaction between risk factors, determine if an individual will develop disease or not.

1.2 MULTIPLE SCLEROSIS

Multiple Sclerosis (MS), "la sclérose en plaques", was first described by the French neurologist Jean-Martin Charcot in 1868 who worked at the Salpêtrière hospital in Paris. MS is a chronic inflammatory disease of the central nervous system (CNS) with a prevalence in Sweden of approximately 0.1-0.2% [12]. MS preferentially affects women, with a gender ratio of 2.5:1 [13], and the onset of disease is usually between 20-40 years of age. With half of patients unable to work 10 years after diagnosis [14], there is an apparent socio-economic impact and a marked reduction in quality of life for those affected.

1.2.1 CHARACTERISTICS

MS is characterized clinically by the breakdown of blood-brain barrier (BBB) integrity [15] and immune-mediated destruction of oligodendrocytes which produce myelin sheaths. These sheaths surround the nerve axons in the CNS and enable the conduction of action potentials. Axons are damaged during demyelination, the conduction of neuronal impulses is reduced and there is progressive disability [14,16,17]. Furthermore, there are changes in white and grey matter and a loss in brain volume over time [18,19].

Symptoms of disease vary, most probably depending on the location of the multifocal demyelinated plaques in the CNS [20]. Common neurological symptoms are problems with coordination and balance disturbance (ataxia) and muscle weakness, as well as loss of sensation, pain, fatigue, visual problems, cognitive impairment and in severe cases, impaired mobility. Pathogenic mechanisms are mediated by immune cells, antibodies and degeneration [21]. The most common plaque is characterized by perivascular inflammation, infiltrating lymphocytes, macrophage-associated deposition of complement and deposition of immunoglobulin G (IgG) around lesions. In subsets of patients there is remyelination, although resultant myelin sheaths are thin [22].

1.2.2 CLINICAL COURSE

MS is heterogeneous and unpredictable in its clinical course, which is classified in four sub-types (Figure 2). A greater portion of patients initially display inflammatory bouts defined by relapses and remissions [23], where symptoms can resolve completely between attacks. However, the majority of these patients will eventually progress to a secondary-progressive phase with accumulated neurological problems. Some patients display an aggressive form of disease in which symptoms continuously become worse over time.
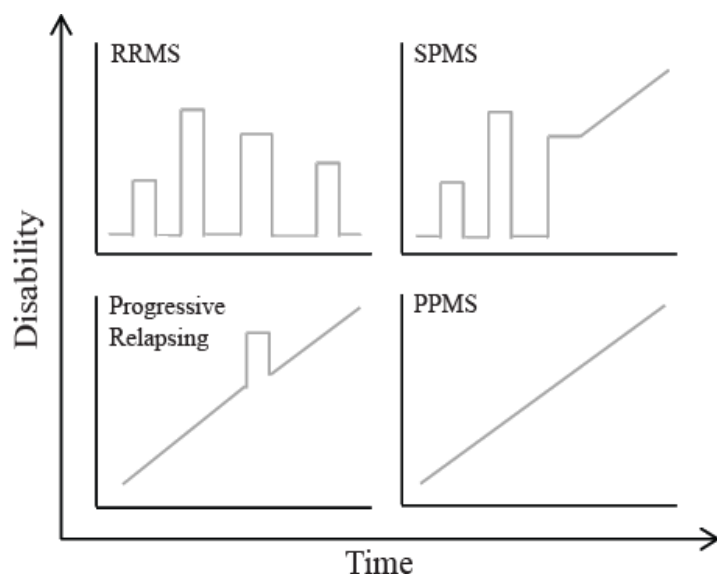
Figure 2. MS is heterogeneous and unpredictable in its clinical course. A majority of patients initially display inflammatory bouts defined by relapses and remissions (RRMS). A majority of these patients later develop a secondary progressive disease (SPMS). Some patients display a progressive-relapsing form of disease or a primary progressive MS (PPMS).

MS is defined by bouts of neurological symptoms, presence of oligoclonal bands in the cerebrospinal fluid (CSF) generated as a consequence of clonal expansion of B cells, and lesions evident using magnetic resonance imaging (MRI) [24,25]. Measurement of the functional status of MS patients is most commonly performed using the expanded disability status scale (EDSS) [26].

1.2.3 CURRENT HYPOTHESIS FOR AUTOIMMUNITY

Autoimmune diseases are characterized by a loss of immunological tolerance whereby autoreactive T cells or antibodies are activated against self-antigens. Tolerance refers to the immune system tolerating the body's own tissue antigens. In organ-specific autoimmune diseases the damage is restricted to a specific tissue, for MS the target organ being the CNS. As the trigger and target antigen for MS are currently unknown, it has not been formally proven that MS is an autoimmune disease. However, there is strong evidence for autoimmune mechanisms in MS pathogenesis [27]. Many of the postulates for MS being autoimmune are thus fulfilled. This includes the presence of autoreactive cells and autoantibodies specific for CNS proteins in the affected tissue [28-31]. Additionally, the active immunization of specific myelin autoantigens and the passive transfer of autoreactive T cells can induce disease in experimental models [32-34].

One hypothesis for the initiation of MS is that myelin-specific T cells escape negative selection during development and these then becoming activated in the periphery and cross over the BBB. They are reactivated in the CNS and release proinflammatory cytokines that further help in recruiting immune cells to the CNS. Consequently, an inflammatory process, also involving microglia, macrophages and astrocytes, results in demyelination and axonal damage [35]. Autoimmune disease and autoreactivity is not synonymous, and healthy individuals also have circulating self-reactive T lymphocytes specific for CNS antigens [36-39]. Several peripheral tolerance processes, including regulatory T cells, probably keep the activation of these under control [40,41]. In addition, it has been reported that autoreactive T cells from patients have an increased inflammatory capacity [42,43].

1.2.4 GENETIC RISK FACTORS

The genetic component of MS has been established by evidence of familiar clustering [44,45]. In addition, twin studies have demonstrated an increased MS concordance among monozygotic twins compared to dizygotic twins [46]. The human leukocyte antigen (HLA) region was for many years the only region associated with MS, and is still the strongest genetic factor [47,48]. This region encodes key molecules of the immune system, molecules involved in antigen presentation, among others. The strongest risk alleles for MS are the HLA Class II, namely DRB1*15:01 alleles [49,50]. The strongest established protective influence is from the HLA-A*02 [51,52].

During recent years numerous additional non-HLA genes have been associated with MS risk, the interleukin receptor 7 receptor (*IL7R)* and IL2 receptor alpha (*IL2RA)* being among those first identified [53-56]. To date, more than 50 genetic variants that contribute to MS susceptibility have been identified [57]. It has long been debated whether inflammation or neurodegeneration is the primary cause of MS symptoms [58,59]. Recent genetic studies demonstrate that genetic determinants in immune genes predispose for disease susceptibility and are significantly over-represented. However, these risk alleles do not explain the full disease heritability and variance [57] and it is likely that additional risk variants that confer small effects also contribute to heritability. Additionally, little is known about the functional outcomes of risk-associated variants.

1.2.5   THE ENVIRONMENTAL COMPONENT

Evidence for a genetic predisposition in MS is strong. Nevertheless, in complex diseases there are other factors generally contributing to susceptibility. There is a 70% discordance rate between monozygotic twins, and an apparent environmental influence on MS. There is a latitude gradient [60] and individuals who in adolescence move between regions with varying prevalence of disease acquire the risk of the new geographical area [61]. High prevalence areas include northern Europe, northern USA and Canada, southern Australia and New Zealand. Surely, both genetic and environmental factors could explain this distribution. Environmental explanations could be sunlight exposure alone or its role in generating active vitamin D (VitD) [62]. VitD has several immunomodulatory effects, currently under investigation in our lab, and high levels associate with protective effects against MS [63,64]. Another explanation is the so-called 'hygiene hypothesis'.

A number of viruses have also been implicated in pathogenic mechanisms of MS, including in particular human herpes virus 6 (HHV6) and Epstein Barr virus (EBV) [65,66]. There is no confirmation of a direct role of viral infection in MS, although bystander activation via epitope spreading and molecular mimicry could represent mechanisms through which inflammation is triggered. In this way viral infections might contribute to pathogenesis [67-69]. Additionally, other environmental factors such as smoking and obesity also increase susceptibility to MS [70,71].

1.2.6 AVAILABLE THEURAPEUTICS

Therapeutic agents available for MS patients relieve symptoms (spasticity, fatigue, tremor or pain) or are disease-modifying drugs (DMDs) that can reduce the number and magnitude of relapses. There is no cure for MS and current therapies target relapses for individuals with RRMS, but fail to slow disease progression in both PPMS and SPMS [20]. The available drugs have different mechanisms of action (MOA): in the periphery, at the BBB or in the CNS. For effective treatment outcomes it is of great value to define biomarkers in order to identify patients who will benefit from a certain treatment.

The most commonly used therapies today are injections of recombinant interferon beta (IFNβ) and polypeptide glatiramer acetat [72]. IFNβ is a first-line treatment for RRMS. However, its MOA is not fully understood, although there are many proposed mechanisms, including a reduction of T helper (T_H) cell 1 pathology [73,74], inhibition of T_H17 differentiation [75,76] and increased production of regulatory cytokines [77]. Importantly, around two thirds of patients do not respond to treatment [78], and in some individuals IFNβ can exacerbate clinical symptoms [79]. A proposed explanation for this is that IFNβ therapy is only effective in MS patients with a T_H1- but not a T_H17-driven disease. In the experimental model of MS, IFNβ reduce symptoms induced by T_H1 cells but exacerbates disease induced by T_H17 cells [80]. Additionally, high serum levels of IL17F, produced by T_H17 cells, is present in RRMS patients who do not respond to IFNβ. IL7 serum concentrations are associated with MS driven by T_H1 cells [81]. IL7 is thus proposed as a potential biomarker to identify MS patients who would benefit from IFNβ therapy.

Natalizumab, a humanized monoclonal antibody directed against the α4-integrin of the adhesion molecule very late antigen 4 (VLA-4) on leukocytes, is designed to block the migration of potentially damaging immune cells over the BBB into the CNS [82,83]. Recently, an oral therapy has become available to patients, known as Fingolimod [84,85]. Current approved therapeutics also includes corticosteroids to reduce inflammation [86] and the immunosuppressant Mitoxantrone.

The available drugs and DMDs in final-stage development for RRMS act by modulating immune aspects of MS. However, they do not halt disease, induce the repair of damaged tissue, or revert disease progression [20]. There is endogenous remyelination to repair axonal damage as evident in so-called shadow plaques,

although this process appears to slow down as the disease progresses. Better understanding of the pathogenic mechanisms involved could help identify new approaches for currently unfulfilled needs in neuroprotection and repair.

1.3 EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS

1.3.1 EXPERIMENTAL MODELS OF HUMAN DISEASE

Experimental models can be useful to overcome some of the confounding effects and other challenges encountered in human studies. For genetic studies in animal models, one can overcome the heterogeneity by generating genetically identical individuals. The sample size is also virtually unlimited and the environment can be controlled. An identified risk gene does not have to be identical in animals and humans, but it can contribute to a pathogenic mechanism of importance in both an experimental model and human disease. The models also provide opportunities for altering the genome, for instance through congenic breeding or gene targeting. An important advantage is the accessibility to relevant tissues to study, especially within the target organ (the CNS in MS), which is difficult to sample. CSF from patients is routinely collected and is used as a surrogate for the target organ. However, it is difficult to say if the cells and proteins in CSF accurately reflect events within the spinal cord and brain tissue. The kinetics of pathogenic mechanisms, including early events before disease onset (before diagnosis in the corresponding human disease) can be studied in detail in a model, as achieved in this thesis.

1.3.2 EAE MODEL

The most widely used model to characterize the genetic basis and disease mechanisms of relevance for MS is experimental autoimmune encephalomyelitis (EAE). EAE has been important for establishing fundamental understanding of inflammatory mechanisms [87]. The model was discovered in the 1930s when complications of a Rabies vaccine were investigated in primates [88]. EAE can actively be induced in other species including rats, mice, guinea pigs and rabbits [89]. There are several models of EAE, each developed to mimic various disease courses (relapsing-remitting and progressive forms) and pathogenic mechanisms of MS (Figure 3). These models depend on which specific CNS antigen and adjuvant is injected and on what genetic background. In rats, the myelin basic protein (MBP)-induced EAE, with the Freund's adjuvant containing mineral oil and *Mycobacterium tuberculosis*, is acute and resolves

after the first disease bout [90]. The injection of myelin oligodendrocyte glycoprotein (MOG)$_{35-55}$ peptide in C57BL/6 mice induces a chronic form of EAE [91]. Proteolipid protein (PLP) peptide immunization in SJL mice induces a relapsing-remitting disease course [92]. Passive immunization of EAE can also be achieved through transfer of myelin-specific autoreactive T cells, giving rise to transient demyelination and motor impairment [93]. While EAE does not occur spontaneously there are transgenic mice harboring CNS-antigen-specific T cells or B cells. These mice will develop spontaneous EAE [94-96].

MOG-induced EAE in rats mimics MS in many respects and is the most extensively used model in this thesis. In this model immune cells infiltrate into the CNS, with ensuing demyelination, an autoantibody response and axonal damage, and a relapsing-remitting clinical course [97,89]. Benefits of using a rat model compared to a mouse model include larger animal (tissue) size and milder induction protocols without the need of *Mycobacterium tuberculosis* or pertussis toxin (used to induce BBB permeability and to skew T effector cells towards a T_H17 response [98,99]).
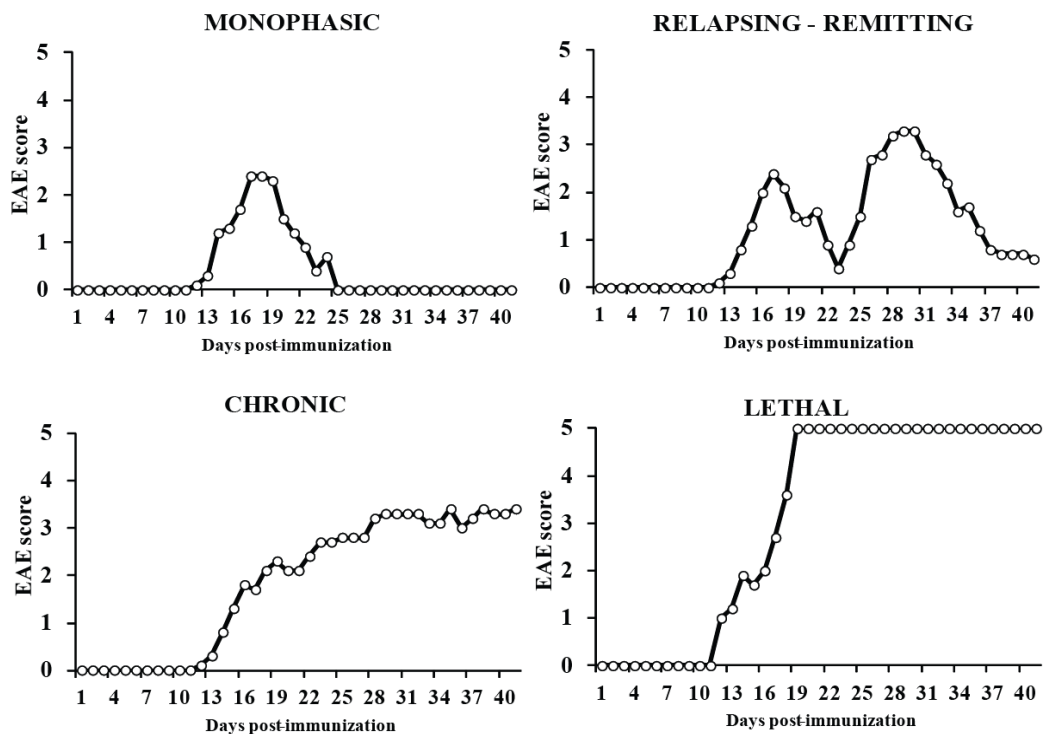

Figure 3. Representative graphs of various clinical disease courses observed in the EAE model. The y-axis represents EAE severity, graded as follows: 0, no clinical signs of EAE; 1, tail weakness or tail paralysis; 2, hind leg paraparesis or hemiparesis; 3, hind leg paralysis or hemiparalysis; 4, tetraplegia or moribund; 5, death. The x-axis represents days after immunization.

Studies in this thesis focus on the relapsing-remitting model of EAE in inbred Dark Agouti (DA) rats, induced by immunization with rMOG and incomplete Freund's adjuvant [97]. Following a subcutaneous immunization local antigen presenting cells (APCs) take up the emulsion and migrate to secondary lymph nodes where they activate autoreactive T cells. These T cells migrate to the CNS by crossing the BBB with the help of adhesion molecules [100]. Next, the cells are reactivated by resident APCs and this is followed by the release of various proinflammatory cytokines. This activates resident cells and increases the attraction of additional effector cells such as macrophages that primarily destroy myelin [89]. Clinical signs of disease begin around 10-12 days post-immunization (p.i.), corresponding to infiltration and demyelination in the CNS [97,101], and are usually recorded until 35-40 day p.i..

While DA rats display a worsening of disease over time, with neurological deficits and ascending paralysis, the major histocompatibility complex (MHC) identical inbred Piebald Virol Glaxol (PVG.1AV1, hereby referred to as PVG) strain is relatively resistant to the same induction protocol (Figure 4). The varying EAE-susceptibility demonstrates a difference originating from the genetic background, a variation in non-MHC EAE risk genes. These differences are used in our studies to identify the disease risk genes, and to characterize how they functionally influence EAE susceptibility. MOG-EAE is a model that displays many disease characteristics similar to MS. However, it is important to recognize that there are indeed differences. These include EAE mainly being $CD4^+$ T cell-driven, whereas in MS lesions, $CD8^+$ T cells outnumber $CD4^+$ T cells [102,103]. Additionally, the balance of T_H1 and T_H17 cells may be critical in EAE although their effect in MS pathogenesis is less clear [104].
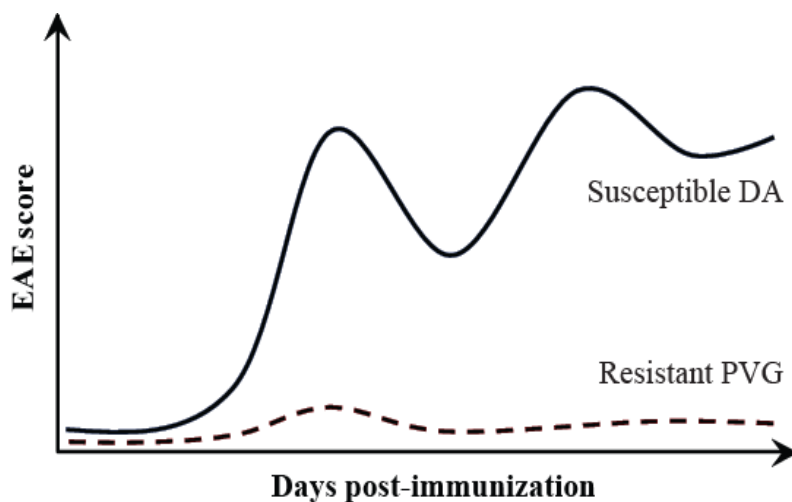

Figure 4. The DA and PVG rat strains have inherent differences in susceptibility to the relapsing-remitting model of MOG-EAE. In DA, clinical signs begin 10-12 days after immunization while PVG rats are relatively resistant to the same induction protocol.

1.3.3 GENETICS OF EAE

There are great similarities in the genetic regulation of EAE and MS. The major genetic determinant of EAE is the MHC locus [105-108], homologous to the HLA locus in humans. There is also significant influence from non-MHC genes, at least 50 genetic regions regulating EAE in rodents [101,109-117]. There is a significant overlap of these genes between EAE and MS [118,119], and this reflects that the polygenetic nature of MS is also captured in EAE. We focus on the identification of disease-regulating non-MHC genes to ultimately better understand pathogenic mechanisms of EAE and MS. For gene identification we take advantage of the difference in EAE-susceptibility of the DA and PVG inbred rats. Various experimental crosses between these strains help identify genetic regions that regulate disease phenotypes. This strategy has helped us in the past to identify a number of genes involved in the pathogenesis of EAE and MS. This includes several β-chemokines [120,121], the MHC Class II transactivator (*Ciita*) [122,123], repulsive guidance molecule A (*Rgma*), *Il21r* [124] and *Vav1* [125]. Furthermore, there are numerous candidate disease-regulating genes currently under investigation in our lab.

1.4 TRANSCRIPTOMICS

When performing experimental crosses or studies in congenic lines we often use susceptibility and severity of disease as read-outs to identify causal genetic variations. For significant differences to be observed the underlying genetic variation has to be strong, which is not always the case. Other than having well-powered experiments in our genetic studies, other phenotypes can be used to determine a genetic variation influencing pathogenic mechanisms. Based on the hypothesis that clinical disease represents an outcome of earlier events, the measurement of messenger ribonucleic acid (mRNA) transcript levels, protein levels or various cellular phenotypes, which are less complex, can be employed. Using mRNA as a marker for immune responses, for instance the expression of inflammatory genes, has the benefits of ease of tissue handling, reproducibility, sensibility and the possibility to handle large data sets.

Genes are encoded by coding sequences in deoxyribonucleic acid (DNA). A gene encodes mRNA molecules (Figure 5). The starting point for transcription is the promoter region located upstream of the gene. Upon transcription, a complex assembles at the promoters. In the ribosome, the mRNA sequence is read and translated into amino acids that build up proteins.
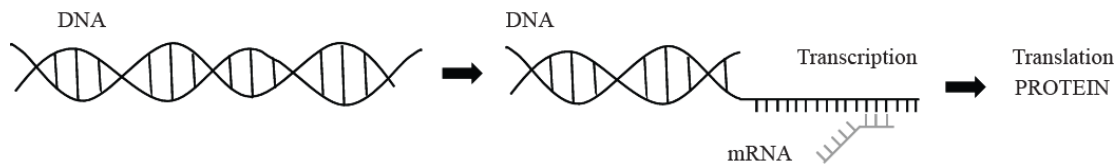
Figure 5. Simplified schematics of the central dogma of molecular biology. Transcription factors unwind the DNA strand and allow the enzyme RNA polymerase to attach to the promoter region and transcribe mRNA. Mature mRNA is translated to protein in the ribosome.

All studies in this thesis include measurement of clinical phenotypes and determination of mRNA levels to define disease-regulating mechanisms. Gene expression in the presented studies has been defined by using Affymetrix arrays and real-time quantitative polymerase chain reaction (qPCR). The Affymetrix technology uses hybridization techniques and qPCR utilizes fluorescent signals upon detection of target mRNA [126-128]. The microarray technologies have made it possible to study the expression of genes at a whole-genome level. In study III we used Affymetrix Gene Arrays to explore the expression of all genes across the genome, in order to further explore if genes or network of genes correlated with clinical EAE phenotypes. Splenic tissue was therefore collected for expression analysis at day 35 p.i.. In study I we used Applied Biosystems TaqMan Low Density Arrays (TLDAs), a qPCR methodology, in order to study expression of pre-selected genes [129]. qPCR analysis in this thesis has been used for a relative expression, where a target gene is related to a reference gene to minimize noise and experimental variance between samples.

The process of 'DNA makes RNA makes protein' is strongly regulated at all levels. There are mechanisms of enhancing or silencing transcription. In addition, the true effector molecule is the protein and not all mRNA is translated. There are endogenous mechanisms to control for RNA levels, for instance mRNA degradation pathways or mechanisms preventing mRNA from being translated. miRNAs are post-transcriptional regulators and ongoing research in our lab demonstrate a role of these in regulating pathogenic responses of EAE. The use of several methods to confirm a true effector molecule is thus essential.

1.5 IMMUNE CELLS AND CYTOKINES

Various immune cells have been described as being important in the pathogenesis of MS. We do not fully understand how disease is regulated, and there is continuous effort

to evaluate the protective or detrimental effects of cell types in pathogenic mechanisms. Genetic studies have demonstrated a causative role for the immune system in MS and EAE, and inflammation has a clear contribution to the pathology of disease [58,59]. Autoreactive T cells and antibodies are detected in the blood and CSF of patients, as well as in experimental models [20,32,33,130,131]. Together with the fact that immunomodulatory drugs are effective in disease treatment [20,132,133] this strongly suggests that MS is an immune-mediated disease.

1.5.1 IMMUNE CELLS

Several observations, including the major genetic risk factor in both MS and EAE being the HLA/MHC locus, have established the adaptive immune response as being central for the experimental model and human disease. Additionally, transfer of myelin antigen-specific T cells can induce EAE [134] and T cell infiltrates are evident in both MS and EAE lesions [34,135-138].

The most studied T cells in EAE are $CD4^+$ T cells, and most EAE models are $CD8^+$ T cell-independent. However, in MS lesions, $CD8^+$ T cells outnumber $CD4^+$ T cells, suggesting their involvement in pathogenesis [139]. $CD8^+$ T cells may be a main mediator of neuronal damage as they can directly kill oligodendrocytes [140]. Nevertheless, $CD8^+$ T cells have been attributed both disease-promoting and -protective roles in MS [141,142] and evidence from EAE studies has suggested a disease protective role [143]. In addition, there is less mortality but more relapses in CD8 knockout (KO) mice [144] and populations of $CD8^+$ regulatory T cells are enhanced during the remission phase in EAE [145].

During recent years several $CD4^+$ T_H lineages have been described, which have added a complexity to the earlier 'T_H1/T_H2' balance hypothesis. New concepts have challenged the previous paradigm and have enabled a better understanding of how immune mechanisms can function in EAE. Nonetheless, how the balance of these subsets influences immunopathogenesis of diseases such as MS is still under investigation.

Naïve $CD4^+$ T cells can differentiate into a variety of effector cells, depending on the local cytokine environment (Figure 6) [146-150]. Lineages include IFNγ- and TNF-producing T_H1, IL17-, IL21-, IL22- and granulocyte-macrophage colony stimulating factor (GM-CSF)- producing T_H17 cells, as well as T regulatory cells (Tregs). Both T_H1

and T_H17 can induce EAE; however, their combination and relative quantities will determine distribution of CNS infiltration [104,134]. In addition, there is plasticity in lineage choice [151] and Tregs, which are often $CD4^+CD25^+FoxP3^+$, can convert into pathogenic cells if not appropriately epigenetically controlled [152].

Differences in T cells could predispose to MS susceptibility, and there are several lines of evidence for this. Autoreactive T cells from patients have an increased inflammatory capacity [42,43]. Additionally, genetic variants in *IL7R* and *IL2RA*, associated with MS, affect T cell development, proliferation and Treg function [153,154]. The functions of Tregs, which maintain peripheral tolerance by suppressing autoreactive T cells [87], are impaired in MS patients compared to in healthy controls [154-156].
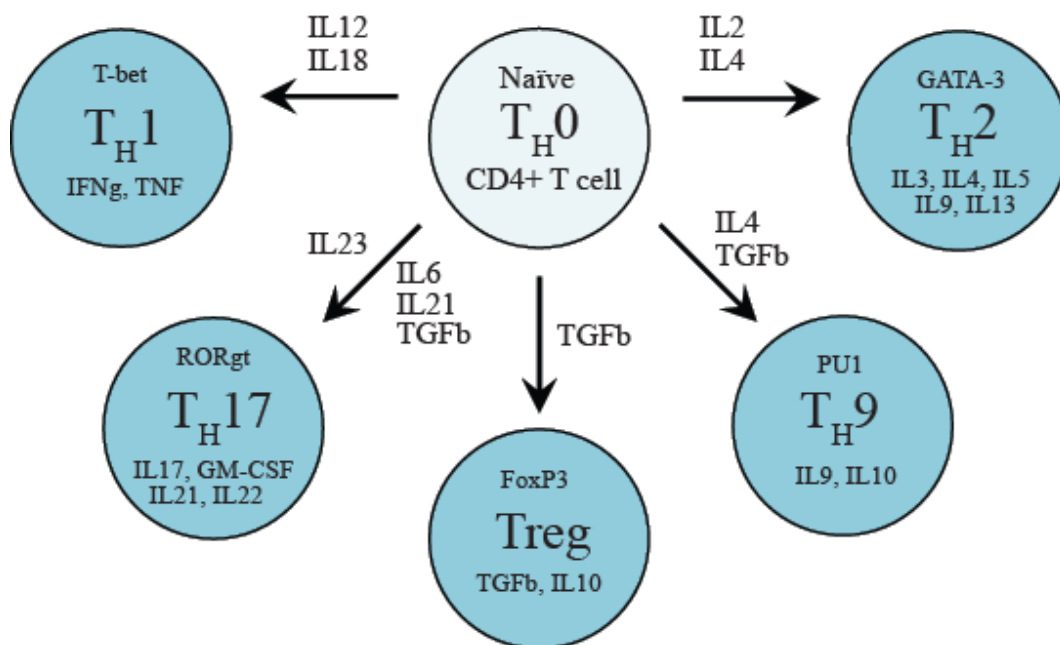
Figure 6. Differentiation of naïve $CD4^+$ T cell precursors (T_H0) into various effector cells. The cell lineage differentiation is controlled through several regulatory factors, such as specific transcription factors and the local cytokine milieu. The effector cells have specialized functions and a distinct pattern of cytokine production. Imbalance can lead to immunopathogenesis and diseases such as MS.

While MS is regarded as a T cell-mediated disease, there are many other cell types involved in disease. B cells contribute to MS pathogenesis through antigen presentation and production of autoantibodies [157,158]. Autoantibodies are present in most demyelinating lesions [159]. Myelin-specific B cells are present in the periphery and in CSF [30] and there is evidence for B cell migration between the blood and CSF compartments, especially during MS relapses [160]. Recently, demyelinating and

axopathic autoantibody responses have been identified in a subset of MS patients. These observations could explain why some patients benefit from antibody depleting treatments [161]. Conversely, there are reports suggesting disease-ameliorating functions of B cells [162,163].

Both innate and adaptive immune responses have been associated with EAE. Infiltrating macrophages and activated CNS-resident microglia are the CNS effector cells which are primarily responsible for destroying CNS tissue, including stripping the myelin from neurons in MS [164-166]. Both macrophages and microglia can present antigen, phagocytose and produce cytokines [167]. Macrophages are recruited to sites of injury and produce inflammatory cytokines [139,164]. However, there are different subsets of macrophages, of which some also have roles in the resolution of inflammation [168].

Dendritic cells (DCs) are professional APCs, and these accumulate in the CNS upon inflammation. They produce various cytokines that can determine if naïve $CD4^+$ T cells differentiate into suppressor or effectors cells [169,170]. Depletion of natural killer (NK) cells in EAE leads to worsening of disease, and decreased numbers of NK cells have been observed in MS patients [87]. They have been attributed both disease-protective and -pathogenic roles in the past. However, recent reports, including evidence from our lab, point to their main disease-protective effect in both MS and EAE [171,172]. Additionally, the role of NK T cells [173] and $\gamma\delta$ T cells [174] are being elucidated.

1.5.2 CYTOKINES

Cytokines, which are molecules that transfer signals between cells to shape an immune response, have been implicated to play a role in MS pathogenesis [175]. In study I of this thesis we identified a differential expression of cytokines characteristic of the T_H1 and T_H17 immune response between the susceptible DA and resistant PVG strains.

The main producers of IFNγ, a type II interferon, are T cells and NK cells [176]. IFNγ has been attributed both disease-protective and -promoting roles in autoimmunity, and MS patients display elevated IFNγ production in the CSF [177]. IFNγ induces T_H1 differentiation, activation of innate immune cells and MHC expression [176,178-180]. Treatment with IFNγ in MS worsens disease [181], while IFNγ deficient mice develop severe EAE [182]. Type I interferons, INFα and IFNβ, are produced in response to viral infection [183], and IFNβ is a first-line treatment for MS.

Tumor necrosis factor (TNF) is central in immune mediation. The cytokine regulates a range of biological processes, including cellular differentiation and expansion, apoptosis and inflammation [184]. Elevated levels of TNF are observed in MS and RA patients and in septic shock [185-187], while decreased levels associate with infections [188]. Blocking TNF has proven successful in RA and Crohn's IBD therapy [189,190], while this exacerbates MS disease [191]. This illustrates a dual role of TNF [192].

IL18 is a member of the IL1 superfamily and promotes T cell, macrophage and NK cell inflammatory responses [193,194]. It has been associated with the activation of $CD8^+$ T cells and T_H1 cell differentiation [195,196]. IL18 is up-regulated in MS patients compared to in healthy controls [197-200]. IL18 is not essential for EAE initiation, but may still play a role in disease [194,201]. IL18 signals through the IL18 receptor heterodimer, which is part of the Toll/IL1 receptor (TIR) superfamily. It consists of an IL18-specific IL18Rα, which is encoded by the *IL18R1* gene, and IL18Rβ, a common signaling subunit. The IL18R1 is expressed on macrophages, astrocytes, NK cells, T cells and DCs [196]. Activation of the receptor activates NK cells and induces T_H1 and T_H17 differentiation [193,194]. The cytokine receptor was recently described to regulate EAE via both IL18-dependent and -independent mechanisms [194].

1.6 AUTOPHAGY AND IMMUNITY

Autophagy is a degradation pathway that regulates the quantity and quality of cytoplasmic content. Autophagy has long been recognized as a pathway in response to nutrient deprivation in order to maintain energy homeostasis [202]. However, autophagy can additionally be a response to endoplasmic reticulum (ER) stress, immune cell signaling and oxidative stress [203,204].

1.6.1 AUTOPHAGY

Autophagosome biogenesis is initiated by the formation of an isolation membrane that elongates. Cargo is randomly or selectively engulfed and the two membrane ends will thereafter fuse to form a double membrane structure termed the *autophagosome* (Figure 7). Next, the vesicle fuses with a lysosome to form an autophagolysosome, and this leads to the degradation of the cargo [205]. It is unknown where the autophagosomal membrane originates from, although proposed sources include the plasma membrane, the ER and the outer mitochondrial membrane [206]. The pathway

engages over 30 autophagy-related proteins (Atgs) [207] and autophagic complexes with distinct functions in autophagosome nucleation, assembly, and maturation have been identified. The covalent conjugation of Atg5 to Atg12, which subsequently form part of a large complex with Atg16l1, is necessary for membrane localization of the autophagic machinery [208] and autophagosome formation. Atg7, which has been the focus of study IV in this thesis, is required for this conjugation, and is therefore essential for autophagy [209].
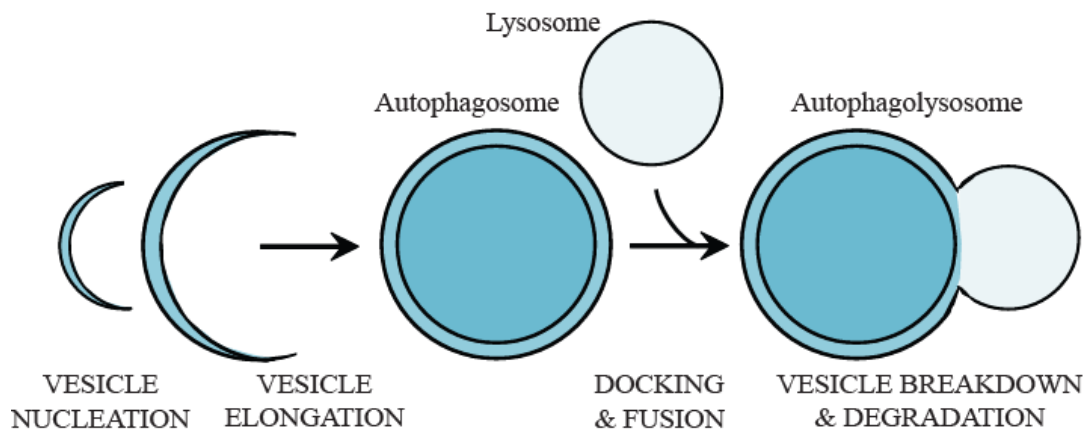

Figure 7. Autophagy involves the formation of double membrane vesicles called autophagosomes. These vesicles engulf proteins and organelles and deliver them to lysosomes for degradation. Adapted figure [210].

The regulation of autophagy in changing environments such as during cytokine signaling and the presence of pathogens is still being elucidated, but likely involves complex crosstalk between pathway components. Research on autophagy has increasingly begun to focus on how perturbations in the pathway could contribute to immune response and inflammation.

1.6.2 AUTOPHAGY IN IMMUNITY

Expanding roles for autophagy genes, in both autophagy-dependent and -independent processes have been demonstrated [211], although substantial questions remain regarding specific mechanisms. Many processes have effects on the immune system and the roles of autophagy bridge both innate and adaptive immunity [212,213]. Autophagy is implicated in neurodegenerative disorders and autoimmunity and defects in the pathway can lead to increased susceptibility to infection [214,215]. Functions of autophagy also include MHC class II cross-presentation of endogenous antigens in which autophagosomes can play an active role [216,217]. Regulation of the inflammasome and intracellular pathogen sensing [218-220], as well as the clearance of

apoptotic corpses [221], are further roles of autophagy in immunity. In specific cell types the impaired expression of autophagy genes can result in enhanced proinflammatory cytokine production [222]. Furthermore, studies of conditional KO mice, in which T cells are deficient in autophagy proteins have emphasized the importance of autophagy in thymic selection [223,224], promotion of T cell homeostasis (including their survival and proliferation) and control of ER homeostasis [225-228]. Autophagy proteins also play a role in B cell development [229] and survival, as deletion of *Atg7* in the hematopoietic system of mice results in decrease in peripheral T and B lymphocyte numbers [230].

1.6.3 AUTOPHAGY IN DISEASE

Dysregulation of the autophagy pathway has been implicated in numerous human diseases including autoimmunity, neurodegenerative disorders, infections and cancer [231]. Genome-wide association studies (GWAS) have revealed gene variants that predispose for Crohn´s IBD. These variants are found in the essential autophagy protein Atg16L1 and in the autophagy-associated proteins IRGM and NOD2 that are important in antibacterial responses [232]. Suggested functional outcomes of these single nucleotide polymorphisms (SNPs) are defects in bacterial clearance, impaired antigen presentation through MHC class II and impaired regulation of proinflammatory cytokines. Additionally, GWAS studies in SLE, a systemic autoimmune disease, have found SNPs in *ATG5* associated with disease susceptibility [233-235], although the functional outcomes are unknown.

Abnormal autophagic activity has been observed in common neurodegenerative diseases such as Alzheimer's disease and Huntington's disease [236] and it is established that loss of *Atg5* or *Atg7* in the CNS leads to neurodegeneration in mice [237,238]. Rapamycin, an immunosuppressant drug that can induce autophagy in neurons, has been suggested as a therapeutic agent for peripheral myelin protein 22-associated demyelinating neuropathies [239]. Stimulation of autophagy by rapamycin also protects neurons from degeneration, and promotes remyelination following acute focal brain damage in mice [240]. In MS, several autophagy genes, including *ATG5* and *ATG16L1*, are more highly expressed in the CSF of MS patients compared to in controls [241]. There is one study suggesting that expression of *Atg5* may contribute to inflammatory demyelination in MS [242]; however, autophagy has not been well studied in MS. A detailed insight into the function of autophagy in health and disease may help in development of novel strategies to treat inflammatory disorders.

2   THE PRESENT INVESTIGATION

2.1   METHODOLOGY

Detailed descriptions of methods used in the presented studies I-IV are included in the respective Methods sections.

2.1.1   CASE-CONTROL COHORT

For many years the HLA was the only genetic region linked to MS with a relative risk increase, odds ratio (OR), of around 3 [243,244]. Using linkage analysis, the search for co-segregation of phenotype with a marker linked to a risk gene, it has been difficult to identify any non-HLA risk loci [245-247]. However, this has changed with technical advancements and using GWAS, in which the aim is to compare frequency of genetic variants between patients and unrelated controls (non-affected individuals). In 2007 the first GWAS results in common diseases [3] and MS [56] were published identifying the first non-HLA genes associated with MS. Numerous additional non-HLA genes have now been identified, more than 50 genetic variants being associated with MS susceptibility to date [57]. In these studies association is tested between genetic markers, usually SNPs, across the genome and disease in a case-control cohort. The number of required cases and controls is determined by the frequency and expected effect of risk genes [248]. The latest GWAS conducted by the International MS Genetic Consortium (IMSGC) included around 10,000 cases and 17,000 controls and over 450,000 SNPs were available for analysis.

The design of association studies is based on the common-disease common variant hypothesis (common variant defined as having minor allele frequency >1% in the population) where the combination of allele variants, each with weak effects, contribute to disease [1,249]. Advantages of association studies include more fine-localization of a disease-casual variant. Identified associated SNPs may constitute true disease causative variations or be surrogate markers used as location markers. Linkage disequilibrium (LD) is the non-random association of alleles at two or more loci [250]. If SNPs are in LD, indicating that they are likely to co-segregate, it may be possible to genotype only one of them to limit the number of markers tested. This is referred to as *tagging*. Identified risk alleles in MS GWAS have weak ORs and together explain only a fraction of

disease heritability and variance [57]. It is likely that additional risk variants that confer small effects also contribute to heritability. Additionally, association studies do not take into account the rare alleles with strong effects or the interaction between genes or genes and environment [251]. Future studies should aim at discerning the functional outcomes of risk-associated variants.

2.1.2 LINKAGE MAPPING

To identify regions in the genome, and ultimately genes, which regulate EAE, we perform linkage studies in experimental crosses of inbred strains that differ in their susceptibility to disease [252] (Figure 8). A phenotype, may it be clinical symptoms, gene expression or IgG titers, can be mapped as a quantitative trait. The crossing between susceptible DA and resistant PVG strains results in the generation of a genetically heterogeneous population. The goal is to identify genomic regions in which allelic variation is associated with phenotypic variation. Once the genotype and phenotype of interest have been ascertained for individuals in the population we use linkage analysis, whereby disease phenotypes are linked to genetic inheritance of regions of the genome.

The objective of linkage analysis is to define markers that are linked to the phenotype in order to define quantitative trait loci (QTLs) where the disease genes are located. We use microsatellite markers, which are di-, tri- or tetranucleotide repeats, to identify different inherited alleles from the strains that have been crossed. If a marker correlates with a disease phenotype it will lie close to the causative loci. The closer the marker is, the larger the difference between the two alleles will be where the maximum difference will be reached when the marker coincides with the QTL for the trait. We use the logarithm of odds (LOD), given as log value with the base of 10, as the measure for confidence of linkage. A higher LOD gives higher certainty that a genetic locus regulates the clinical phenotype of interest. As an example, with a LOD of 5, a QTL is 100,000 times more likely to be truly linked than not.

Crosses used in study I, III and IV include an N_2 backcross (BC) and an advanced intercross line (AIL) of the 10th generation (G_{10}) (Figure 8). To create the BC, F_1 individuals were crossed with the inbred DA strain. To create the AIL, an F_2 intercross was generated by breeding of F_1 individuals, followed by continuous breeding for several generations [253].
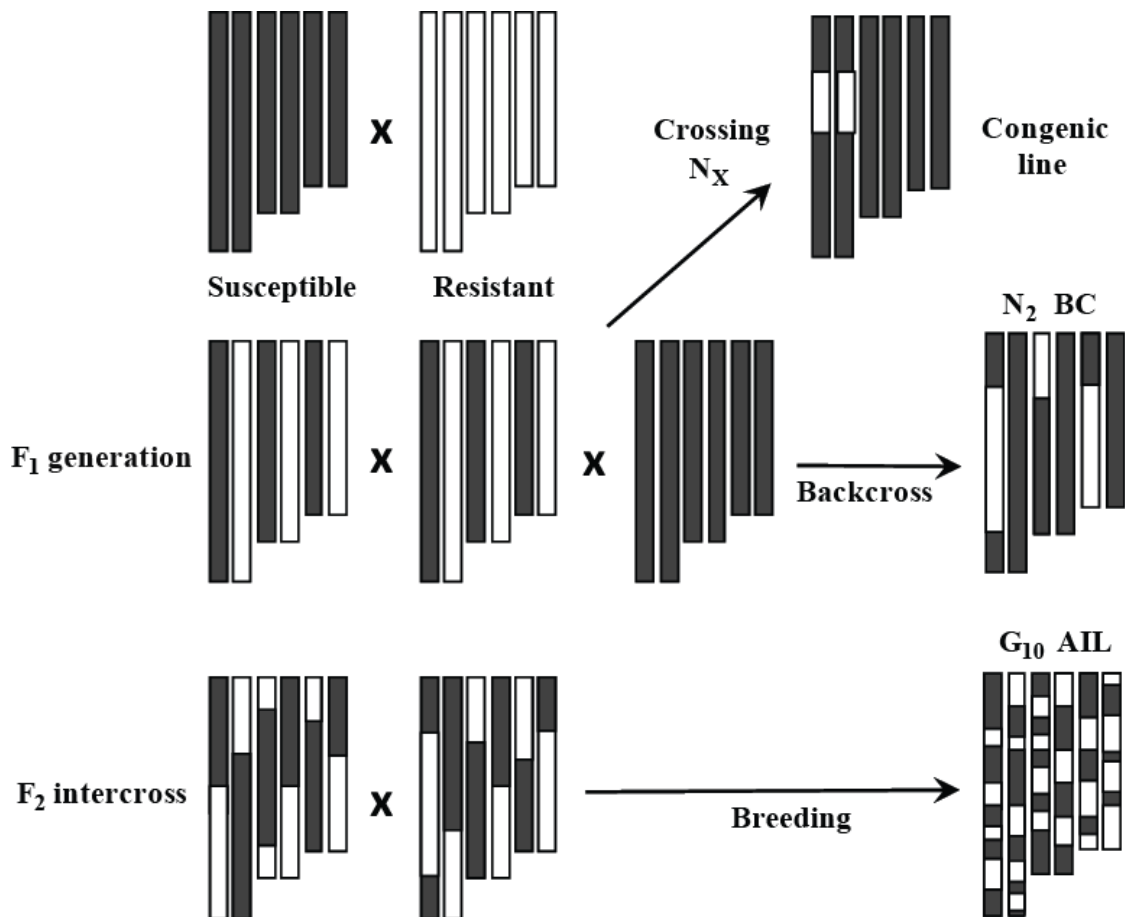
Figure 8. By breeding EAE-susceptible DA and EAE-resistant PVG parental strains various experimental populations can be generated and used for genetic mapping of phenotypes. In a first step, a heterozygous F_1 progeny is obtained. The F_2 is generated by crossing F_1 individuals, or alternatively, a backcross (N_2) is generated by crossing F1 with either parental strain (in this thesis with the susceptible DA). Congenic lines, which are valuable tools for functional studies, are generated by a similar backcrossing for several generations with the selection for a region of interest. An AIL (G_{10} used in this thesis) is produced by the intercross of F_2 individuals for several generations.

In F_2/N_2 populations the QTLs typically comprise several hundreds of genes, thus making it difficult to point to the causing variant. Additionally, a larger QTL can harbor several QTLs [254]. Because of a greater number of recombination events, linkage mapping in an AIL enables a higher resolution of loci that regulate phenotypes compared to linkage mapping in an F_2 populations or a BC (Figure 9). Alternatively, a heterogenous stock with mapping resolution exponentially higher than an AIL can be used [255,256].
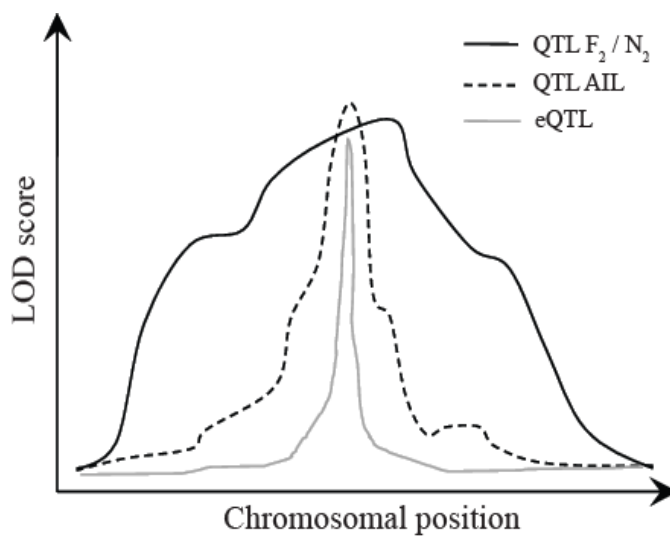
Figure 9. Comparison of the QTL region obtained when mapping phenotypes in an F_2 / N_2 or an AIL. The QTL and candidate gene list can be considerably reduced by using the strategy of fine-mapping. With an eQTL approach and the mapping of *cis*-regulated transcripts, one can further identify the plausible candidate gene/s underlying the trait QTL effect.

2.1.3 CONGENIC STRAIN

To confirm a QTL biologically we often use a congenic approach. The congenic strain is also used to explore the functional role of the genes within the isolated QTL region (study III). The breeding of congenic animals includes backcrossing for several generations, and we have used a marker-assisted speed-congenic approach to this end [257] (Figure 8). A congenic strain was established for the EAE-regulating QTL *Eae34* on rat chromosome (RNO) 13 (study III). We isolated the genomic region by transfer of PVG alleles from the locus onto the genetic background of the DA strain. The phenotypic difference between the congenic line and the parental DA strain will result from the effect derived from the isolated region. The advantages of using a congenic strain compared to KO strategies include the comparison of different natural variants as opposed to a presence or absence of a specific gene. The former approach has frequently been used in our lab and has helped in the isolation of genes and resolution of pathogenic mechanisms in neuroinflammation. This includes *Vav1* [125], *Il21r* and *Rgma*[124], *Ciita* [122,123], the genetic regulation of TNF production in macrophages that in turn regulates inflammation [258] and the balance between splice variants of *Zeb1*[259]. Through functional experiments in the congenic lines, the importance of each candidate gene in the region can be evaluated and ultimately the gene responsible for the EAE phenotype can be identified. Recent studies in our lab using a congenic approach have revealed the genetic regulation and importance of epistasis and NK cells, chemokines, lipid peroxidation and protein modification and anti-MOG antibody response in EAE pathogenesis.

2.1.4 CONDITIONAL KO

Conditional gene targeting in mice can be used to study the impact of a gene in a cell type-specific way [260]. We applied this approach and studied *Atg7* deficiency in T cells and its impact on EAE (study IV). To generate the conditional KO we used the Cre-*loxP* system [261-263] (Figure 10). Two transgenic mouse lines are required for a recombinase-dependent conditional genetic experiment. One mouse line expresses the recombinase Cre in a selected cell lineage or tissue; in our study *Lck-Cre* transgenic mice were used [264]. The other, *Atg7*[Flox/Flox][265], carries the conditional allele, an exon flanked by two *loxP* recognition sites. After intercrossing the two lines the cells in the offspring expressing the recombinase will have a deleted conditional allele, while the target gene remains intact and functional in all other cells and tissues.
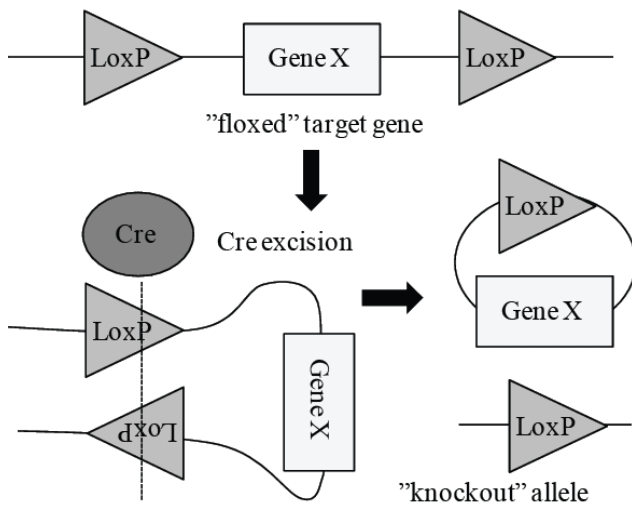

Figure 10. Simplified schematics of conditional gene targeting. The box represents an exon of the gene of interest (*Atg7* in study IV). In cells expressing Cre (T cells in study IV) the gene fragment in-between two LoxP sites will be excised, resulting in cell-specific deletion of the gene.

KO of a gene in a cell type-specific or inducible way is a powerful technology for analyzing gene function and can help circumvent limitations of conventional gene inactivation. A gene can have multiple roles at different time points and in various tissues. The technique is especially useful if the conventional gene KO leads to a lethal phenotype, as in the case for *Atg7*[265], or if the phenotype affects multiple tissues.

2.1.5 EXPRESSION QTL APPROACH

Natural genetic variation contributes to variation in gene expression and identifying determinants of gene expression may give insights into pathogenic mechanisms of complex traits. The regulation of expression as a quantitative trait can be characterized by expression QTL (eQTL) mapping introduced in 2001 [266-269]. Linkage analyses in experimental animal crosses have detected numerous QTLs related to clinical traits of complex diseases, although it has been challenging to define single quantitative trait

genes [270]. The eQTL approach can facilitate identification of genes and genetically driven networks of genes important for regulation of complex diseases [271-275]. eQTLs influence expression of transcripts either in *cis* or in *trans*, where *cis*-acting eQTLs are located in close proximity of the target gene itself, while *trans*-acting eQTLs are located in a region distant to the target gene[276]. *Cis*-regulation could be explained by a variation in DNA sequence in the regulatory regions of the target gene [277] and a *cis*-eQTL overlapping a trait QTL could be considered a likely causal gene underlying the disease QTL. In study III we mapped eQTLs in a BC during the chronic phase of EAE and characterized several *cis*-regulated transcripts as positional candidate genes for known EAE QTLs. To rule out false positive *cis*-regulated transcripts due to technical artifacts (e.g. hybridization differences) [278] we confirmed the linkage using qPCR.

2.1.6   NETWORK ANALYSIS

One way of characterizing the great amount of information yielded in genome-wide eQTL analysis is to build networks. The emphasis lies on describing pathways as opposed to individual genes and this exercise permits identification of characteristics within the data set that may not be obvious and could otherwise go unnoticed. A strong correlation between expression levels of genes suggest that they might participate in similar biological processes or are regulated by similar mechanisms. We utilized the Weighted Gene Co-Expression Network Analysis (WGCNA) method to construct gene networks [279]. Moreover, the Pearson correlation coefficient was used to determine eight gene networks that significantly correlated with EAE phenotypes. There are several analytic tools to infer functional properties for these networks of genes or to explore the role of genes with yet unknown molecular function. The eQTL data set, including disease-correlated networks and positional candidate genes with their respective correlated transcripts, was described using Ingenuity Pathways Analysis (IPA).

2.2   RESULTS AND DISCUSSION

Findings presented in this thesis (studies I-IV) include the characterization of genetically predisposed pathogenic mechanisms in neuroinflammation, the identification of candidate genes that may play important roles in regulating these mechanisms, and the validation of MOG-EAE as having similar pathogenic mechanisms to those operative in MS.

2.2.1 MOG-EAE IS REPRESENTATIVE OF MANY ASPECTS OF MS

Substantial progress in deciphering genetic variants contributing to MS susceptibility has been made, especially with the recent GWAS. These studies demonstrate that the immune system plays a causative role in MS pathology, as genetic determinants in immune genes predispose for disease susceptibility. However, knowledge of the functional outcomes of these candidate genes requires further studies, which may be difficult in humans. Validation of the MOG-EAE model for relevance to MS is crucial so that functional studies can be carried out in the appropriate model.

MOG-EAE mimics MS in many respects including the involvement of pathogenic responses of T and B cells, the clinical chronic relapsing-remitting clinical course and similar histopathology. In studies I and III we demonstrated that the genetic regulation of MS and EAE is similar. From previous studies it is known that the major genetic determinant of both MS and EAE is the HLA/MHC. In study I we assessed expression of MS candidate genes (the genes confirmed at that time) and disease mechanisms of relevance for MS in the susceptible DA and resistant PVG inbred strains. Using this approach we identified genetically driven differences in expression of several MS candidate genes. The first to identified non-HLA associated MS genes *IL2RA* and *IL7R* [53,54,56,280] were up-regulated in the lymph nodes of susceptible DA compared to resistant PVG rats (study I), which is in accordance with previous studies in humans [281,282].

Following induction of MOG-EAE the susceptible DA rats exhibited up-regulation of known MS-associated immunological pathways such as T_H1 and T_H17 in lymph nodes. Upon restimulation with MOG autoantigen the susceptible DA rats displayed augmented marker molecules of these pathways and had higher expression of effector molecules such as IFNγ (T_H1), IL17F and IL22 (T_H17) (Figure 11). DA autoreactive lymphocytes also had a higher proliferative capacity (study I). Our results support a model whereby pathogenic T_H1 and T_H17 cells are induced in the periphery and infiltrate the CNS. Humoral and adaptive responses are mounted against CNS myelin and inflammation consequently leads to demyelination and EAE induction in the susceptible rats. We characterized a genetic regulation of immune response development in DA and PVG rats following immunization of MOG.

In study III (with more non-HLA genes associated with MS at this point in time) we could identify additional similarities of shared susceptibility genes between MS and

EAE and we further defined disease correlated gene networks enriched for pathways involved in cell-mediated immune mechanisms of relevance for both EAE and MS. Our work validates the model being representative for many aspects of human MS regarding pathological mechanisms and candidate gene involvement. Studies of EAE in DA and PVG strains can give insight into genetically driven disease mechanisms of relevance for MS.
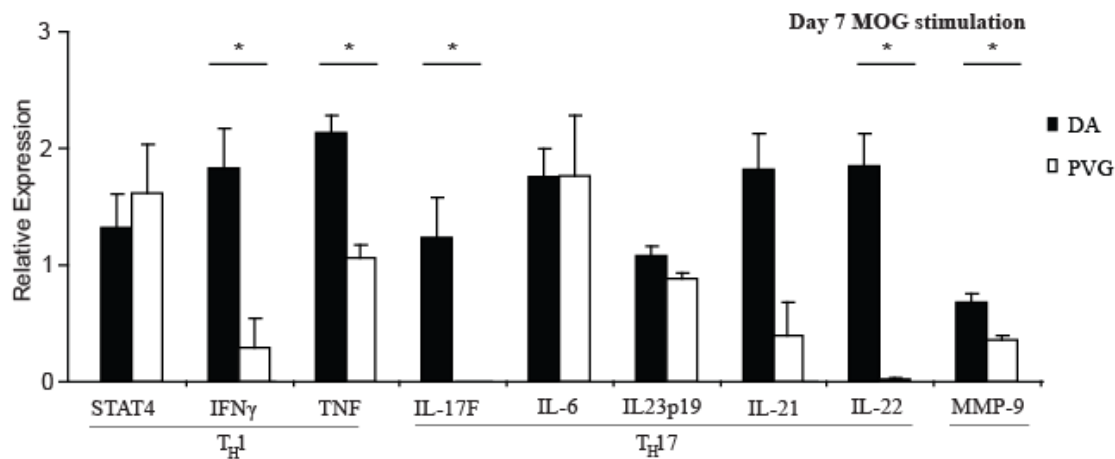

Figure 11. T_H1 and T_H17 pathways are enhanced in susceptible DA lymph nodes day 7 after MOG restimulation. DA has increased expression of IFNγ and TNF and of the T_H17 effector molecules IL17F and IL22 as well as the proinflammatory macrophage activation marker MMP9.

A translational approach can be used to evaluate candidate genes that regulate neuroinflammation in rat and humans, in order to better understand underlying disease mechanisms. We took advantage of a human biobank of peripheral blood mononuclear cells (PBMCs) and CSF cells. CSF has been used as a surrogate for the target organ while profiling of expression in PBMCs can indicate important events outside of the CNS e.g. the triggering of relapse or remission [241]. Using qPCR we determined that *IL18R1* and *ATG7* expression (studies II and IV, respectively) is dysregulated in EAE and altered in MS patients. These projects demonstrate that findings in MOG-EAE likely represent true pathogenic mechanisms of human disease.

IL18 has been studied in several autoimmune diseases and the cytokine is up-regulated in MS [197-200], although it is not of absolute necessity for disease in experimental models [194]. There is little known about the influence of IL18R1 on neuroinflammation, and we thus explored its role in MOG-EAE and MS using a translational approach. The cytokine receptor is necessary for EAE via both IL18-dependent and -independent mechanisms [194]. *Il18r1* expression was up-regulated in the susceptible DA strain

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compared to the resistant PVG during initiation of EAE (study I), indicating that related mechanism could be of importance for pathogenesis. *IL18R1* expression was increased in PBMCs and CSF of MS patients compared to controls with other neurological diseases (OND) (study II, Figure 12). However, expression of *IL18R1* did not reflect disease course or severity. An elevated *IL18R1* expression was observed in the CSF of clinically isolated syndrome (CIS) patients. Given that a majority of these patients will eventually develop MS [283], *IL18R1* could potentially serve as an early disease biomarker.
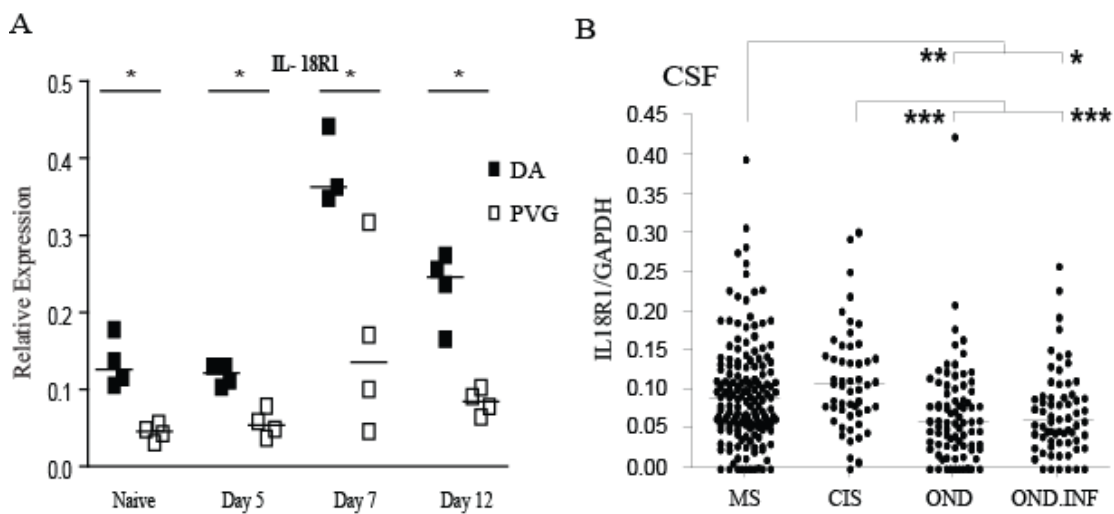
Figure 12. Increased *IL18R1* expression is evident in A) EAE-susceptible DA rats and B) MS patients.

We next tested if polymorphisms in the candidate *IL18R1* gene associated with MS, but recorded no evidence for association (study II). Although *IL18R1* genotypes do not determine susceptibility to MS, levels of the receptor may be important for disease initiation and ongoing inflammation.

Taken together, our results suggest a mechanism by which dysregulation of IL18 and IL18R1 mediates an immune activation in patients through T cell differentiation and activation and through activation of macrophages. The pathway has been targeted therapeutically, including IL18 blockade [284], IL18-binding protein delivery and caspase-1 inhibitors. However, these have not been developed into treatments due to weak and off-target effects [285,286]. Nevertheless, monoclonal antibodies against IL18Rα can effectively ameliorate EAE [194]. The increased expression of *IL18R1* in both EAE (study I) and in human disease (study II) makes it an attractive potential therapeutic target and this deserves further investigation.

2.2.2 FINDING CAUSATIVE GENES AND REGULATED MECHANISMS

Defining a genetic determinant of a distinct pathogenic mechanism can provide better understanding of the studied disease. Importantly, it can also point to potential novel targets for treatment. The unbiased approach we use to identify risk genes by establishing experimental animal crosses can detect QTLs related to clinical traits of complex diseases [109]. However, to define single quantitative trait genes can be difficult [270]. There are several methods to identify causative alleles, one of them being expression analysis, which have successfully been demonstrated in studies of neuroinflammation [122]. Associated genotypes often control expression of their own gene, as in the case of *IL2RA* and *IL7R* [54,287]. The mapping of eQTLs can aid in the identification of candidate genes [271-273]. We used this approach by combining classical QTL mapping with genome-wide expression profiling in an experimental backcross between EAE-susceptible DA and EAE-resistant PVG rat strains (study III).

We identified 60 *cis*-regulated transcripts that overlap previously described EAE QTLs in the same DA and PVG strain combination. These genes, including the major facilitator superfamily domain containing protein 4 (*Mfsd4)* (study III) and autophagy-related gene 7 (*Atg7)* (study IV), constitute *cis*-regulated positional candidate genes for the trait QTL effect (Figure 13). Further verification and biological characterization of these genes and other candidates may give insight into mechanisms dysregulated in EAE. The identified molecular pathways can thereafter be evaluated in human disease. Results presented in study III support the hypothesis that *Mfsd4* is a candidate gene for *Eae34* that influences pathogenesis of EAE.
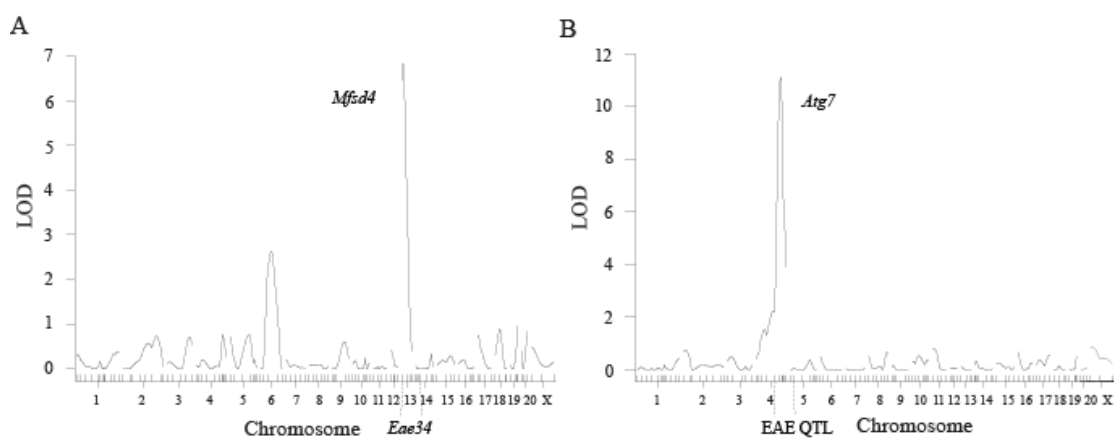

Figure 13. *Cis*-regulation of A) *Mfsd4* (study III) and B) *Atg7* (study IV) overlap EAE-regulating QTLs. A *cis*-eQTL that maps to an EAE QTL can be considered a likely causal gene for the disease QTL.

Mfsd4 is a transmembrane transporter of the major facilitator superfamily (MFS), which are secondary carriers transporting solutes by using chemiosmotic ion gradients [288,289]. There is little functional knowledge of *Mfsd*; however, we provide the first evidence of its involvement with T lymphocyte biological processes. Our combined effort of classical QTL mapping, eQTL mapping, pathway analysis and *in vitro* functional studies in a congenic rat strain that carries PVG alleles in *Eae34* demonstrated a functional effect on T cell proliferation and activation conferred by the locus. Genetic variants in *Eae34* impact the clinical course of disease and congenic rats exhibited both higher T cell proliferative and activation capacities. Congenic rats, which displayed more severe EAE compared to DA rats, also expressed lower *Mfsd4*. We identified a SNP in the promoter region that might explain the lower expression of *Mfsd4* in the DA.PVG-*Eae34* congenic rats. Overall, our data suggests that the exacerbation of autoimmune responses in congenic rats may be a result of a differential T cell expansion and activation upon disease induction, which in turn may be influenced by variation in *Mfsd4* levels. The congenic strain provides an important tool for future studies of how *Mfsd4* levels functionally contribute to influence disease phenotypes.

Other *cis*-regulated transcripts mapping to previously described EAE QTLs that are being investigated in our lab as candidate genes for EAE are C-type lectins and NK cell receptors and ligands. Results have demonstrated that both NK cell frequency and activity and EAE severity are regulated by epistasis between multiple clustered NK cell receptors and the NK cell ligand cluster. Using the eQTL approach we also identified highly significant *cis*-regulated transcripts that did not overlap EAE QTLs, but that could still denote functions important for EAE. Particularly interesting could be genes or family members of genes previously known to associate with MS. The regulator of G-protein signaling 4 (*Rgs4*) is one of these *cis*-eQTLs and its family member *Rgs1* is associated with MS [290]. Up-regulation of certain RGS proteins decreases immune cell migration and reduces chemokine-dependent calcium fluxes. They are also being discussed as potential important drug targets. Further studies in MOG-EAE of the functions and pathways affected by MS family member genes could add to the understanding of the genetic contribution of susceptibility and pathogenesis of neuroinflammation.

Our unbiased whole-genome approach helped in detecting *cis*-regulated transcripts, both in EAE QTLs and outside, which may play important roles in regulating key mechanisms in both EAE and human disease. These can serve to generate novel hypotheses useful in further dissecting pathogenic molecular mechanisms that are dysregulated during chronic inflammation. Using various analyses and experimental strategies, characterizing these pathways could ultimately help direct new therapeutic approaches.

2.2.3   NETWORK ANALYSIS CAN DISCLOSE RISK GENE FUNCTIONS

When performing microarray-based experiments, as in study III, the amount of data generated is often large and effort has to be made in dissecting relevant information to build new hypotheses about disease relevant processes. Several strategies can be employed for this purpose, one of them being the search for candidate genes for physiological QTLs (study III). With the whole-genome expression profiles available we also had the opportunity to explore the role of genes with yet undescribed functions and to link them to functional pathways. As exemplified with *Mfsd4*, and its association with T cell functions (study III), this can be achieved by investigating interacting molecules or pathways the gene of interest correlates with.

The microarray technologies have been essential for GWAS studies, which have proven successful in identifying risk alleles. However, these risk alleles do not explain the full disease heritability and variance [57] and it is likely that additional risk variants that confer very small effects can contribute to heritability of complex diseases. Supporting this hypothesis is the clustering of genes, below thresholds for significant association with MS, into functional networks [291]. Combining genome-wide expression traits with clinical information enables identification of gene networks that correlate with clinical phenotypes and reveal mechanisms central in disease regulation. Our mapping of eQTLs in the chronic stage EAE enabled correlation of transcripts with clinical EAE phenotypes. With gene clustering analysis we defined several disease correlated gene networks.

We performed pathway analysis to identify functional properties of these gene networks. The most significant disease-correlated network was associated with molecular functions including T cell-mediated immune mechanisms that are implicated in MS [57]. It included multiple genes associated with MS, and by prediction of causal

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relationships between transcripts we described both established and novel gene interactions (Figure 14). Several genes in this network, including *Themis,* lymphoid enhancer-binding factor 1 (*Lef1*), lymphocyte-specific protein tyrosine kinase *(Lck)*, cytotoxic and regulatory T cell molecule (*Crtam*) and IL2-inducible T-cell kinase *(Itk)*, have previously been linked to functions of the adaptive immune system. *Themis* act through T cell receptor (TCR) signaling and has been linked to Treg functions and inflammation [292]. In our gene network analysis *Themis* was found to interact with *Lef1* (study III) that binds to a functionally important site in the TCR-alpha enhancer. *Lef1* in turn was predicted to interact with *Lck*, known to be a key player in TCR signaling. A directed interaction was found between *Crtam*, which influence the adaptive immune response [293], and *Itk* with a suggested role in T cell proliferation and differentiation [294]. Additionally, our analyses served to reveal novel players that influence specific molecular pathways (study III). *Mfsd4* was predicted to directly affect phosphodiesterase 3B (*Pde3b*). *Pde3b* is an enzyme that hydrolyzes cAMP for cell metabolism and is regulated downstream of Foxp3 to support Treg cell homeostasis and lineage stability [295,296].
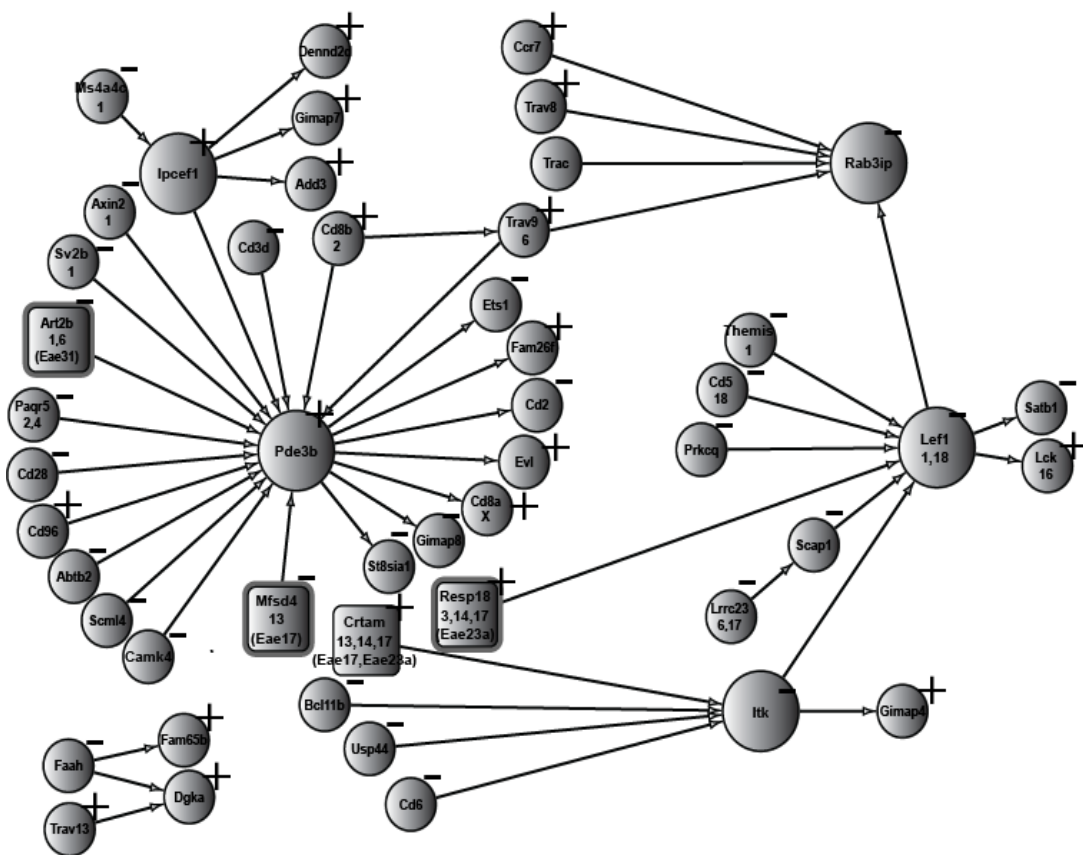

Figure 14. A gene network of interacting genes that correlates with EAE severity and associates with molecular functions including T cell-mediated immune mechanism (study III).

In study III we identified several networks of genes involved in specific immune related pathways, presumably of importance for both EAE and MS pathogenesis. Through our co-expression analyses we gained novel insights into the cause-effect interactions of several genes that have not previously been linked to immune-related pathways.

2.2.4 PATHOGENIC MECHANISMS - AUTOPHAGY IN MS AND EAE

Evaluating risk genes and pathogenic mechanisms using a disease model can be important in understanding the pathogenic mechanisms of human disease (studies I and II). An unbiased approach of examining genetically regulated disease phenotypes in an experimental model can uncover genes or pathways that have not yet been associated to immune specific mechanisms or are unexplored in human disease. Using the eQTL approach we established a genetic predisposition for expression of *Atg7* (studies III and IV), which we further identified as a positional candidate for an EAE-regulating locus on RNO4 [297]. Autophagy, which is a mechanism regulating quantity and quality of organelles and proteins, has been linked to innate and adaptive immunity as well as numerous human diseases [231].

In study IV we applied a translational approach to investigate the involvement of *Atg7* in the pathogenesis of EAE and MS. We used rat and mouse EAE models and PBMCs and CSF cells from MS patients and control groups. *Atg7* expression was regulated in *cis* and we identified numerous SNPs in *Atg7* between DA and PVG rat strains that could potentially affect gene expression. Resistant PVG alleles at the *Atg7* locus on RNO4 conferred higher levels of *Atg7* and less severe EAE. *Atg7* expression was higher in T cells of EAE resistant PVG compared to susceptible DA rats both in naïve state and after induction of EAE. Furthermore, we observed differences in the levels of key autophagic proteins between strains following EAE induction.

We then investigated the effect of conditional deletion of *Atg7* on T cells of mice on the T-cell compartment and on EAE susceptibility and severity. Consistent with previous work by others [227], *Atg7* KO in T cells decreased CD8$^+$ T cell frequencies in naïve lymph nodes and spleen. This was also seen after EAE induction (study IV). The conditional KO mice further displayed higher CD8$^+$ T cell activation markers in lymph nodes. The deletion of *Atg7* in T cells did not affect susceptibility or severity of EAE. This could potentially be explained by the fact that the utilized EAE model

predominantly is CD4$^+$ T cell-driven and CD4$^+$ T cells were not significantly affected in the *Atg7* conditional KO mice.
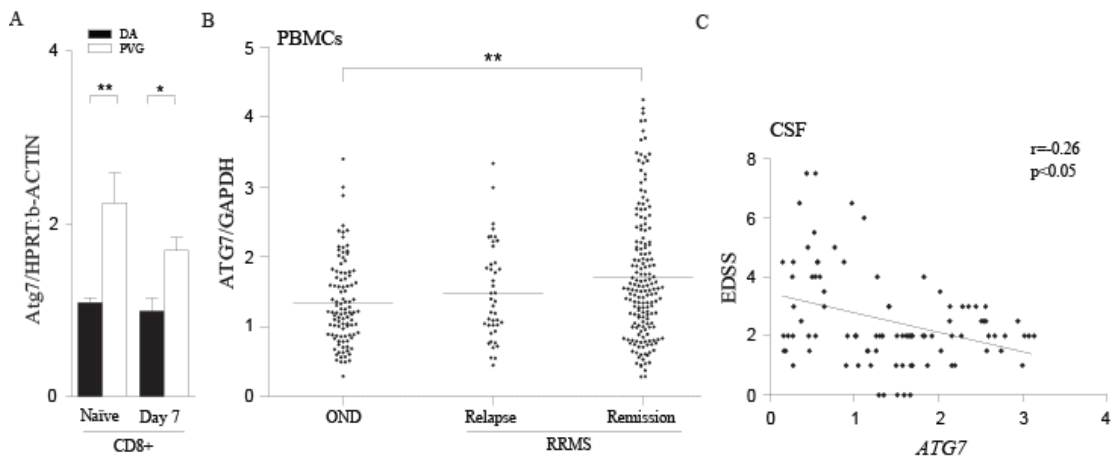

Figure 15. A) *Atg7* expression is higher in T cells of EAE-resistant PVG compared to EAE-susceptible DA rats. B) *ATG7* expression in PBMCs is higher in MS patients experiencing a clinical remission when compared to controls. C) MS patients with less clinical severity have higher expression of *ATG7*.

Based on our findings in the animal models we next explored the findings in MS. Higher expression of several autophagy genes, including *ATG5* and *ATG16L1*, have been observed in CSF of MS patients compared to controls [241]. We compared *ATG7* expression in MS patients and controls, and at different disease stages. *ATG7* expression was increased in PBMCs of MS patients compared to controls. Interestingly, *ATG7* expression was higher in the remission phase in RRMS patients. Furthermore, expression of *ATG7* correlated negatively with disease severity as measured by EDSS (Figure 15) and the chemokine (C-X-C motif) ligand 13 (CXCL13), a biomarker for disease activity and unfavorable prognosis [298].

ATG7 was co-expressed with *ATG16L1*, a gene that has previously been associated with Crohn's disease. Crohn´s IBD and MS share some immunological characteristics, and we thus hypothesized that SNPs in autophagy-related genes may also predispose for disease susceptibility in MS. In this regard we investigated SNPs in the genes encoding *ATG7* and *ATG16L1* in a Swedish MS case-control cohort. Importantly, we identified SNPs in both *ATG7* and *ATG16L1* that showed evidence for association with MS. To our knowledge, this is the first suggested association of *ATG7* and *ATG16L1*, or any other autophagy-related gene, with MS and thus further investigation in larger cohorts is warranted.

The autophagy pathway has not been studied in neuroinflammation and we do not know how it contributes to pathogenic mechanisms. Some of the potential mechanisms are hereby shortly summarized. Populations of CD8^{+} regulatory T cells have been identified, and have also been shown to be enhanced during the remission phase of disease in a model of MS [145]. Autophagy may be involved in shifting CD8^{+} T cells into a more regulatory phenotype or to promote their survival. Using both clinical measurement and a biomarker of disease severity, we could observe that high *ATG7* expression in CSF cells of MS patients correlated with less severe clinical symptoms. This indicates that the autophagy pathway may be triggered as a consequence of an ongoing inflammation. This idea is supported by the recent discovery that autophagy can be induced when inflammasomes are activated in a way to dampen inflammation [299]. Another hypothesis is that autophagy is involved in neuroprotective effects. It is possible that the increased expression of *ATG7* observed in patients with RRMS can be involved in preventing or delaying a development into a progressive disease stage. However, these hypotheses all require further experimental testing.

In conclusion, we demonstrate a potential involvement of Atg7 in MS and EAE. Studies in this thesis provide a good base for further functional studies involving Atg7 and the autophagy pathway to add to the understanding of the molecular basis of autophagy in autoimmune mechanisms.

2.3 CONCLUSIONS AND FUTURE PERSPECTIVES

The projects in this thesis have aimed to identify and evaluate risk genes and pathways in order to better understand pathogenic mechanisms of neuroinflammation. They have included unbiased approaches, such as identifying loci that regulate clinical traits in experimental populations as well as whole-genome profiling of gene expression regulation. We have performed hypothesis-driven experiments in models of human disease using a variety of methodologies and translated findings to human disease. Results presented in these studies add to the understanding of pathogenic mechanisms involved in EAE and MS, and importantly serve as a basis for the scientific community for formulation of new hypotheses.

A complex inflammatory disease such as MS is challenging to study and an experimental model can be useful to overcome some of the confounding effects since conditions can be controlled and relevant tissue is more freely available. However, it is critical to continuously evaluate the model of disease. Our results validate MOG-EAE in DA and PVG as being good models for studying MS in many aspects. We demonstrate that similar pathogenic mechanisms are present in MS and EAE and that several MS-associated genes are also regulated in EAE. Conversely, identification of EAE risk genes enables testing of hypothesis in MS cohorts. We concluded that while *IL18R1* genotypes do not determine susceptibility to disease, increased *IL18R1* expression might contribute pathogenically to disease. Our translational studies collectively support the usefulness of studying genetic regulation of experimental models to better understand human disease.

We refined an EAE-regulating QTL on RNO13, *Eae34*, and confirmed its biological importance in a congenic line. It can be difficult to obtain a narrow QTL with few candidate genes by mapping in experimental crosses, and yet, if this is successfully done, the challenge remains in how to exclude candidate genes in an unbiased way. Complementary strategies need to be utilized to this end, and one such approach was implemented in this thesis through the use of eQTL mapping. This strategy of combining conventional linkage analysis of physiological QTLs and genome-wide transcript array analysis has successfully identified several candidate genes [271-273]. By using the eQTL approach, functional clustering and gene network analysis, we identified *Mfsd4* as a strong positional candidate for *Eae34*.

Furthermore, we could predict and demonstrate a functional effect on T cell proliferation and activation conferred by *Eae34*. In study III we defined several candidate genes that motivate future translational studies. As for *Mfsd4*, future studies are needed in order to fully characterize the function of the gene in biology and disease. The use of congenic animals, with natural variation in selected regions, is a valuable resource when discerning mechanisms controlled by risk genes. A combination of approaches that complement congenic lines to define critical genetic variations are various gene-targeting technologies, for instance knock-in or knock-down of genes using small interfering RNA.

Genetic components and pathogenic mechanisms are to some degree shared between diseases [122,300-302], and we should therefore make use of our congenic tools to study risk genes in other chronic inflammatory diseases. Although risk genes do not have to be shared, they can influence important central pathogenic mechanisms and it is therefore essential to continue the dissection of mechanisms regulated by risk alleles. Disease mechanisms and risk genes that have been identified in other diseases should therefore be evaluated in EAE and MS. A shared genotyping effort to replicate and define functional SNPs of established risk genes for various autoimmune disorders, including MS, has recently been performed with the Immunochip [303]. This included the *ATG7* and the *ATG16L1* genes, the latter having previously been associated with Crohn's IBD.

Based on our finding of a predisposition for *Atg7* expression in EAE and the fact that autophagy genes have been linked to Crohn's IBD, both conditions which share immunological features with MS, we engaged in the project to explore the potential importance of autophagy in MS and EAE pathogenesis. We determined that *ATG7* levels are altered and correlate with clinical parameters in MS patients. However, the impact of autophagy pathways in MS remains to be elucidated. Focused studies on the interplay between cytokines and autophagy proteins, and the influence these interactions have on immune cells, may give significant insights to the role of autophagy in immunity and inflammation. Studies III and IV in this thesis illustrate how experimental models can contribute in identifying pathways that may be pathogenic but are not yet explored in the studied disease.

High throughput technologies and large international efforts have enabled identification of risk alleles for common complex diseases. GWAS has given us new insights and confirms MS as being a complex disease, with many genes contributing to susceptibility. Emerging deep sequencing of genetic regions surrounding associated SNPs brings the promise of more accurate positioning of a causative polymorphism. Results from studies I and III demonstrate how MS risk genes translate well to EAE and efforts should now be put into combining clinical and experimental research to evaluate candidate genes and to functionally study their mechanisms of action.

Association studies have identified many risk variants; however, there are a few variables that must be taken into account when interpreting results from these studies. There are probably numerous genes imposing relatively individual small effects. Even these can denote important pathogenic mechanisms of relevance to disease. Rare variants are also not detected by current approaches. Genetic and phenotypic heterogeneity of the studied population is a major challenge. Although the recent GWAS studies comprise many thousands of patients and controls, there is a possibility that sample sizes are still too small [304]. In addition, it is of importance that future studies take into account epigenetic and parent-of-origin effects, as well as the influence of gene-gene interactions and gene-environment interactions. These factors have recently in our lab all been assigned a significant contribution to the etiology of EAE, and by taking these effects into account a network of risk genes with stronger effects on disease may be discovered.

There is no question that with emerging technologies we will gain a deeper understanding of MS. There will be more sequencing data, large-scale expression profiling projects in many subjects, many tissues and at several time-points, and there is an eQTL study comprising 200 human samples with RNA sequencing ongoing in our lab. With this approach a new challenge arises, and resources will have to be put into the field of computational bioinformatics and to develop new advanced analytical tools to interpret and combine large-scale scientific efforts. Specifically for EAE and MS, it would be of interest to conduct a translational study aimed at identification of shared genetic regions associated to neuroinflammation in humans and the experimental models. These studies have in the past provided evidence for overlap of QTLs [118] and support the validity of EAE as a model of MS.

Finding a way of combining recent GWAS, several expression mapping studies in human and model systems and sequencing data, could contribute to the understanding of shared genetic regions and pathogenic mechanisms in multiple species. Ultimately, these integrated findings could provide insight into possible diagnostic and prognostic biomarkers or to potential therapeutic interventions for inflammation.

Several new MS therapeutics have recently been approved, most of these being focused on the immune system. They have been developed in part with the aid of EAE [305] and experimental research will in the future probably also have its focus on finding strategies for drug development. Although an identified risk gene does not have to be identical between rats and humans, studies in MOG-EAE can still resolve a pathogenic mechanism of importance in both EAE and MS. The genes identified in this thesis, including *IL18R1*, *Mfsd4* and *Atg7*, provide exciting new candidates for understanding disease mechanisms, but they, or pathways regulated by them, may also serve as potential targets for novel therapeutics.

To conclude, at date we do not know the etiology of MS, but the long-term goal of our research is to develop better means of prediction and prevention and ultimately to develop a cure. A deeper understanding of immunological events in MS pathogenesis must be achieved in order to identify better therapeutic targets and to select the best suitable therapy for individual patients. Combining various research fields, genomic and transcriptomic studies, epigenetics, interaction and epidemiological studies, in both human cohorts and experimental models is a promising approach to increase our capacity to define susceptibility genes and pathways to target for treatment.

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