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GENETIC REGULATION OF AUTOIMMUNITY IN EXPERIMENTAL NEUROINFLAMMATION

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Genetic Regulation of Autoimmunity in Experimental Neuroinflammation

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Στη γιαγιά κ τον παππού μου,
Σεβαστή και Βαγγέλη

To my grandmother and grandfather,
Sevasti and Vaggeli

"Όσο πιο πολλά μαθαίνω, τόσο πιο πολύ καταλαβαίνω πόσο λίγα ξέρω"

Σωκράτης, 470/469-399 π.Χ.

"The More I Learn, The More I Realize How Little I Know"

Socrates, 470/469-399 BC

ABSTRACT

Multiple sclerosis (MS) is a chronic inflammatory disease that affects the central nervous system causing axonal demyelination and leads to significant disabilities. MS affects mainly young adults and incidence of disease in Sweden is around 600 new cases/year. Genetic factors together with lifestyle/environmental influences contribute to MS pathogenesis, though their functional roles and mechanisms leading to disease are incompletely understood and current treatments exhibit modest effect and harmful side effects. Identification of risk genes and elucidation of molecular mechanisms will shed light on disease pathophysiology.

In this thesis, we used experimental autoimmune encephalomyelitis (EAE), an animal model mimicking clinical and pathological features of MS, to genetically dissect and study the role of antibodies (Abs) against myelin and neuronal antigens. Additionally, we attempted to pinpoint risk genes and investigate molecular pathways controlling antibody response and EAE.

Following a hypothesis-free approach, we identified that Ab response against myelin oligodendrocyte glycoprotein (MOG) was under polygenic control and *antigen-presenting lectin-like* (*APLEC*) genes, encoding C-type lectin receptors (CLRs), were the major regulators. Likewise, Ab response against neurofascin was also genetically regulated and correlated with EAE severity, encouraging further attempts to study MS genetics in relation to severity as well. Moreover, genetic regions controlling Ab response overlapped with EAE-regulating regions, implying common molecular pathways, and Ab titers correlated with disease susceptibility and severity implicating Abs in disease pathogenesis. We identified differential genetic regulation of Th2-related IgG1 isotype versus Th1-related IgG2b isotype. Moreover, the protective effect of IgG1/IgG2b ratio that we observed during EAE may have a potential significance in relation to therapeutic agents that promote Th2 predominance.

Functional studies were performed on congenic lines to elucidate the role of CLRs, able to sense also endogenous alarm signals, in EAE pathogenesis. We identified that macrophage C-type lectin (Mcl) and macrophage inducible C-type lectin (Mincle) receptors, mainly expressed on myeloid cells, are essential for the amplification of the inflammatory response in the CNS and are able to modulate EAE by skewing the immune response towards Th17 pathway. Unraveling molecular mechanisms underlying CLRs signaling is particularly important as these receptors may serve as novel therapeutic targets.

Combining genetic and immunological studies on experimental models is a promising approach that gives insight in disease mechanisms and facilitates the development of effective prognostic, diagnostic or therapeutic tools/agents.

LIST OF SCIENTIFIC PAPERS

I. **Anti-MOG antibodies are under polygenic regulation with the most significant control coming from the C-type lectin-like gene locus.**
Flytzani S, Stridh P, Guerreiro-Cacais AO, Marta M, Hedreul MT, Jagodic M, Olsson T.
Genes and Immunity., 2013 Oct;14(7):409-19

II. **MOG-induced experimental autoimmune encephalomyelitis in the rat species triggers anti-neurofascin antibody response that is genetically regulated.**
Flytzani S, Guerreiro-Cacais A, N'diaye M, Lindner M, Linington C, Meinl E, Stridh P, Jagodic M, Olsson T.
*Journal of Neuroinflammation***,** 2015 Oct 29;12:194.

III. **Regulation of anti-MOG antibodies is isotype-specific: IgG1 is MHC-dependent while non-MHC genes direct IgG2b and IgG2c**.
*Stridh P, ***Flytzani S**, Baud A, Diez M, Johannesson M, Beyeen AD, Guerreiro-Cacais AO, Abdelmagid N, Ockinger J, Gillett A, EURATRANS phenotyping and genotyping group, Holmdahl R, Mott R, Flint J, Jagodic M, Olsson T.
Manuscript

IV. **C-type lectin receptors (Mcl and Mincle) modulate experimental autoimmune encephalomyelitis.**
*N'diaye M, ***Flytzani S,** Brauner S, Warnecke A, Guerreiro-Cacais AO, Kular L, Piket E, Adzemovic M, Daws MR, Fossum S, Jagodic M, Olsson T
Manuscript

***Authors contributed equally to the work**

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Journal of Inflammation Research. 2015 Nov 13;8:211-25. Review

II. **Rat bone marrow-derived dendritic cells generated with GM-CSF/IL-4 or FLT3L exhibit distinct phenotypical and functional characteristics.**
*N'diaye M, *Warnecke A, **Flytzani S**, Abdelmagid N, Ruhrmann S, Olsson T, *Jagodic M, *Harris RA, *Guerreiro-Cacais AO.
Journal of Leukocyte Biology. 2016 Mar;99(3):437-46.

III. **Parent-of-origin effects implicate epigenetic regulation of experimentalautoimmune encephalomyelitis and identify imprinted Dlk1 as a novel risk gene.**
*Stridh P, *Ruhrmann S, Bergman P, Thessén Hedreul M, **Flytzani S**, Beyeen AD, Gillett A, Krivosija N, Öckinger J, Ferguson-Smith AC, Jagodic M.
PLoS Genetics. 2014 Mar 27;10(3):e1004265.

IV. **Combining genetic mapping with genome-wide expression in experimental autoimmune encephalomyelitis highlights a gene network enriched for T cell functions and candidate genes regulating autoimmunity.**
Thessen Hedreul M, Möller S, Stridh P, Gupta Y, Gillett A, Daniel Beyeen A, Öckinger J, **Flytzani S**, Diez M, *Olsson T, *Jagodic M.
Human Molecular Genetics. 2013 Dec 15;22(24):4952-66.

V. **Genetic variability in the rat Aplec C-type lectin gene cluster regulates lymphocyte trafficking and motor neuron survival after traumatic nerve root injury.**
Lindblom RP, Aeinehband S, Parsa R, Ström M, Al Nimer F, Zhang XM, Dominguez CA, **Flytzani S**, Diez M, Piehl F.
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***Authors contributed equally to the work**

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LIST OF ABBREVIATIONS

Ab Antibody
ADCC Antibody-Dependent Cellular Cytotoxicity
ADEM Acute Disseminated Encephalomyelitis
AIL Advanced Intercross Line
ALS Amyotrophic Lateral Sclerosis
APC Antigen-Presenting Cell
BAFF B-cell Activating Factor
BC Backcross
BBB Blood Brain Barrier
BDNF Brain-Derived Neurotrophic Factor
BMMa Bone Marrow Macrophages
CII Collagen Type II
CFA Complete Freund's Adjuvant
CI Confidence Interval
CIA Collagen Induced Arthritis
CIS Clinically Isolated Syndrome
CLR C-type Lectin Receptor
cM Centimorgan
CNS Central Nervous System
CRD Carbohydrate Recognition Domain
CSF Cerebrospinal Fluid
DAMP Damage-Associated Molecular Pattern
DC Dendritic Cell
Dcar Dendritic Cell Immunoactivating Receptor
Dcir Dendritic Cell Immunoreceptor
DUR Duration
EAE Experimental Autoimmune Encephalomyelitis
EBNA EBV Nuclear Antigen
EBV Ebstein-Barr Virus
EDSS Expanded Disability Status Scale
FcRγ Fc Receptor-γ
G10 10th Generation
GA Glatiramer Acetate
GWAS Genome-Wide Association Study
HLA Human Leucocyte Antigen
HS Heterogeneous Stock
IFA Incomplete Freund's Adjuvant
IFNβ Interferon β
IM Infectious Mononucleosis
ITAM Immunoreceptor Tyrosine-Based Activating Motif
ITIM Immunoreceptor Tyrosine-Based Inhibitory Motif
IVIG Intravenous Immunoglobulin
JCV JC Virus
KO Knockout
LN Lymph Node
mAb Monoclonal Antibody
MAG Myelin-Associated Glycoptotein
MAX Maximum Score
MBP Myelin Basic Protein
MCL Macrophage C-type Lectin
MHC Major Histocompatibility Complex
MINCLE Macrophage Inducible C-type Lectin
MO Monocytes
MOG Myelin Oligodendrocyte Glycoprotein
MRI Magnetic Resonance Imaging
MS Multiple Sclerosis
NF Neurofascin
NLR Nod-like receptors
OCB Oligoclonal Band
OGD Oligodendrocyte
ONS Onset
PAMP Pathogen-Associated Molecular Pattern
PBMC Peripheral Blood Mononuclear Cells
pDC Plasmacytoid Dendritic cell
PLP Myelin Proteolipid Protein
PML Progressive Multifocal Leukoencephalopathy
PNS Peripheral Nervous System
PPMS Primary Progressive Multiple Sclerosis
PVM Perivascular Macrophages
QTL Quantitative Trait Locus
RA Rheumatoid Arthritis
RLR RIG-I like receptors
RRMS Relapsing Remitting Multiple Sclerosis
SLE Systemic Lupus Erythematosous
SNP Single Nucleotide Polymorphisms
SPMS Secondary Progressive Multiple Sclerosis
SUM Cumulative Score
Syk Spleen Tyrosine Kinase
T1D Type 1 Diabetes
TBI Traumatic Brain Injury
TCR T Cell Receptor
TDB Trehalose-6,6-dibehenate
TDM Trehalose Dimycolate
TLR Toll-Like Receptor
TMEV Theiler's Murine Encephalomyelitis Virus
TRAIL TNF-Related Apoptosis-Inducing Ligand
UVR Ultraviolet Radiation
WL0 Weight Loss day 0
WT Wild Type

1 INTRODUCTION

1.1 COMPLEX DISEASES

The revolution in medical genetics came almost 35 years ago when genetic mapping was applied to identify genes responsible for simple Mendelian diseases [1]. However, most of the diseases do not follow simple Mendelian monogenic inheritance patterns. These are polygenic diseases where multiple genes, each having a small and/or interactive role, contribute to disease susceptibility [2]. These diseases are also characterized by complexity, meaning that nutritional, geographical, infectious and other environmental influences will add to disease risk or interact with gene variants, so-called gene-environment interactions. The complexity of these diseases applies also in their genetic dissection, where predisposing genes might have a small or a moderate effect to the overall risk, same genes might result in different phenotypes or disease risk may be influenced by post-genomic modifications or epigenetic changes [3].

The underlying susceptibility of an individual to develop disease may be defined by the liability threshold model (Figure 1) [4,5]. According to the threshold model hypothesis, the members of a population have a normal (Gaussian) distribution of genetic liability for a particular disease and a threshold value exists. Several environmental and stochastic factors and epigenetic interactions contribute to the disease, each having small effects. The effects add up and once the cumulative effects of the factors pass the threshold, an individual becomes affected.

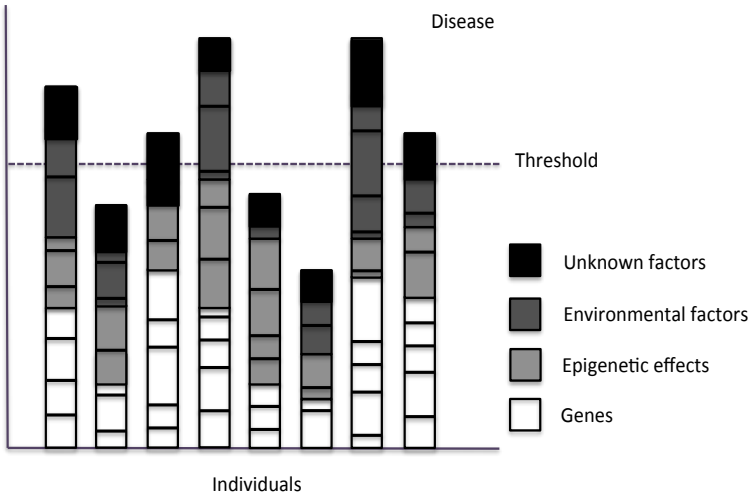

Figure 1. The threshold model for susceptibility to complex disease. An individual will develop a complex disease once the accumulation of predisposing factors (e.g. genetic, epigenetic, environmental and unknown) and the interaction between them will exceed the threshold level for total risk.

Examples of common complex immune-mediated diseases include type 1 diabetes (T1D), rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), Crohn's disease and multiple sclerosis (MS). In this thesis, we try to identify and functionally characterize gene variants contributing to MS by using a translational research approach. Great advances in the field of genetics have given insight in disease pathogenesis, however resolving the complexity that underlies the susceptibility of such a polygenic human disorder remains a great challenge.

1.2 MULTIPLE SCLEROSIS

MS is a chronic inflammatory disease where the first comprehensive clinical and pathological description was made by neurologist Jean-Martin Charcot in 1868. MS affects the central nervous system (CNS) i.e. the brain and spinal cord causing neuronal axon demyelination and subsequent axon loss. MS is a common disorder among young adults in the Western world (around 2.5 million people are affected worldwide [6]). Disease prevalence in Sweden is approximately 0.19% and incidence is around 600 new cases/year [7]. The disease usually starts in early adulthood, between 20 and 40 years of age, and preferentially affects women with a ratio of almost 2.35:1 man. MS patients not only have lower life expectancy but also their quality of life is markedly reduced as a result of their significant disabilities. To date and in line with the complexity of MS, a long series of gene variants and lifestyle/environmental factors have been associated to increased risk for disease, though their precise functional roles are not clear. Furthermore, treatments available for MS are fairly effective, however comprise potential risks.

1.2.1 Clinical features and course

MS is characterized by demyelination, inflammatory cell infiltration and neurodegeneration [8]. Axonal demyelination leads to defect saltatory conduction of action potentials resulting in various clinical symptoms and signs that reflect the functional anatomy of affected sites. MS patients may comprise a variety of clinical symptoms such as balance and vision disturbance, bowel and bladder function impairment, loss of sensation, cognitive impairment, fatigue and motor disturbances [9]. A common method of evaluating the functional status of MS patients is the expanded disability status scale (EDSS) [10]. EDSS is based on quantification of neurological signs of different functional groups i.e. pyramidal, sensory, cerebellar, brain stem, bowel and bladder, visual and cerebral or mental.

Besides dissemination in space, another key characteristic of MS is dissemination in time, i.e. appearing episodically over time [11,12]. Clinically isolated syndrome (CIS) is characterized the

first clinical presentation of a demyelinated disease that could potentially be MS but still does not fulfill the criteria of dissemination in time. The majority of MS patients appear with the relapsing remitting subtype of MS (RRMS) characterized by acute clinical attacks of demyelination followed by periods of remission with complete or partial recovery (Figure 2). Most of RRMS patients will enter the secondary progressive phase of MS (SPMS) where gradual worsening of the symptoms takes place without remissions and neurological disabilities are accumulated (Figure 2). Some MS patients experience continuous worsening of their symptoms upon disease onset featuring primary progressive form of MS (PPMS).

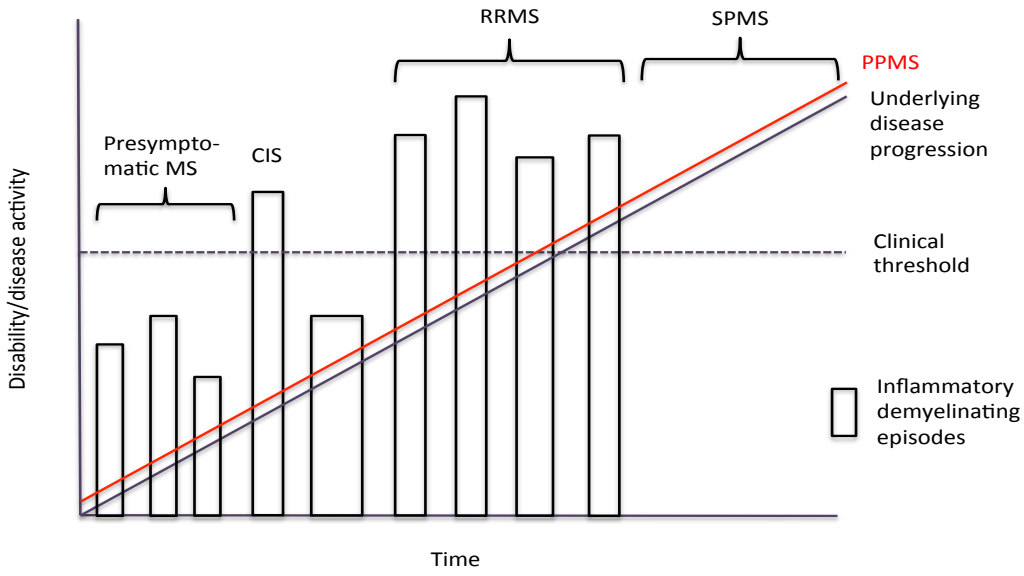

Figure 2. Multiple sclerosis clinical course. The spectrum of clinical multiple sclerosis (MS) is variable, ranging from a monotonically progressive course (PPMS) to relapsing remitting MS (RRMS) with prominent inflammation, possibly preceded by a clinically isolated syndrome (CIS) years before RRMS sets in. A majority of RRMS patients will later develop a secondary progressive disease (SPMS). Figure adapted after [13].

1.2.2 Immunopathology

The pathologic hallmark of MS is demyelinated plaques within the CNS accompanied by inflammation, gliosis, oligodendrocyte (OGD) death, neurodegeneration, axonal loss and remyelination [8,14]. Damage in the blood brain barrier (BBB) enables the infiltration of inflammatory cells, and macrophages containing myelin debris, CD8 and CD4 T cells, B cells, plasma cells, antibodies (Abs) and complement have been observed in active MS lesions. Early active MS lesions are characterized by a profound pathologic heterogeneity suggesting that diverse mechanisms may account for MS pathogenesis in different disease

subgroups. Lucchinetti et al have categorized MS lesions into four distinct patterns according to demyelination and pathological features of the analyzed MS brains [15,16]:

- Pattern I (15%): perivascular demyelination with activated macrophages, microglia and T cells, and extensive remyelination.
- Pattern II (58%): similar to Pattern I. Additional deposition of immunoglobulin (Ig) and complement at sites of active myelin destruction.
- Pattern III (26%): Inflammatory T cells and macrophages with distal OGD apoptosis, preferential loss of the periaxonal myelin components and limited remyelination.
- Pattern IV (1%): Similar to Pattern I with primary OGD injury, extensive OGD degeneration and limited remyelination.

Even though MS has been considered a white matter disease, demyelinated plaques have been observed also in the gray matter [17] and they may reflect the pathologic substrate of cognitive impairments that MS patients might comprise [18]. Besides the focal demyelinated lesions, MS brains, mainly in the progressive forms of disease, are characterized by a diffuse injury of the normal-appearing-white matter illustrated by perivascular and parenchymal inflammatory infiltrates [19].

Axonal loss may be due to inflammatory but also non-inflammatory factors such as loss of trophic support by glia cells [20], and over time the pathological changes become dominated by neurodegeneration that leads to progressive accumulation of disabilities [9]. Remyelination varies among patients and is characterized by thinly myelinated axons depending on the availability of OGD precursor cells and the balance between pro- and anti-inflammatory processes [15,21].

1.2.3 Diagnosis

Diagnostic criteria for MS include both clinical and paraclinical evaluations in order to assess the main features of disease i.e. dissemination of lesions in space and time [22]. The MacDonald criteria, based on clinical, radiologic and laboratory assessments, are the most commonly used criteria to diagnose and differential diagnose MS. Magnetic resonance imaging (MRI) discriminates active from older demyelinated plaques and identifies lesions reflecting BBB damage and brain atrophy [23]. Around 90% of MS patients comprise IgG oligoclonal bands (OCB) in cerebrospinal fluid (CSF) that are not detectable in the serum, thereby providing a powerful diagnostic tool for MS [24]. Lastly, delayed nerve evoked potentials are also useful in identifying clinically silent lesions in MS patients [25].

1.2.4 Treatment

Despite the significant advances occuring in the last 20 years in understanding MS pathogenesis, there is still no fully effective treatment available for the disease, and all approved therapeutics (Table 1) aim to aleviate symptoms or to prevent rather than repair tissue damage.

Until 1993, when interferon β (IFNβ) was first approved to treat MS relapses [26], exacerbations of disease were symptomatically treated only with high doses of intravenous methylprednisolone [27]. Interferons are not only anti-viral drugs but also endogenous cytokines with the capacity to immunologically modulate cellular signaling, gene transcription and innate and adaptive immunity [28]. IFNβ treatment is complicated by frequent development of neutralising antibodies resulting in lower drug efficacy [29]. Another drug for MS is glatiramer acetate (GA) that is believed to induce tolerance or anergy of myelin-reactive lymphocytes [30]. IFNβ and GA are first-line agents for MS patiens and give approximately 30% reduction in annual relapse rate [31]. However, none of these have emerged as superior treatments given the relatively low efficacy and the variable adverse effects they may cause.

Much better efficacy in MS treatment was achieved with the approval of natalizumab to treat MS patients in 2003. Natalizumab is a humanized monoclonal antibody (mAb) that targets α4-integrin in leucocytes leading to reduced cell infiltration into the CNS through the BBB [32]. Despite its high efficacy (it is the most effective treatment for MS to date), natalizumab has a major side effect which is the development of progressive multifocal leukoencephalopathy (PML), a rare, but often fatal opportunistic infection that is caused by polyoma JC virus (JCV) [33 34]. That led to the introduction of measurement of JCV Abs, that predict individuals at high PML risk, as a routine in clinical practice. A recently (2009) approved drug for MS with the advantage of being orally administered is fingolimod [35], and with natalizumab represent the second-line agents for MS treatment. Fingolimod downregulates S1P receptor on leukocytes resulting in T cell trapping in the lymph nodes (LN). Alemtuzumab, a leucocyte-depleting mAb was recently approved as a second or third line treatment, however, connected to frequent development of other autoimmune diseases [36].

Two more immunomodulatory agents have been recently approved as first-line treatment for disease, teriflunomide and dimethyl fumarate, and few more such as ocrelizumab (CD20+ B cell-depleted Ab), laquinimod, daclizumab and atumumab are under advanced

clinical studies to verify their efficacy and safety [31]. Despite the wide range of therapeutics for RRMS, there are no available effective treatments for SPMS and PPMS.

Table 1. Efficacy ranking of approved therapies for multiple sclerosis. Table adapted after [31].

Drug	Era of development	Mechanism of action
Most effective		
Natalizumab	Second	Monoclonal antibody against integrin-α4 in leukocytes
Highly effective		
Fingolimod	Second	Sphingosine S1P receptor modulator
Dimethyl Fumarate	Third	Immunomodulator
Alemtuzumab	Second or third	Leucocyte-depleting monoclonal antibody
Moderately effective		
IFNβ	First	Immunomodulator
Glatiramer Acetate	First	Immunomodulator
Teriflunomide	Third	Pyrimidine synthesis inhibitor

1.2.5 Aetiology

Familial/genetic, twin/adoption and environmental/migration studies have a major contribution on our understanding of MS aetiology. MS is considered to be a complex disease, where genetic and environmental/lifestyle factors together with epigenetic changes and stochastic events contribute to disease pathogenesis (Figure 3).

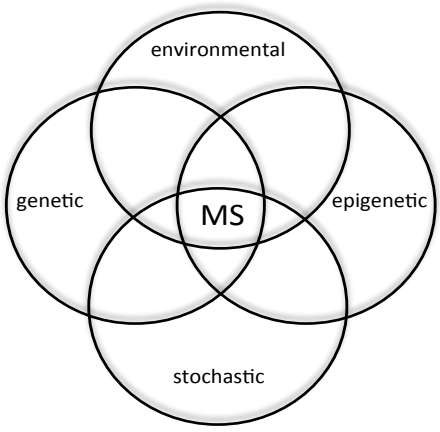

Figure 3. Multiple sclerosis risk factors. Genetic, environmental/lifestyle factors together with epigenetic changes and stochastic events contribute to disease pathogenesis.

1.2.5.1 Genetic risk factors

The genetic component of MS has become evident by familial studies of MS aggregation [37]. Approximately 20% of MS patients have a familial history of the disease, and concordance rate in monozygotic twins is higher compared to dizygotic twins. The genes strongest associated to MS are the ones encoding human leucocyte antigens (HLAs). The HLA class II, HLA-DRB1*15:01, has been associated with increased risk of MS whereas the HLA class I, HLA-A*02:01, has been shown to confer protection [38]. The contribution of other HLA haplotypes, such as DRB1*13:03, DRB1*03:01 and DRB1*08:01, in MS pathogenesis has recently been confirmed. Despite the several efforts on non-HLA risk gene identification during the last few decades, little progress was made until gene centered and genome wide association studies (GWAS) were implemented. The first GWAS identified variants in both *IL-7R* and *IL-2RA* as heritable risk factors for MS [39]. Since the first GWAS, in total 14 GWASs have been executed in MS and collectively they have identified approximately 110 non-HLA variants to increase risk for MS, though having a relatively smaller impact compared to HLA genes [40]. Interestingly, the majority of identified variants are involved in immune functions providing compelling evidende supporting the inflammatory versus neurodegenerative component on disease pathogenesis [41].

1.2.5.2 Environmental risk factors

The relatively modest familial risk, the low concordance rate of MS in monozygotic twins [42] and the uneven geographical distribution of disease (prevalence is increased with distance from the equator) [43] highlight the environmental influences as contributors in disease pathogenesis. Migration studies show that adolescents who move between regions with diverse disease prevalence acquire the risk of the new geographical area, and argue for the great impact of environmental exposures, during childhood and early adolescence, on disease susceptibilty [44].

MS incidence and prevalence increase with the distance from the equator and high prevalence areas include Europe, North America, Australia and New Zealand [45]. A possible explanation for this latitude-dependent gradient of disease is the lower exposure to sunlight/ultraviolet radiation (UVR) and the subsequent decreased vitamin D levels [46].

Other environmental triggers include active and passive smoking, obesity during adolescence, night shift work before the age of 20, exposure to organic solvents and Epstein-Barr virus (EBV) serology [47]. Interestingly, EBV is a common herpes B-cell tropic

virus and more than 90% of all individuals worldwide become affected usually during early childhood, though only a small percentage of EBV carriers will develop infectious mononucleosis (IM). Individuals with IM have a higher risk of developing MS compared to both EBV carriers and non-carriers and the latter have an extremely lower risk of MS compared to EBV carriers [48]. Importantly, the consistent finding that MS patients have increased anti-EBNA (EBV nuclear antigen) antibody titers prior to MS onset has led to a debate of whether or not EBV is the causal factor for MS development. Though, the underlying mechanisms behind viruses and other environmental triggers are still unknown and under rigorous investigation [47].

1.3 EXPERIMENTAL ANIMAL MODELS

An alternative approach to study human diseases is the use of experimental animal models. In the recent years a number of animal models have been developed mainly to elucidate the basic biology behind the disease and to monitor the development of new therapeutic approaches. The main advantages of the use of animal models is the availability of tissues and the relatively unlimited sample size. In genetic studies the use of animals enables the development of experimental crosses and genetically identical individuals (inbred and congenic strains) and permits the control of environmental influences.

1.3.1 EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS

Especially for MS in which the target organ of the disease, the CNS, is not easily accessible in humans, the use of an animal model is even more imperative compared to other diseases. It was almost a century ago [49], when researchers first attempted to reproduce the encephalitogenic effect of rabies vaccination on monkeys, that the whole concept of experimental autoimmune encephalomyelitis (EAE) as an animal model for MS initiated. Since then EAE has become the most powerful tool to study clinical, neuroimmunological, genetic and other aspects of human MS in the laboratory. EAE has been elicited in a wide range of species such as mice, rats, rabbits, monkeys and guinea pigs and a number of diverse models have been developed with the aim to reproduce different aspects of MS [50]. Classic EAE can be actively induced by injecting CNS homogenate or a CNS antigen together with an adjuvant. The most commonly injecting antigens are myelin proteins or peptides e.g. myelin oligodendrocyte glucoprotein (MOG) and myelin proteolipid protein (PLP) [51]. The use of mineral oil (Freund's) as an adjuvant leads to faster induction of disease and the addition of heat-inactivated mycobacteria tuberculosis to the adjuvant (complete Freund's adjuvant) or injections of pertussis toxin are necessary in many models

to augment or even induce disease [52]. The clinical outcome, the CNS pathology and the effector cells of the elicited disease depend on the animals' genetic background and the immunization protocol i.e. selected antigen and adjuvant.

Alternatively, passive EAE can be induced by transfer of myelin specific T cells and results in a short monophasic disease with minimal demyelination and rapid recovery [53]. Even though most of EAE models are induced, spontaneous EAE has been observed in transgenic mice over-expressing myelin basic protein (MBP)-specific T cell receptor (TCR) [54,55].

In general, EAE has been useful mainly in dissecting immunogenetic, histopathological and therapeutic aspects of MS. For example, GA and natalizumab were first developed based on their success in treating EAE. Nonetheles, EAE is not MS and there are major differences between these two [56]. First of all, MS is a spontaneous disease while the majority of EAE models are induced. Moreover, EAE, which is studied in inbred animals living under controlled laboratory conditions, does not reflect the complexity of human MS. Despite these limitations, much of our current knowledge for MS pathogenesis derives from studies on EAE and when EAE is used appropriately is a great tool for investigating such a complex disease [57].

1.4 IMMUNOLOGY

1.4.1 ADAPTIVE IMMUNITY

1.4.1.1 T cells

The clinical heterogeneity of MS reflects both the genetics and the complex, multicellular pathophysiological processes.

The similarities between CD4+ T cell mediated EAE and MS [58] and the identification of certain major histocompatibility complex (MHC) class II as risk genes for MS [39] promoted studies on the potential role of CD4 T cells in disease immunopathogenesis. As demyelination is the main characteristic of MS pathology, it has been hypothesized that a myelin antigen should be the potential auto-reactive antigen [59]. Indeed, T cells from MS patients recognize a wide range of myelin proteins including MOG [60], MBP [61] and PLP [62] among others. However, auto-reactive T cells, although of a different phenotype, are found also in healthy individuals [63,64], and there is a controversy on the evidence regarding differences in T cell frequency and avidity between the two groups [59]. Furthermore, T cells

have been shown to recognize non-myelin antigens as well such as aB crystalline [65] and contactin-2, which is a neuronal protein [66]. It is likely that T cells recognize a diverse and extensive array of antigens during MS development, however how and why these T cells become abnormally activated towards CNS antigens remains to be elucidated [67].

Until recently, several data indicated the prominent role of CD4+ Th1 cells, that require IL-12 for their differentiation and they secrete IFNγ, IL-2 and TNF, in MS pathogenesis. Among other findings, MS was exacerbated by IFNγ administration [68], increased MS clinical activity correlated with IFNγ and IL-12 expression in the CNS and CSF [69], and adoptively transferred Th1 cells could induce EAE [70]. The observation that mice deficient in IL-12, IFNγ and TNF develop severe EAE [71], while IL-23-deficient mice [72] are completely resistant led to the identification of Th17 cells, that need TGFβ, IL-6 and IL-1 for their differentiation, produce IL-17 and IL-22 and induce GM-CSF and IL-6 synthesis, as important cellular mediators in neuroinflammation [58]. Increased IL-17 expression has been detected in blood and CSF of MS patients as well as in MS brain lesions [73,74]. The observation that Th17 cells induce more severe EAE compared to Th1 cells and neutralization of IL-17 activity could ameliorate EAE [75-78] led to the notion that Th17 cells might be the true effector cells of CNS autoimmunity [58]. Even though the relative contribution and importance of Th17 versus Th1 cells is not clear to date, these T cell subsets comprise a preferential distribution in the CNS during EAE suggesting differential regulation of neuroinflammation in the brain and spinal cord [79].

Even though CD4+ T cells contribution in MS and EAE has been extensively studied, CD8+ T cells outnumber CD4+ T cells in MS lesions and their numbers correlate with axonal damage, a finding that stronlgy suggests their implication in disease immunopathogenesis [80]. Furthermore, oligoclonal expansion of CD8+ memory T cells have been detected in CSF and brain tissue from MS patients [81,82], and adoptively transferred CD8+ T cell induced severe EAE in mice [83]. Taken together, there is no doubt that both CD4+ and CD8+ T cells contribute to disease pathogenesis, although they may have distinct roles at different steps.

1.4.1.2 B cells and autoantibodies

Even though there is a "T cell centric" approach of MS pathophysiology, the prominent role of B cells and autoantibodies has become solid by a wide range of evidence. Intrathecal antibody production and CSF IgG OCB are hallmark findings of MS patients [24] and they serve as diagnostic and prognostic markers [84]. These intrathecal IgG are synthesized by

local plasmablasts and plasma cells but their antigenic target is not known to date [85]. B cell follicle-like aggregates had been detected in the meninges of RRMS and SPMS patients and their presence correlated with cortical pathology and more aggressive clinical course [86-88]. Furthermore, elevated levels of B-cell activating factor (BAFF), the major survival factor for B cells and CXCL13, a B-cell chemoattractant chemokine have been detected in MS patients [89,90]. Taken together the previous observations suggest that CNS in MS patients provide a fostering inflammatory milieu for the survival of B cells and Ig-producing plasma cells [91]. Lucchinetti et al has categorized MS brain lesions into four distinct patterns (for detailed description see before "Immunopathology"), and Pattern II lesions are characterized by prominent immunoglobulin deposition and complement activation. Furthermore, therapeutic plasma exchange, that depletes autoantibodies, has successfully treated MS demyelinating attacks and MS patients with pattern II lesions are more likely to improve after plasmapheresis [92]. The therapeutic effect of rituximab, an anti-CD20 mAb that depletes B cells but not antibody producing plasma cells indicates that pro-inflammatory features of B cells may dominate in many MS patients [93]. Additional evidence for the fundamental role of B cells in MS are provided by genetic studies where B cells showed significant enrichment of genomic regions associated with MS [94] and by environmental studies that have indicated the B-cell tropic EBV as a strong risk factor for MS [48].

In addition to antibody production, B cells exert not only pro-inflammatory functions through production of cytokines such as IL-6 [95] but also have anti-inflammatory features through the production of anti-inflammatory cytokines such as IL-10 [96] and neurotrophic factors such as brain-derived neurotrophic factor (BDNF) [97]. Likewise, antibodies may not only be damaging through mechanisms such as activation of complement and antibody-dependent cellular cytotoxicity (ADCC) but have also regulatory roles [98] as indicated by the successful intravenous Ig (IVIG) treatment of patients suffering from autoimmune or inflammatory diseases [99].

Anti-MOG auto-antibodies

The oligoclonal nature of the IgG OCB, found in the majority of MS patients, suggests that MS is an antigen-driven disease and identifying the auto-antigen would offer significant insight in disease pathogenesis. Despite that several autoantigens have been proposed as potential targets in MS, their clinical relevance remains controversial. As MS is a demyelinating disease, a substantial amount of studies have focused on CNS myelin-

specific autoantigens [100] such as MOG [101], MBP [102], PLP [103] and myelin-associated glycoprotein (MAG) [104]. However, numerous non-myelin antigens have been also identified as potential Ab targets including autoantigens of oligodendrocytes (e.g. CNPase, transaldolase and transketolase), astrocytes (e.g. potassium channel KIR4.1), cellular ion channels (e.g. anoctamin 2), microbial (e.g. viral) and many more [105,106]. The recent detection of axon-specific autoantibodies such as against neurofascin and contactin has contributed in the long list of MS candidate autoantibodies [66,107]. Despite the numerous studies on the field of MS autoantigens, none were ultimately identified to be MS specific.

Unlike intracellular antigens such as MBP and PLP, MOG is unique due to its localization on the surface of oligodendrocytes and the myelin sheath (Figure 4), thereby available for antibody binding [108]. Demyelinating MOG autoantibodies were first identified in EAE 30 years ago [109] and when co-transferred together with MBP-specific CD4+ T cells accelerated clinical signs of EAE and induced demyelination [110-113]. Moreover, demyelination observed

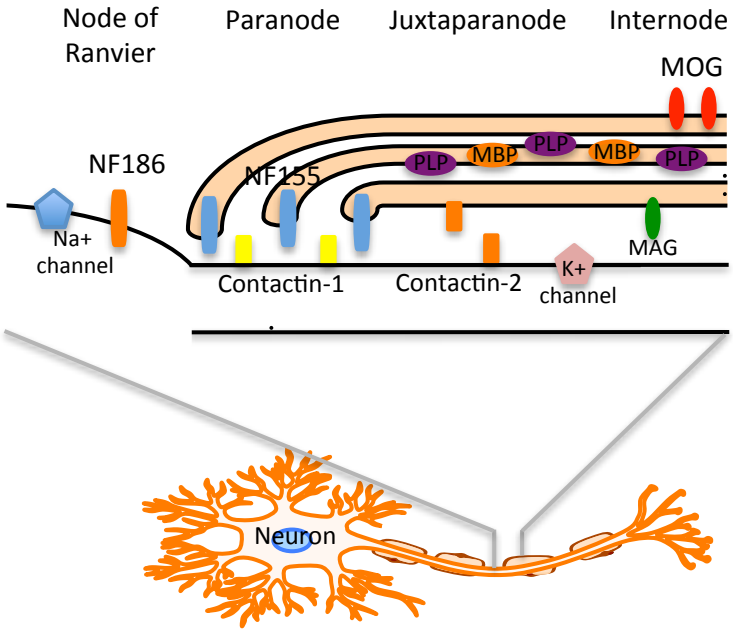

Figure 4. Distribution of CNS myelin proteins. A neuron with a myelinated axon is depicted. Myelin enwraps the axon at intervals called internodes omitting small opening termed nodes of Ranvier. Adjacent to the nodes of Ranvier are the paranode and the juxtaparanode. MOG, myelin oligodendrocyte glycoprotein; NF186, neurofascin 186; NF155, neurofascin 155; MBP, myelin basic protein; MAG, myelin associated glycoprotein; PLP, proteolipid protein. Figure adapted after [114].

in these models was associated with immunoglobulin and complement deposition [101,115] reflecting the immunopathology of Pattern II MS lesions according to Lucchinetti's classification. Notably, myelin injury is not induced by anti-MBP or anti-PLP Abs transfer [110].

The identification of the demyelinating nature of anti-MOG Abs in EAE, initiated a rigorous research on the evaluation of these Abs in MS, albeit with controversial and conflicting results. MS patients were reported to have elevated serum and/or CSF anti-MOG Ab levels in some studies [116,117], while in other studies no differences in the anti-MOG Ab titers were detected between MS patients and controls [118,119]. The lack of reproducibility between the research groups highlighted the necessity of developing techniques to selectively detect pathogenic MOG-specific autoantibodies that would recognize the native protein as it exists *in vivo*. For an antibody to be pathogenic it has to fulfill several criteria. Among them, the antigenic epitope the antibody recognizes has to be expressed at a site within the CNS easily accessible to the antibody, and, at the molecular level, the epitope recognized must also be accessible in vivo [120]. Until ten years ago, that was not the case in the majority of the studies where anti-MOG Abs recognized the denatured recombinant MOG. Since then a successful approach to identify potential pathogenic MOG-specific autoantibodies in MS has been the use of live MOG-transfected cell lines expressing MOG on their surface [121,122]. Notably, there is a consensus that anti-MOG Abs recognizing native MOG are not present in the sera of the vast majority of MS patients implying that they are not involved in the primary pathogenic mechanism of MS [120,123,124]. Nonetheless, Abs recognizing native MOG have been detected in a subgroup of pediatric-onset demyelinated disorders such as MS, acute disseminated encephalomyelitis (ADEM) and CIS, and the presence of Abs correlated with a younger age of disease onset [125-130]. Other CNS diseases where anti-MOG Abs have been detected include neuromyelitis optica (NMO), bilateral optic neuritis and anti-NMDA-receptor (NMDAR)-Ab-associated encephalitis [131] (Figure 5).

Despite the controversy in the field of anti-MOG Abs, it is yet to be elucidated whether these antibodies have a pathogenic role in demyelination, as indicated by animal studies, or whether they are a secondary epiphenomenon to myelin damage [132].

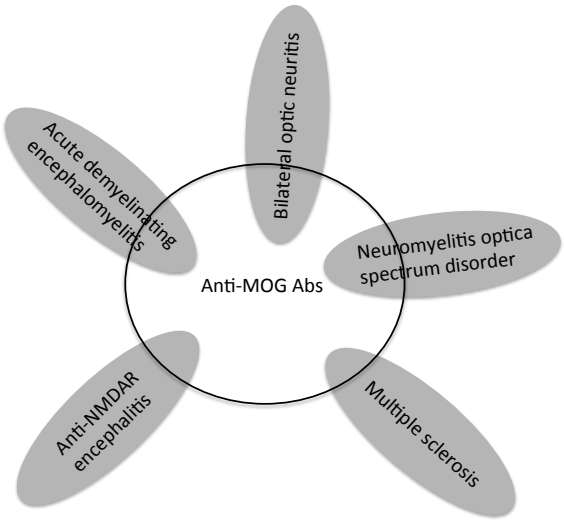

Figure 5. Overlapping features of MOG-antibody-associated CNS disorders. Anti-MOG Abs are detected in a proportion of patients with different clinically defined CNS diseases and Abs frequencies in these disorders are indicated by the extent of overlap in the diagram. Figure adapted after [131].

Anti-neurofascin auto-antibodies

There have been laborious attempts to identify and characterize candidate autoantigens in MS using different strategies. Using an unbiased proteomics approach two axo-glial antigens, neurofascin [107] and contactin [66], were recently recognized as novel targets of the autoimmune response in MS. Neurofascin is an adhesion molecule and exists in two isoforms: neurofascin 155 (NF155) that is expressed by oligodendrocytes and located at the paranodal axo-glial junction and neurofascin 186 (NF186), a neuronal protein located at the node of Ranvier (Figure 4). Both neurofascin isoforms contribute to the voltage-gated potassium and sodium channels clustering.

Mathey et al detected Abs against neurofascin approximately in one-third of MS patients, and in a T-cell adoptive transfer model of EAE neurofascin-specific Abs exacerbated EAE associated with increased axonal injury caused by the antibody binding to the node of Ranvier [107]. Anti-neurofascin Abs had also the capacity to impair neuronal transmission *in vitro*. Thus, these novel mechanisms of axonal injury observed in EAE might contribute to axonal damage in MS patients as well. Since then, anti-neurofascin Abs have been detected in patients with demyelinated disorders of the CNS and/or peripheral nervous system (PNS) such as chronic inflammatory demyelinating polyneuropathy [133,134], chronic inflammatory demyelinating polyradiculoneuropathy [135] and Guillain-Barre syndrome [136].

Contactin exists also in two isoforms: contactin-1 that is located at the paranodes and contactin 2, expressed by both neurons and glial cells and is localized in the juxtaparanodes

(Figure 4). Derfuss et al found that contactin-2 was recognized by both autoantibodies and Th1/Th17 cells in MS patients [137]. Additionally, adoptive transfer of contactin-2-specific T cells induced inflammation in the rodents' brain preferentially localized in the gray matter of the cortex and spinal cord. These observations paved the way for research on antibody-mediated cortical demyelination.

1.4.2 INNATE IMMUNITY

1.4.2.1 Antigen presenting cells (APCs)

APCs are involved in multiple stages during MS and EAE immunopathophysiology and comprise a wide range of functions from antigen uptake and presentation to T cells to cytokines secretion that drives T cell differentiation [138].

Perivascular macrophages (PVM) are able to express MHCII and it is now clear that their strategic location enables them to initiate and maintain neuroinflammation [139]. During MS and EAE, PVM up-regulate MHCII and co-stimulatory molecules such as CD80, CD86 and CD40 and their activation is mediated by IFNγ and TNF [139]. In addition, elimination of PVM suppresses clinical signs of EAE [140,141]. The finding that depletion of microglia in EAE was followed by reduced disease onset, progression and demyelination highlights the importance of microglia in EAE and MS pathogenesis [142]. Due to the abundance of microglia within the CNS, they are a powerful source of pro-inflammatory cytokines and chemokines important for maintenance of immune response. Phagocytosis of myelin by microglia and macrophages *in vitro* triggers pro-inflammatory cytokines, such as IL-6, IL-1 and TNF, and NO release [143-145]. It has also been shown that during EAE, IL-23 expression in CNS CD11b+ cells (macrophages, microglia and DCs) peaks early in disease, suggesting a critical role of these cell subsets during initiation of CNS inflammation prior to T cell infiltration [146]. Likewise, increased IL-23 and iNOs has been detected in microglia/macrophages of active MS lesions [147,148]. MS plaques have also been characterized by the presence of chemoattractant proteins secreted by microglia and macrophages, thus contributing in leukocyte infiltration into the CNS and enhance neuroinflammation [149].

The importance of DCs in MS has become evident by studies showing the presence of immature and mature DCs in the meninges and parenchyma of MS patients [150]. Furthermore, DCs contained myelin antigen deriving from demyelinating lesions has been observed in the cervical LNs of MS patients and monkeys affected with EAE implying that

DCs are able to process and present endogenous myelin [151,152]. The prominent role of DCs in promoting CNS autoimmune pathology was shown by adoptive transfer EAE experiments where CD11c+ DCs were the only APCs required for disease initiation and their numbers were correlated with disease severity [153].

1.4.2.2 C-type lectin receptors (CLRs)

A growing number of studies highlight the importance of innate sensors in MS pathogenesis [154]. Evidence suggests that recognition of pathogen-associated molecular patterns (PAMPs) and/or damage-associated molecular patterns (DAMPs) by altered pathogen recognition receptors (PRRs) might be key events during disease development [155]. PRRs, mainly expressed on myeloid cells, consist of four main categories: Toll-like receptors (TLR), Nod-like receptors (NLRs), RIG-I like receptors (RLR) and C-type lectin receptors (CLRs) [156].

CLRs are either transmembrane or soluble proteins characterized by a carbohydrate recognition domain (CRD) that is highly conserved among species [157] and has a calcium-binding pocket essential for carbohydrate binding. The CLR family now includes receptors that have domains homologous to CRD that do not necessarily bind carbohydrates or calcium [158].

Dcir1, Dcir2, Dcir3, Dcir4 and Dcar1 receptors

Dendritic cell immunoreceptor 1 (Dcir1), Dcir2, Dcir3, Dcir4 and dendritic cell immunoactivating receptor 1 (Dcar1) are highly conserved among species and mainly expressed in neutrophils and APCs [158]. CLRs consist of a CRD, a transmembrane region and a short cytoplasmic tail. The cytoplasmic tail of Dcir1-4 receptors contains an immunoreceptor tyrosine-based inhibitory motif (ITIM) that upon ligand binding becomes phosphorylated and regulates immune signaling via recruitment of SHP1 or SHP2 phospatases (Figure 5) [159]. On the other hand, Dcar1 is functionally associated with immunoreceptor tyrosine-based activating motif (ITAM)-bearing Fc receptor-γ (FcRγ) chain and signals through spleen tyrosine kinase (Syk) (Figure 6) [158,160]. The ITAM versus ITIM dichotomy has been useful in understanding the signaling of immunoreceptors but does not necessarily indicate activating or inhibitory outcomes [161]. Functional studies have made clear that CLRs serve as PRRs and upon activation they initiate a signaling cascade resulting in shaping innate and adaptive immune mechanisms, although their natural ligands have not yet been elucidated.

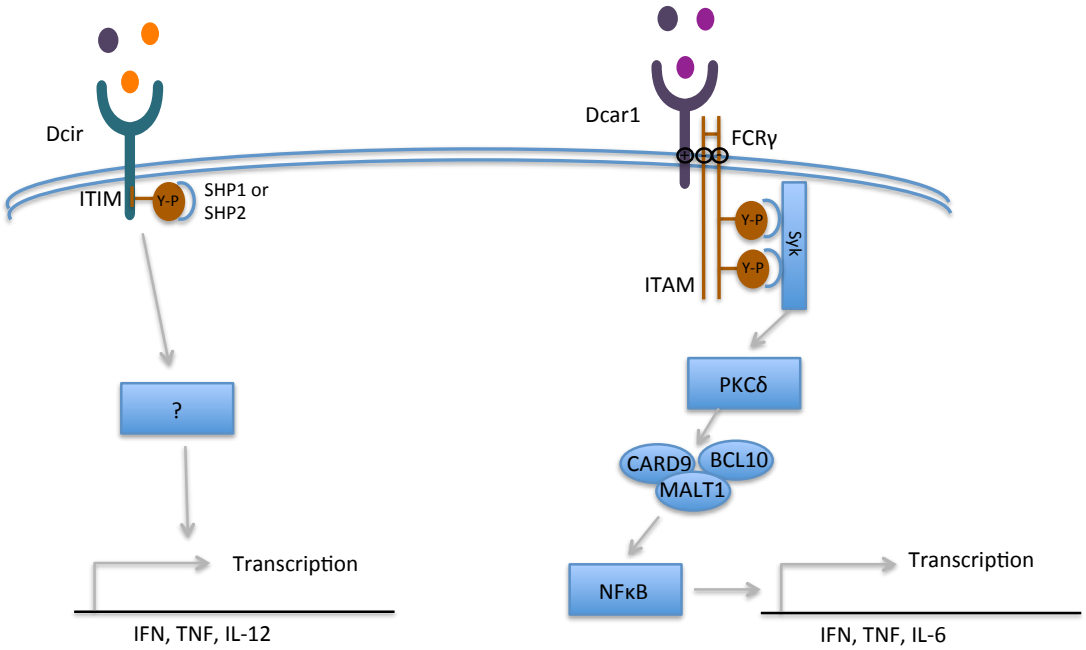

Figure 6. Schematic representation of signal transduction pathways of Dcir and Dcar1. Activation of Dcir leads to phosphorylation of its ITIM and SHP1 or SHP2 recruitment that in turn induces the activation of an unidentified signalling pathway leading to production of immunoregulatory cytokines. Activation of Dcar1 leads to SYK recruitment to the phosphorylated ITAM of the paired signaling adaptor FcRγ and induces a signaling cascade through the Card9-Bcl10-Malt1 complex. NFκB-driven transcription results in the production of immunoregulatory cytokines. FCRγ, Fc receptor-γ; Syk, phosphotyrosine kinase; PKCδ, protein kinase C-δ; ITAM, immunoreceptor tyrosine-based activation motif; ITIM, immunoreceptor tyrosine-based inhibitory motif; NFκB**,** Nuclear factor-κB. Figure adapted after [158,160].

Dcir receptors have been shown to be important in a number of autoimmune diseases (Table 2). A recent publication assessed the impact of Dcir2 in the initiation and progression of EAE [162]. They showed that *Dcir2* Knockout (KO) mice displayed more severe disease and had increased anti-MOG_{35-55} IgG titers compared to wild type (WT) mice. *Dcir2* KO mice also exhibited higher production of IFNγ and IL-17 and increased frequency of Th1 and Th17 cells. Interestingly, *Dcir1* KO mice were more severely sick during collagen-induced arthritis (CIA) and they displayed higher anti-collagen type II (CII) IgG1 Abs compared to WT [163]. Lack of Dcir1 predisposed aged *Dcir1* KO mice to develop spontaneous sialadenitis and enthesitis followed by increased IgG and IgM Ab levels, suggesting a protective role of the receptor. On the contrary, the pathogenic role of Dcir1 was demonstrated by studies showing that Dcir1 was essential for the development of experimental cerebral malaria [164]. Evidently, Dcir may exert both pathogenic and regulatory functions. Human *DCIR* polymorphisms have been associated with RA susceptibility and

primary Sjögren's syndrome in Swedish and Asian populations [165-167]. BDCA2, the human homologous to Dcar1 receptor, has been shown to exert regulatory functions during SLE by inhibiting type I IFN production [168] and a recent study suggested that lack of surface expression of BDCA2 receptor may be one of the genetic pathophysiological features in amyotrophic lateral sclerosis (ALS) [169].

Mcl and Mincle receptors

Macrophage C-type lectin (Mcl) and macrophage inducible C-type lectin (Mincle) are transmembrane proteins containing a short cytoplasmic tail, a transmembrane domain, an extracellular region and a single CRD [170]. *Mcl* and *Mincle* genes have been mapped to a cluster on rat chromosome 4, on mouse chromosome 6 and on human chromosome 12p13 [171,172].

Mcl and Mincle are mainly expressed on myeloid cells such as macrophages and neutrophils, DCs, monocytes, but their expression has also been observed in B cells and T cells and Mincle has been detected in neurons and endothelial cells as well [160-162]. Mcl has been shown to be a monocyte/macrophage endocytic receptor [173,174] and Mincle senses fungi and mycobacteria. It has been shown that both Mcl and Mincle are essential receptors for the recognition of mycobacterial glycolipid trehalose dimycolate (TDM) [175,176], a known innate immune stimulus that possesses strong adjuvant activity. Yamasaki et al showed that Mincle is also a macrophage sensor for damaged cells by recognizing a self ribonucleoprotein SAP130 released from dead cells and promotes inflammatory cell infiltration into the damaged tissue [177].

Mincle is associated with an ITAM-bearing FcRγ through a positively charged amino acid in its transmembrane region [177]. PAMPs or DAMPs recognition leads to ITAM phosphorylation and Syk recruitment that induces Bcl10-Malt1-Card9 signaling cascade resulting in NF-κB activation and induction of inflammatory cytokines such as TNF, IL-6, CXCL1 and CXCL2 (Figure 7) [176-178].

Lobato et al showed that Mcl interacts with FcRγ in an indirect way, as lack of a positively charged amino residue in Mcl transmembrane domain renders it incapable of directly associating with FcRγ [179]. They further showed that Mcl interaction with FcRγ requires the formation of Mcl-Mincle heterodimers and that co-expression of Mcl and Mincle led to a dramatic synergistic increase of anti-Mincle bead phagocytosis [180]. On the other hand, Miyake et al observed a Mincle-independent Mcl-FcRγ co-immunoprecipitation attributed

to the hydrophilic nature of threonine 38 that corresponds to the positively charged arginine of Mincle at this site [181]. The previous findings do not necessarily contradict each other, however crystal structure analysis of Mcl-Mincle heterodimers will hopefully shed more light on Mcl signaling. Subsequently, Miyake et al observed that Mcl positively facilitates both Mincle expression and its signaling [182]. In addition to this finding, Zhao et al have shown that Mcl serves as a molecular sensor of TDM leading to Mincle expression and that depends on NF-κB pathway activation (Figure 7) [183]. Whether formation of heterodimers between Mcl and Mincle expand ligand specificity or confer complementary functions to a single receptor [184] remains to be investigated in future studies.

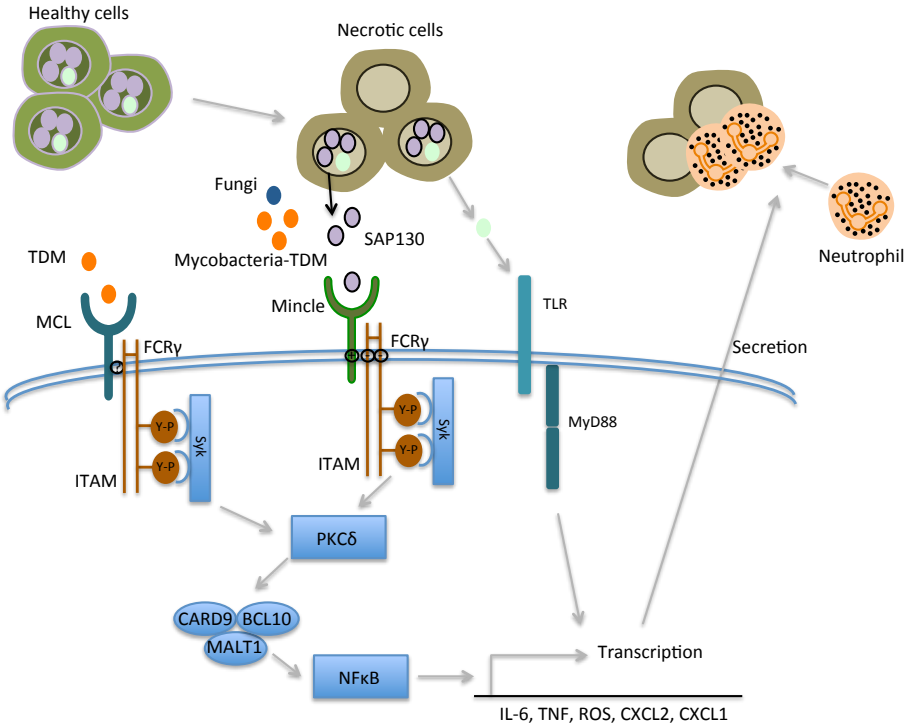

Figure 7. Schematic representation of signal transduction pathways of Mcl and Mincle. Activation of Mcl and Mincle leads to SYK recruitment to the phosphorylated ITAM of the paired signaling adaptor FcRγ and induces a signaling cascade through the Card9-Bcl10-Malt1 complex. NFκB-driven transcription results in the production of immunoregulatory cytokines and chemokines. FCRγ, Fc receptor-γ; Syk, phosphotyrosine kinase; PKCδ, protein kinase C-δ; ITAM, immunoreceptor tyrosine-based activation motif; TLR, Toll-like receptor; TDM, trehalose dimycolate; NFκB**,** Nuclear factor-κB. Figure adapted after [160,185,186].

Beyond fungal and mycobacterial infections, Mcl and Mincle have been implicated in a number of other CNS diseases, including EAE (Table 2). Miyake et al have suggested that Mcl but not Mincle has a prominent role during EAE development [181]. They observed that *Mcl* KO mice were almost completely protected against MOG-EAE whereas *Mincle* KO

Table 2. Summary of data on C-type lectin receptors and their genes involvement in autoimmune, inflammatory or CNS diseases.

Gene	Role in disease
Dcir1	*Dcir1* KO mice are more severely sick during CIA compared to WT [163].
	Aged *Dcir1* KO mice are predisposed to develop sialadenitis and enthesitis [163].
Dcir2	*Dcir2* KO mice are more severely sick during EAE compared to WT [162].
DCIR (*Dcir1-2* human orthologue)	Association to RA and primary Sjögren's syndrome [166,167].
BDCA2 (*Dcar1* human orhtologue)	Potential implication in amyotrophic lateral sclerosis [169].
	BDCA2 receptor has regulatory function in SLE [168].
Mcl	*Mcl* KO mice are protected during MOG peptide-EAE compared to WT [181].
Mincle	*Mincle* KO mice are partially protected during MOG peptide-EAE compared to WT [181].
	Mincle KO mice showed better outcome after ischemic stroke compared to WT [187]. Mincle, SAP130 and Syk levels are up-regulated after cerebral ischemia in mice. Mincle inhibition, by piceatannol, rescued cerebral ischemic damage [188].
	Mincle KO mice are less severely sick during experimental autoimmune uveitis [189].
	Mincle, Syk and SAP130 expression is increased after subarachnoid hemorrhage [190].
	Mincle receptor, SAP130 and Syk levels are elevated in patients with TBI [191].
	Mincle KO mice are protected against autoimmune hepatitis [192].

mice exhibited a partial protection after MOG peptide immunization compared to WT mice. In the CNS, it has been shown that Mincle plays a pivotal role in the pathogenesis of cerebral ischemia, subarachnoid ischemia and traumatic brain injury (TBI) both in animal models and humans. Specifically, *Mincle* KO mice showed a better disease outcome after stroke induction and the absence of *Mincle* conferred protection to neurons [187]. In addition, protein expression levels of Mincle, its ligand SAP130 and the downstream molecule Syk were upregulated after cerebral ischemia in mice. Moreover, up-regulation of both Mincle

and SAP130 followed similar patterns over time implying that SAP130 is the primary ligand of Mincle in this context [188]. In the same study, they also showed that Mincle is expressed in immune cells as well as in neuronal and endothelial cells in ischemic brains of both mice and humans. Mincle and SAP130 levels were elevated in the CSF of patients with TBI as well as Mincle and Syk protein levels were higher in injured brain tissue compared to controls [191].

Lastly, *MALT1* and *BCL10*, the downstream molecules that are induced after Mcl and/or Mincle activation, were found to be strongly associated to human MS in GWAS [41,193]. Though, how Mcl and Mincle receptors or the signaling pathway they are involved in, contribute to disease pathogenesis remains to be investigated.

1.4.3 DISEASE MOLECULAR MECHANISMS

Although the primary trigger of MS remains unknown, potential MS etiological mechanisms include autoimmune, infectious and degenerative causes with a focus on the immune responses [194]. Upon encountering an environmental stimulus e.g. an infectious agent, genetically susceptible individuals may respond with an autoimmune attack of CNS myelin based on molecular mimicry mechanisms between infectious and myelin antigens [195]. If this response becomes autonomous it may lead to epitope spreading to other CNS antigens resulting in a chronic recurrent condition such as MS [195,196].

Molecular mimicry is the activation of auto-reactive cells by cross-reactivity between self antigens and foreign antigens [197]. During infections, cross-reactivity of potentially auto-reactive T cells with infectious agents may lead to T cell activation and infiltration into the brain where they can attack CNS antigens. Studies have shown that EBV-specific CD4+ T cells and human herpes virus (HHV)-6 isolated from MS patients' CSF and blood respectively, cross-react with MBP [198,199]. Olson et al directly demonstrated that molecular mimicry did initiate virus-induced demyelination in the Theiler's murine encephalomyelitis virus (TMEV) model of MS [200-202]. They showed that demyelination was initiated by Theiler's virus-specific CD4+ T cells and secondary release of sequestered auto-antigens led to self-reactive T cell activation and damage of multiple myelin epitopes, such as PLP and MOG. Hence epitope spreading mechanisms sustained and enhanced disease progression.

Epitope spreading is the process where epitopes non-cross-reactive with the inducing antigen become major targets of an ongoing immune response [203]. Response may be directed against different regions of the same protein (intramolecular epitope spreading) or a distinct protein (intermolecular epitope spreading). Even though the verification of epitope spreading as a

potential mechanism in MS is not easy because the initial antigen is not known, there are some evidence that the phenomenon occurs in MS. Tuohy et al followed patients with isolated monosymptomatic demyelinating syndrome (IMDS) over the years and when patients progressed to clinically definite MS he detected T-cell autoreactivity to PLP epitopes other than those first observed [204-206]. The contribution of epitope spreading to the progression of autoimmune disease has also been shown in animal models. When mice injected with $PLP_{139-151}$, besides $PLP_{139-151}$-CD4+ T cell response, they exhibited $PLP_{178-191}$-specific CD4+ T cell reactivity during the first relapse and MBP_{84-104}-CD4+ T cell response during the second disease relapse [207,208]. The essential role of B cell epitope spreading in EAE has been shown in mice immunized with PLP antigen. These mice produced autoantibodies recognized not only the immunized antigen but also other myelin epitopes such as MOG and MBP [209].

Although there have been significant advances in MS research and molecular mimicry and epitope spreading are viable hypotheses that could explain how MS is initiated and progresses, we are still lacking strong evidence on MS etiology.

2 THESIS AIMS

The work in the present thesis aimed to investigate the genetic and immunological regulation of autoimmunity in experimental neuroinflammation. Antibodies contribution in multiple sclerosis pathogenesis is still under debate. Identification of antibodies genetic regulation and elucidation of risk gene variants and underlying molecular mechanisms will give insight in disease pathophysiology and may facilitate the development of diagnostic tools and therapeutic agents.

Specific scientific goals:

I. To investigate if anti-MOG and anti-neurofascin antibody response is genetically regulated in experimental autoimmune encephalomyelitis and to detect the genomic locations and/or genes regulating antibodies response.

II. To identify if anti-MOG and anti-neurofascin antibodies correlate with susceptibility and severity of clinical experimental neuroinflammation.

III. To delineate pathogenic mechanisms through which *C-type lectin-like receptor* (*APLEC)* genes, that were linked to anti-MOG antibody response and encode C-type lectin receptors, modulate experimental autoimmune encephalomyelitis.

3 METHODOLOGICAL CONSIDERATIONS

3.1 GENETIC DISSECTION

Although the field of human genetics has been rapidly evolving and GWAS have identified strong candidate genes in human MS, the use of animal models in detecting risk gene variants and elucidating disease molecular pathways is invaluable [210]. In traditional reverse genetics the focus is to investigate the function of certain candidate genes using genetically engineered animals. However, many genes and pathways relevant to disease might not be identified using this hypothesis-driven approach. In our laboratory we are using positional cloning i.e. forward genetics methodology to identify natural genetic variants, likely to be relevant in other species as well, and molecular pathways in an unbiased approach [211]. Positional cloning is a procedure that detects a gene, responsible for a phenotype, based on its location in the genome, using methods such as linkage analysis and association mapping.

3.1.1 LINKAGE ANALYSIS

Genetic linkage analysis, based on the observation that genes physically close have the tendency to remain linked during meiosis, identifies genes that are inherited together with disease [212]. Linkage mapping is the statistical method that is used to identify quantitative trait loci (QTLs) responsible for a certain trait/phenotype. Polymorphic genetic markers e.g. microsatellite (di-, tri- or tetra-nucleotide repeats) or single nucleotide polymorphisms (SNP) are utilized to follow certain genomic locations and link them to the trait.

Linkage analysis is performed in R/qtl package [213]. We typically compare non-parametric, multiple-QTL and Haley-Knott (HK) model and if they all generate the same results we continue with HK. HK which is a simple regression method and the only model that allows introduction of covariates and interactions is a powerful tool for QTL mapping in experimental crosses between inbred lines [214]. In Haley-Knott method, multiple markers are analyzed simultaneously and the genotypes in between are predicted based on their recombination frequency thus providing good estimates of QTL location. The statistical significance of the linkage is expressed by the logarithm of odds (LOD), given as log value on the basis of 10, and estimates the likelihood of “QTL linkage” compared to “no QTL linkage”. For example, an identified QTL with LOD score of 3 indicates that the odds are 1,000 that there is a QTL at this location versus that there is no such QTL. The potential location of a QTL is defined by the confidence interval (CI) [215]. A 95% CI means that there is a 0.95 probability that the true location of the QTL is within the CI.

Genetic linkage mapping in experimental rat crosses is possible due to the differences at a given phenotype/trait i.e. EAE susceptibility and/or antibody response, that different inbred rat strains comprise, reflecting the variations in their genetic background. We use populations that derive mainly from DA and PVG rat strains, EAE-susceptible and resistant respectively, comprising the same MHC genes, thus enabling the detection of non-MHC genomic regions responsible for certain phenotypes.

3.1.2 EXPERIMENTAL CROSSES

3.1.2.1 Backcross (BC) and advanced intercross line (AIL)

The rate of recombination events during meiosis determines the size and position of an identified QTL and the use of experimental crosses between inbred rat strains increases the power of linkage mapping due to the higher recombination events that these populations accumulate [216]. Linkage mapping in a backcross (BC) population (Figure 8), derived from interbreeding F1 hybrids with either one of the parental inbred rat strains and characterized by few recombination events, leads to relatively wide QTLs (Figure 10) with high impact on trait, though with low number of genetic markers. To narrow down a certain QTL mapped in the BC populations, in this thesis we have used advanced intercross line (AIL) in the 10^{th} generation (G10). An AIL is obtained by random breeding of two inbred rat strains for several generations avoiding brother-sister mating (Figure 8) [217]. That increases the recombination events and leads to higher resolution mapping identifying QTLs of smaller confidence intervals (CI) (Figure 9). However, to get sufficient power to detect a QTL in an AIL, higher marker density is required compared to a BC. In Papers I and II, we used two BC populations (DABC and PVGBC) deriving from DA and PVG rat strains to genetically map anti-MOG and anti-neurofascin antibody response and in Paper I additionally we used a DAxPVG G10 AIL to fine map a QTL regulating anti-MOG antibody levels.

3.1.2.2 Congenic strains

Congenic rat strains are used to validate if the positionally-cloned (in BC or AIL) phenotype-regulating QTL has a biological relevance for the trait [218]. Congenic lines are established by repetitive backcrossing the genomic fragment of interest from one of the parental inbred strains (donor) onto the background genome of the other parental strain (recipient) (Figure 8). Approximately ten generations of backcrossing are required to minimize the contamination of the donor genome outside the genomic fragment of interest.

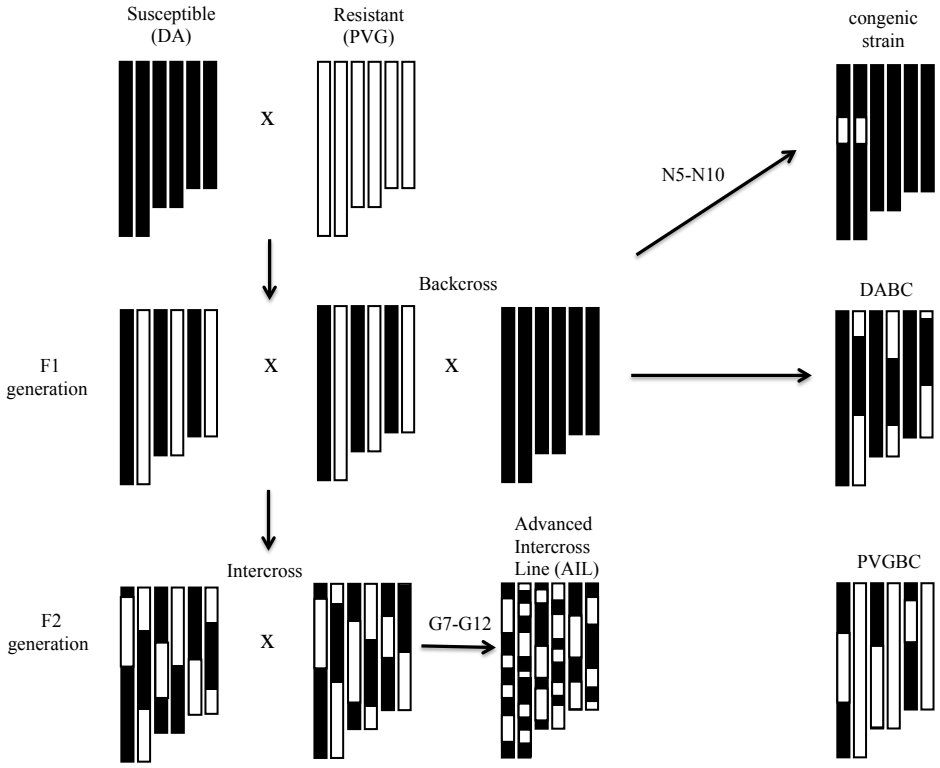

Figure 8. Schematic illustration of population breeding design. Experimental populations can be generated by breeding EAE-susceptible DA with EAE-resistant PVG rat strains. Initial F1 generation is obtained by crossing the parental strains. The backcross population (BC), permitting rough mapping of quantitative trait loci (QTLs), is generated by backcrossing F1 individuals with either parental strain. An advanced intercross line (AIL) can be used to narrow down the identified QTLs and is produced by random breeding of two inbred rat strains for several generations avoiding brother-sister mating. Congenic lines, enabling validation of the linked QTL and functional characterization, are obtained by repetitive backcrossing with the selection of the region of interest.

Besides congenic lines' use in fine genetic dissection and validation of the phenotype, congenic strains also represent a powerful tool for mechanistic studies. The functional role of the region and/or candidate gene(s) can be further investigated in genetically identical congenic rats that are easy to reproduce, and the gene responsible for the phenotype may be determined.

In Papers I and IV, we used congenic rats to validate the Ab response-regulating QTL, identified in BCs and AIL, and to further functionally characterize the region and elucidate the risk gene(s). The congenic rat strains that we used originated from introgression of alleles

of the fragment of interest from the resistant PVG strain onto the genetic background of susceptible DA strain.

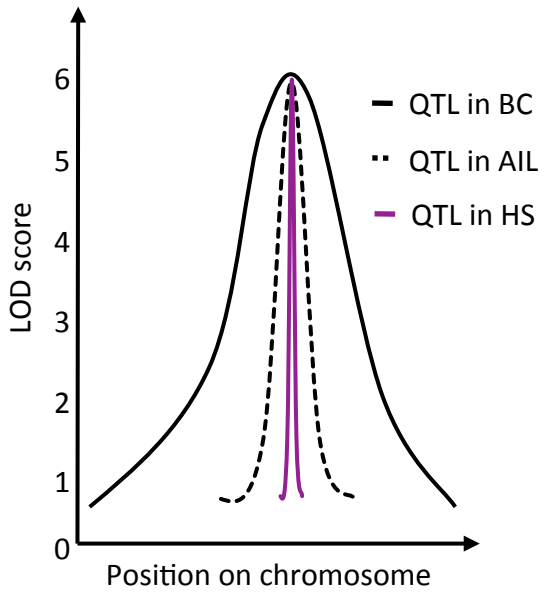

Figure 9. Comparison of QTL mapping resolution in different rat populations. Mapping in backcross (BC-black solid line) results in broad QTLs harboring many genes while an advanced intercross line (AIL-dashed black line) narrows down identified QTLs. A heterogeneous stock (HS-purple line) population enables resolution exponentially higher compared to an AIL and may identify regions smaller than a cM. LOD, logarithm of odds.

3.1.2.3 Heterogeneous stock (HS)

The Heterogeneous Stock (HS) is an outbred rat population descended from eight inbred founder strains: ACI/N, BN/SsN, BUF/N, F344/N, M520/N, MR/N, WKY/N, WN/N [219] (Figure 10). These strains, whose genomic sequences are known, were inter-crossed for 62 generations according to a standard out-breeding rotational schedule to minimize the extent of inbreeding, drift and fixation [220]. The increased number of recombination events that have accumulated over the generations leads to rats with unique genetically random mosaic of the founder genomes, attempting to approach the complexity of human population, and increases the potential for high-resolution mapping [221] (Figure 9).

HS provides resolution of genetic mapping exponentially higher compared to an AIL (Figure 10) and theoretically permits mapping of small-impact regions and QTLs with CI smaller than a centimorgan (cM) [222,223]. Additionally, the variable MHC regions, that the founder strains of HS population carry, enable the identification of both MHC and non-MHC genes. In Paper III we used a rat HS to genetically map anti-MOG and anti-neurofascin IgG response.

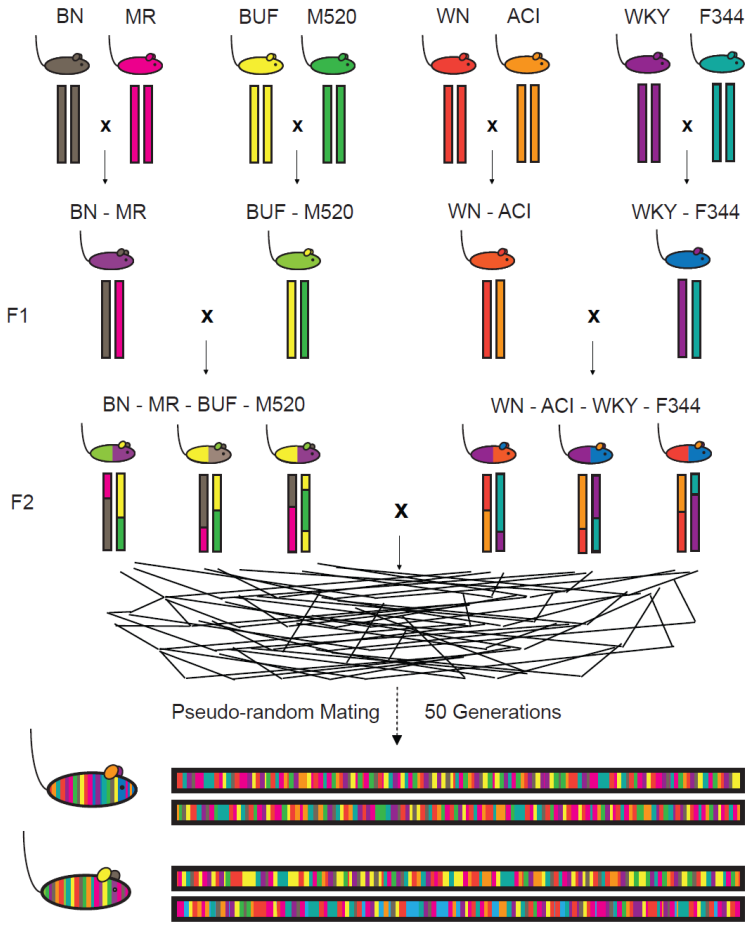

Figure 10. Schematic illustration of rat heterogeneous stock breeding design. The rat heterogeneous stock (HS), offering high-resolution gene mapping, is an outbred population descended from eight inbred rat strains intercrossed for 62 generations according to a standard outbreeding rotational scheme that leads to individuals with increased number of recombination events.

3.1.3 ASSOCIATION ANALYSIS

The population structure of HS rats with different degrees of genetic relatedness requires novel statistical approaches [224]. Analytical methods and statistical packages, developed specifically for mouse HS populations were also used for genome-wide association analysis of our rat HS [225,226]. Anti-MOG and anti-neurofascin antibody response was analyzed using mixed-models, an approach that controls the false positive rate of association by diminishing the estimated effect and significance of loci that are predictive of relatedness [227].

3.2 EAE MODELS

Studies on this thesis are mainly based on the chronic relapsing-remitting model of EAE induced in DA rats by immunization of recombinant MOG_{1-125} protein with incomplete Freund's adjuvant (IFA). MOG is a unique myelin autoantigen as it induces not only encephalitogenic T-cell response but also demyalinating autoantibody response [56]. Upon MOG immunization, CD4+ T cells are primed in the inguinal LNs by DCs that present

MOG and primed T cells migrate through blood circulation into the CNS. T cells get re-activated by local APCs and enter the parenchyma where soluble mediators secreted by T cells, macrophages and microglia, and auto-antibodies secreted by B cells trigger demyelination [58,228].

In the projects of this thesis, we have used two main rat strains, EAE-susceptible DA and EAE-resistant PVG strain, populations and congenic strains that have derived from their intersections. Within 10-12 days after MOG immunization, DA rats develop clinical signs of the disease (Figure 11) characterized by ascending paralysis starting by tail weakness and followed by hind leg and subsequently front leg paresis. A common observation prior to paresis is loss of body weight. The severity of motor signs is scored by a scale ranging from 0 (healthy) to 5 (death) and we assess EAE phenotypes such as incidence, onset, duration, maximum and cumulative score and weight loss, that reflect either susceptibily or severity of disease. Conversely, PVG rats, sharing the same MHC alleles with DA rats, are relatively resistant under the same induction protocol (Figure 11). The differential EAE-susceptibility of DA and PVG rat strains is due to infuences from non-MHC genes and populations originating from DA-PVG intersections facilitate gene identification and their functional characterization.

In one of the studies of this thesis, we have also used the myelin basic protein (MBP)-EAE model. EAE induced by active immunization of guinea pig MBP 63-88 peptide with complete Freund's adjuvant (CFA) is acute, monophasic, self-limited disease (Figure 11), characterized by extensive T cell infiltration in spinal cord and brain stem with, however, very little observable demyelination [229].

3.3 RATS VERSUS MICE

Despite the fact that rat had lagged far behind the mouse as a "genetic model", improvement and development of rat genome resources led to a new era where rat has become a powerfool organism for gene discovery [230]. The most important advantage of using rats in our research is the milder protocol required for EAE induction and the high reproducibility of the model compared to the mice that CFA and/or pertussis toxin [231] are needed to induce disease and the model is not stable. Other benefits of rats versus mice include their bigger size that results in easier animal handling and higher number of acquired cells.

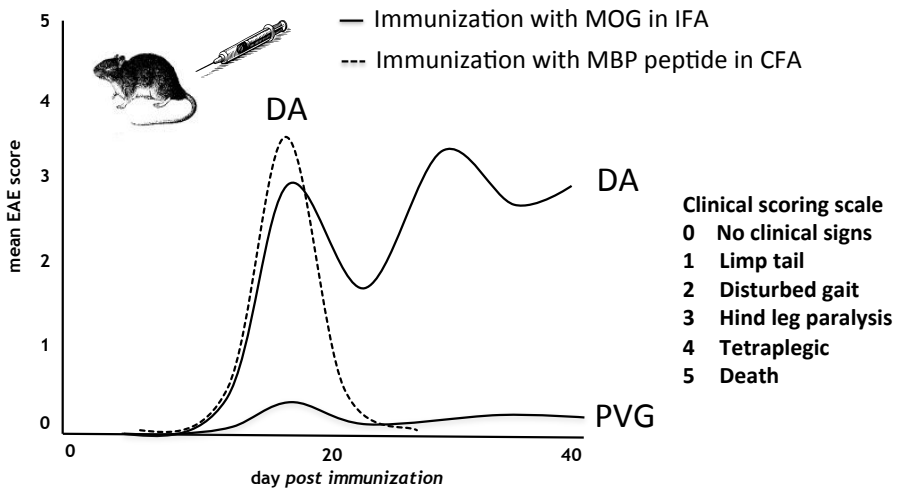

Figure 11. MOG-EAE and MBP-EAE. Upon immunization with MOG in IFA, EAE-susceptible DA rats develop a relapsing-remitting pattern of the disease displaying first clinical signs within 10-12 days while PVG rats are relatively susceptible (black solid line). On the other hand, upon immunization with MBP peptide in CFA, DA rats comprise a short, monophasic disease (dashed black line).

3.4 TRANSLATIONAL APPROACH

Our translational research approach is based on the assumption that there are conserved mechanisms between the species. Thus, once forward genetics approach and functional studies in rat EAE identify a risk gene and/or the underlying molecular pathway, these can be further tested for association in human MS (Figure 9). So far, this approach has been feasible in our laboratory and has proven relevant to human disease, identifying gene variants such as *MHC2TA* [232], *VAV1* [233], *CCL2* [234], *RGMA* and *IL21R* [235] that are important for both EAE and MS. When a disease gene is found relevant for MS, it is possible to go back to the experimental models to explore further the pathophysiological mechanisms, studies not possible in humans, and to develop relevant therapeutics.

4 RESULTS AND DISCUSSION

Autoantibodies role in MS pathophysiology is a field of immense research though antibodies relative functional roles and contribution to disease pathogenesis is not clear. Results presented in this thesis include the characterization of genetic regulation of anti-MOG and anti-neurofascin Abs response and their correlation with disease susceptibility and severity. Additionally, the role of *APLEC* genes, genetically linked to anti-MOG Ab response, and the underlying molecular pathways they control during EAE have been dissected and results are presented and discussed here.

4.1 ANTIBODIES IN EAE

4.1.1 Polygenic regulation of anti-MOG Abs response

To investigate if anti-MOG Ab levels are genetically regulated, we mapped their response in two backcross and one HS rat populations. Genome-wide linkage analysis in 421 DABC and 471 PVGBC rats and genome-wide association analysis in 1524 HS rats revealed a number of QTLs regulating anti-MOG IgG and IgG isotypes. This polygenic and genetically heterogeneous regulation of EAE phenotypes, shown in previous studies as well [236-238], recapitulates the complex nature of human MS, suggesting that similar genetic mechanisms may control susceptibility to develop autoimmune neuroinflammation in several species and providing evidence for the relevance and importance of this experimental model to study human disease. Additionally, we observed that few of the identified anti-MOG Ab-controlling QTLs overlap with EAE clinical phenotypes in the BCs [239] and HS, suggesting that common molecular pathways might be responsible for EAE and Abs regulation and providing evidence for the role of anti-MOG Abs in disease development.

Likewise, few of the detected QTLs overlap with QTLs identified to regulate other inflammatory phenotypes in different experimental models and potentially could be controlled by the same gene. For example, *Amig3* alleles, the major regulators for anti-MOG Abs identified in the BCs, overlap with *Oia2*, *Cia13*, *Pia7* regulating oil-, type II collagen- and pristane-induced arthritis respectively, and *Iddm14* linked to glucose levels in T1D [165,240-242]. Interestingly, in the biggest MS GWAS performed in 2011, one third of the identified loci overlapped with regions previously associated with other autoimmune diseases such as RA, T1D, Crohn's disease and others [41]. During different autoimmune diseases the clinical outcome might be different, though certain pathogenic mechanisms might be common, a notion supported also by data showing that MS patients may suffer from other autoimmune diseases such as T1D and psoriasis [243,244]. Importantly, high homology and conserved

pathways between species, reflected by studies where molecular mechanisms characterized in human disease after being identified in rodents, enables human gene identification through also a translational approach.

4.1.2 Anti-MOG Abs correlate with disease susceptibility and severity

To investigate if anti-MOG IgG and IgG isotypes associate with disease, we first compared Ab levels between rats affected with EAE and healthy rats. In the BC populations, affected rats exhibited higher anti-MOG IgG levels compared to healthy population, at both time points, onset and late phase of the disease, with the differences being more pronounced during onset. Anti-MOG IgG2b and IgG2c response was also increased in the sick rats whereas anti-MOG IgG1 levels were higher in the sick versus healthy rats only at onset of EAE, and at the late phase healthy rats displayed higher anti-MOG IgG1 levels. In the HS population, we observed increased anti-MOG IgG, IgG1 and IgG2b levels in the sick rats versus healthy, but no difference in anti-MOG IgG2c titers between the two groups.

To evaluate if anti-MOG Abs affect EAE severity we studied how Abs correlate with clinical EAE phenotypes. Overall in the BC and HS populations, we observed positive correlations between anti-MOG IgG, IgG2b and IgG2c and clinical parameters of EAE such as maximum and cumulative score. Thus our data strongly supports that the presence of anti-MOG Abs associate with disease susceptibility and severity, though we did not address whether anti-MOG Abs contribute to disease pathophysiological mechanisms or if Abs response is a pure secondary epiphenomenon. Interestingly, the emerging picture is that anti-MOG Abs, measured by ELISA, recognizing linear or denatured epitopes cannot bind MOG on the cell surface in vivo thereof are not pathogenic [245,246], while conformation-specific Abs can directly contribute to demyelination [112,247].

A number of data now implicates MOG as a crucial myelin autoantigen in demyelinated disorders including ADEM, optic neuritis and transverse myelitis mainly in children but also in adults [132]. Although there may be early evidence suggesting anti-MOG autoantibody-dependent demyelinated mechanisms, the literature is still lacking in the area of the effector mechanisms of anti-MOG Abs in MS autoimmunity.

4.1.3 Th2 predominance confers protection in EAE

Interestingly, healthy rats displayed increased ratio of anti-MOG IgG1/IgG2b compared to sick rats at all time points after immunization in the BC populations. In addition, APLEC congenic strains, that were resistant upon EAE induction, displayed increased levels of anti-

MOG IgG1 and IgG1/IgG2b ratio compared to DA control rats (Figure 12). Taken together, these data imply a protective role of anti-MOG IgG1 versus a pathogenic role of anti-MOG IgG2b during EAE development. In rat, IgG1 subtype has been assigned to the IL-4-producing Th2 phenotype while IgG2b and IgG2c are associated to IFNγ-producing Th1 dependent response [248,249]. Additionally, in rodents IgG1 isotype lacks complement-activating functions and exhibits lower activatory/inhibitory ratio compared to IgG2b, and IgG1 protective role has also been shown in experimental models of SLE [250,251].

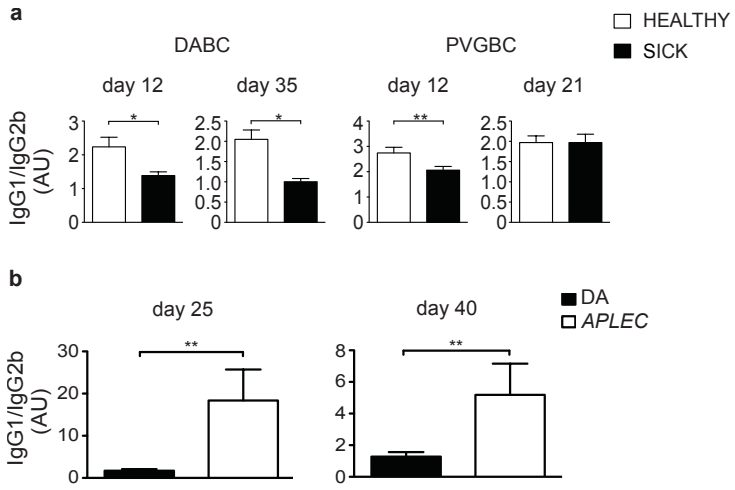

Figure 12. Th2 predominance has protective effect. Anti-MOG IgG1/IgG2b ratio is higher in a) healthy versus sick rats in DABC and PVGBC at onset and late phase of EAE and b) in APLEC congenic rats compared to DA control rats at day 25 and day 40 after MOG immunization. APLEC rats remain unaffected while DA rats get sick upon EAE induction. AU, arbitrary units.

The Th1-Th2 paradigm in EAE and MS has been under extensive research though with contradictory data. Adoptive transfer of Th1 cell clones was shown to be sufficient to induce EAE, while Th2 clones of the same specificity were not [70,252]. Additional studies showing that Th2 cells may inhibit encephalitogenic CD4+ T cells underline the protective role of Th2 subtypes versus Th1 during disease development [253]. Moreover, a variety of immune-modulating agents exert their therapeutic properties by shifting the balance towards a Th2 phenotype [254-256]. On the other hand, Th2 cells have been described in Pattern II MS lesions questioning their protective role [257] and Genain et al reported that shifting autoimmunity towards Th2 pattern exacerbated EAE in marmosets via the production of pathogenic auto-antibodies [258].

Evidently, shifting the balance away from Th1 towards a Th2 response might not be without complications, though our data support the notion that such a Th2 skewed response

may confer protection in the context of EAE. Likewise, the differential genetic regulation of anti-MOG IgG1 and anti-MOG IgG2b and IgG2c by MHC and non-MHC genes respectively in the HS population, suggests that distinct molecular pathways may control Th2 and Th1 responses. Nonetheless, especially after the discovery of Th17 cells as key players in disease pathogenesis, and considering the enormous plasticity of T-cell responses, such a Th2 shift is not a simple option.

4.1.4 Anti-neurofascin Ab titers associate with disease severity during MOG-EAE

To investigate the epitope spreading to neurofascin in different EAE models we immunized DA rats either with MOG or MBP_{63-88} peptide. As previously shown, immunization of DA rats with MOG leads to T and B cell activation as well as monocyte/macrophage recruitment, with extensive demyelination and both immunoglobulin and complement deposition in lesions [259,260]. In our study the prolonged disease course in MOG-immunized rats correlated with the generation of anti-rrNF IgG. On the other hand, the paralysis observed in MBP_{63-88}-induced EAE, which is a monophasic self-limited disease, likely reflects blood-brain barrier breakdown, cellular infiltration and edema, rather than demyelination, [261] and no sizeable epitope spreading to neurofascin could be observed. A key, speculative notion here is that an EAE-driving autoantigen such as MOG (but not MBP peptide) needs to cause a conspicuous damage to myelin [262] to, in turn, expose the immune system to axonal antigens, such as neurofascin, in order to elicit a further deleterious autoimmune response which may be involved in disease progression. Such a scenario is consistent with the previous observations of a more frequent presence of anti-neurofascin antibodies in progressive MS [263]. How and where the spread of reactivities for the B cell compartment occur remains to be established. Our study however, highlights that the intermolecular epitope spread can also occur from an antigen exclusively expressed on oligodendrocytes (MOG) to an antigen whose expression is shared by oligodendrocytes and neurons (neurofascin), which in turn could lead to further epitope spreading to neuronal antigens.

Besides demyelination, MS is characterized by damage to axons/neurons [264]. Furthermore, in a histopathological study neural damage was always connected to inflammation [265], which at some point continues independently from the initial immune attack and can contribute to further, and permanent disabilities. It is currently not completely clear what leads to axonal injury but epitope spreading to axonal antigens, among other factors, might account for the neurodegenerative processes and axonal pathology. Anti-neurofascin

antibodies from MS patients have been shown to cause axonal injury in EAE in a complement-dependent manner [263,266].

Interestingly antibodies reacting to neurofascin in our study do cross-react with another recently identified target in MS, contactin-2, which is located in the juxtaparanodes [267]. If responses to these proteins overlap, it might mean that these would constitute hot-spots for immune reactivity by both T and B cell reactions to oligodendrocytes and neuronal cells, leading to inhibition of nodal function and possibly exacerbation of axonal damage.

It has been shown in animal studies that epitope spreading is responsible for relapses and also leads to increased disease severity and chronicity [203]. In our DA backcross population anti-neurofascin IgG and IgG2b titers significantly correlated with more severe disease, as represented by higher cumulative and maximum score, longer disease duration and higher weight loss in rats with anti-neurofascin reactivity compared to rats with no Ab response to neurofascin (Figure 13). Thus our data strongly supports that the presence of an anti-neurofascin response correlates with heightened severity, though if anti-neurofascin Abs contribute to axonal pathology or if epitope spreading to neurofascin is a reflection of an exacerbated disease is not clear.

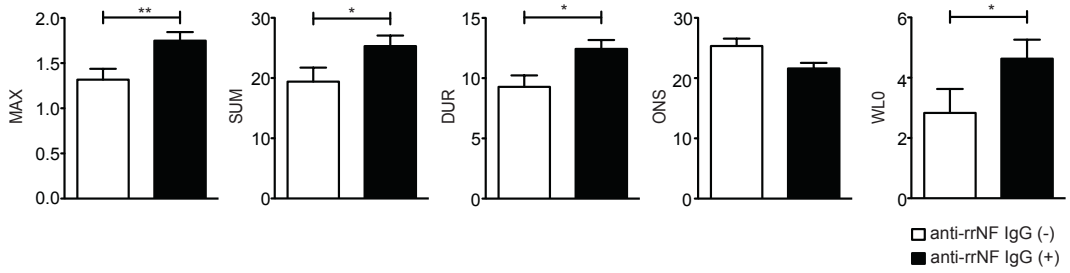

Figure 13. Anti-neurofascin antibodies correlate with EAE severity. DA backcross rats with anti-neurofascin Abs (anti-rrNF IgG (+)) are more severely sick compared to rats without anti-neurofascin reactivity (anti-rrNF IgG (-)) as indicated by higher maximum (MAX) and cumulative (SUM) EAE score, longer duration of disease (DUR), earlier onset of the disease (ONS) and lower weight loss (WL0).

4.1.5 Anti-neurofascin Abs are genetically regulated

In our genome-wide linkage analysis, we identified a QTL on chromosome 3 that is linked to anti-neurofascin IgG2b isotype-specific response in 174 female DA backcross rats, suggesting that anti-neurofascin reactivity is under genetic regulation. Interestingly, this

locus on chromosome 3 overlaps with QTLs that have been detected in different crosses such as DABC, PVGBC, AIL and HS, that regulate various EAE phenotypes such as EAE severity (in the DABC, PVGBC and AIL G10) and anti-MOG IgG (in the DABC), IgG2b (in the AIL G10) and IgG2c (in the PVGBC and HS) response. Our genome-wide association analysis in the HS population identified only MHC regions, and specifically MHC classes III, II and Ia, on chromosome 20, to control anti-neurofascin IgG response, implying that the influence of non-MHC alleles may be diminished when MHC regions exert an effect. These MHC loci were also linked to anti-MOG IgG and IgG1 response in the HS population. Taken together, these data imply that genetic polymorphisms in the loci identified on chromosomes 3 and 20, may influence general, rather than neurofascin-specific auto-antibody response and/or other autoimmune mechanisms that regulate EAE severity.

Our findings are interesting in view of the current development in MS genetics. So far, only risk loci controlling incidence of MS have been described, likely due to poor methods to measure MS severity on a large scale, as well as lack of appropriate intermediate sub-phenotypes. The data shown here encourages further attempts in MS, on a genome-wide level to study genetics in relation to severity and to sub-phenotypes.

4.2 GENETIC MAPPING IN HETEROGENEOUS STOCK

4.2.1 HS identified both MHC and non-MHC regions and pinpointed candidate genes

We also used a unique population, the rat HS, that offered the opportunity to map Abs response in rats with mixed MHC types, not possible in any other population we used in this thesis as they carry the same MHC alleles. Thus HS population enabled genetic dissection of both MHC and non-MHC regions-regulating Abs response. HS rats, that have accumulated high number of recombination events over the generations, offered the potential of high-resolution mapping compared to other populations used, and allowed us to pinpoint candidate risk genes.

Among the five regions within the MHC locus associated to anti-MOG Abs response, MHC class II and Ia alleles were the major regulators and MHC class II displayed the strongest association. That is consistent with findings from human case-control cohorts where MHC II alleles exert the highest impact on disease. Identified MHC class II QTL contains 35 genes, highly conserved among mammals [268], including the ones encoding the beta chain of

RT1-Db1, whose human homologs, HLA DRB1 and DRB5 are the major risk genes for MS. Additionally, MHC II QTL was previously associated to CD4:CD8 T cell ratio in blood from naïve HS rats [269]. *Tap-2* alleles were identified to be responsible for the T cell effect and *Tap-2,* which is at the border of the identified MHC II QTL, may also influence Abs response.

MHC class Ib QTL that mainly controls anti-MOG IgG1 thus Th2-associated response, consists of a single gene, *RT1-CE10*, that encodes for the H2 class I histocompatibility antigen alpha chain, involved in antigen processing and presentation via MHC class I. We identified a SNP in *RT1-CE10* that results in an amino acid change, from arginine to lysine, that in some contexts may be harmful [270].

Non-MHC regions were identified to control mainly anti-MOG IgG2c and IgG2b, related to Th1-driven response. Among the genes in the associated non-MHC QTLs we pinpointed *IgKc* on chromosome 4 and *Ccr7* on chromosome 10 as candidate genes controlling anti-MOG Abs response. *IgKc* encodes the constant domain of kappa light chains of antibodies and in humans *IGKC* has been associated with different types of cancer [271,272]. *Ccr7* encodes for CCR7 chemokine receptor. CCR7+ myeloid cells have been detected in MS brain lesions and CSF [273,274], and CCR7 expression has been shown to be up-regulated in the CNS of EAE animals [275,276]. Though, how candidate genes influence Abs response and/or EAE pathogenesis remains to be investigated.

4.3 C-TYPE LECTIN RECEPTORS IN EAE

4.3.1 *APLEC* (*antigen-presenting lectin-like*) gene cluster was the major regulator of anti-MOG Ab response

Although anti-MOG Ab response was controlled by several loci in the BC populations, *Amig3* alleles were the major regulators in both BCs and both at onset and late phase of EAE. Though *Amig3* locus spanned a region of several Mb harboring several genes. To fine-map *Amig3* QTL, we used an AIL in the 10th generation. The AIL analysis narrowed-down *Amig3* into a region of approximately 0.5 Mb with LOD score of 38. In AIL, *Amig3* was linked to anti-MOG IgG and IgG1 and PVG alleles predisposed for higher Ab levels (Figure 14).

To validate the QTL's effect identified in BCs and AIL populations we used APLEC congenic strain. APLEC congenic originates from introgression of resistant PVG *Amig3* alleles into the DA susceptible background. *Antigen-presenting lectin-like* (*APLEC)* locus

harbors a cluster of genes (*Dcir4*, *Dcir3*, *Dcir2*, *Dcir1*, *Dcar1*, *Mcl* and *Mincle*) encoding C-type lectin receptors (CLRs) that are expressed mainly in APCs and are important innate sensors. Anti-MOG IgG1 and ratio IgG1/IgG2b was increased in APLEC versus DA control rats both at onset and late phase of disease and APLEC rats were drastically protected upon MOG immunization implying common molecular pathways.

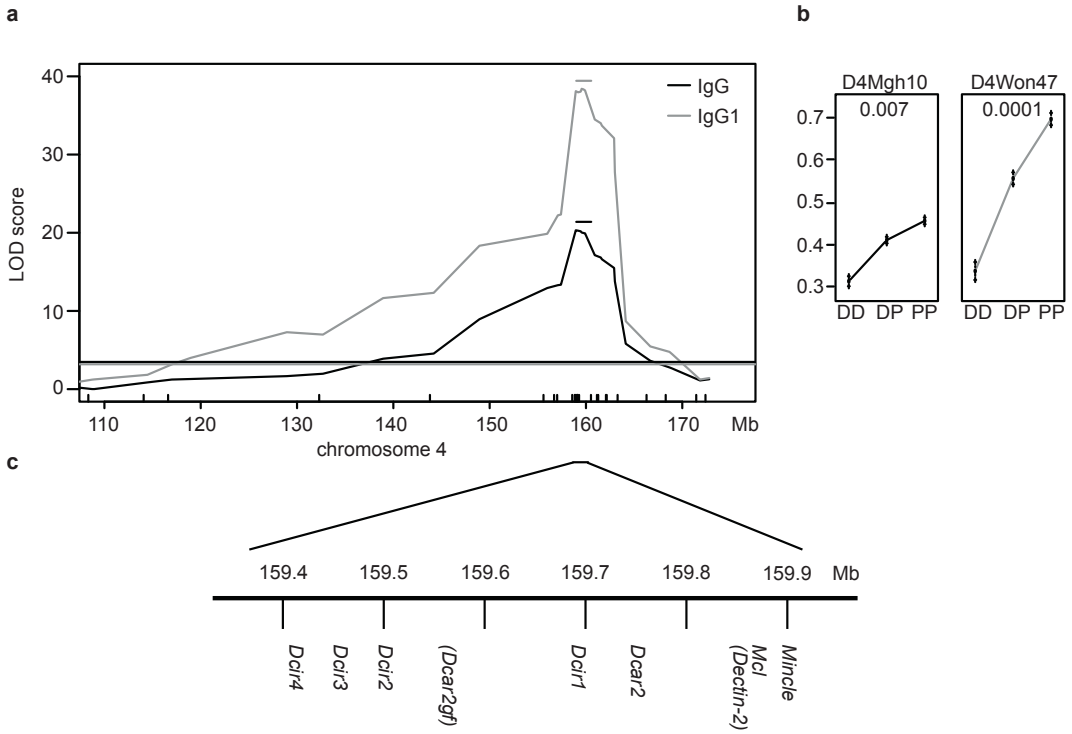

Figure 14. Mapping in AIL G10 narrowed down *Amig3* locus and identified C-type lectin genes as the major regulators of anti-MOG Ab response. a) *Amig3* locus was fine-mapped into a 0.5 Mb region with LOD score of 38 and was linked to anti-MOG IgG and IgG1 response. b) PVG alleles predisposed for higher Ab levels. c) *APLEC* locus harbors a cluster of C-type lectin genes. DD, DA/DA; DP, DA/PVG; PP, PVG/PVG.

4.3.2 *APLEC* genes involvement in EAE

To elucidate how resistant PVG alleles in the *APLEC* locus confer protection upon MOG immunization we analyzed cell numbers in the peripheral compartment i.e. the inguinal lymph nodes, where priming of the disease takes place, seven days p.i.. We observed increased B cell numbers in the APLEC compared to DA control rats, while T cells and TNF-producing T cells were decreased in the APLEC congenic strain. Anti-MOG IgG1 titers and anti-MOG IgG1/IgG2b ratio were also increased in the sera of the congenic rats.

The impact of *APLEC* alleles on the B cell compartment has previously been shown in an arthritis model using different inbred rat strains [277]. Moreover, Guo et al showed that APLEC congenic rats displayed higher serum IgG1 Ab titers against CII in experimental arthritis [278]. In the same study, they showed that PVG alleles in the *APLEC* locus decreased IL-17, IL-1β (associated to Th17) and IFNγ (associated to Th1 response) mRNA levels in the LNs 10 days after CIA. A plausible hypothesis in our model (Figure 15), is that upon MOG immunization PVG alleles in the *APLEC* locus may modulate the expression of molecules such as cytokines involved in T cell differentiation and skew T cells towards a Th2 response as indicated by increased anti-MOG IgG1 and IgG1/IgG2b ratio, thus conferring protection. Since IgG2b isotype but not IgG1 activate the complement system [279], IgG1 may have less encephalitogenic properties thus decelerating EAE development. The diminished Th1/Th17 polarization is also indicated by the decreased levels of IFNγ, IL-1β and IL-17 cytokines [278] and TNF-producing T cells in the APLEC congenic compared to DA rats. Mitsdoerffer et al have shown that IL-17 cytokine can induce little IgG2b production but no IgG1 [280]. Although anti-MOG IgG2b titers did not significantly differ between DA and APLEC congenic rats, significantly lower ratio of anti-MOG IgG1/IgG2b and increased IL-17 in the DA rats implies that besides IFNγ, IL-17 may also promote the shift towards IgG2b isotype (Figure 15).

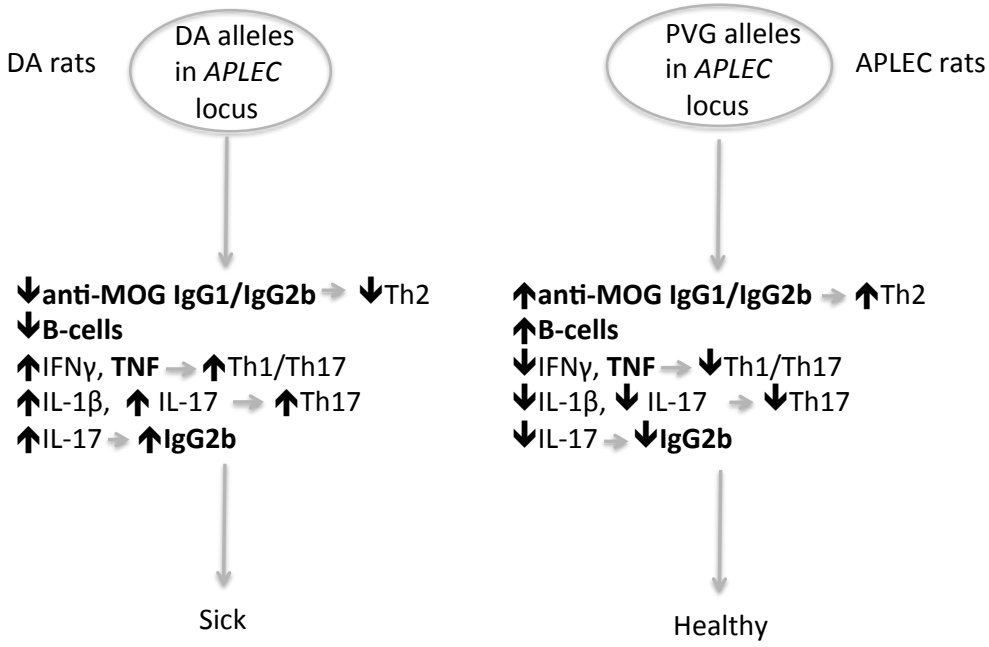

Figure 15. Proposed model of PVG alleles in *APLEC* region conferring protection during EAE.

CLRs have been implicated in a number of autoimmune diseases (Table 2, page 20), though the ligands for most of the receptors and how they exactly modulate autoimmune phenotypes are not clear to date. In our model, it is hard to speculate which of the *APLEC* genes is/are the risk gene/s. The *Dcir2* and *Dcir1* effect on Abs response has been shown in experiments using *Dcir2* KO and *Dcir1* KO that were more severely sick during EAE and CIA respectively, and displayed higher anti-MOG and anti-CII Ab levels compared to WT [162,163]. Hence, *Dcir2* and *Dcir1* serve as attractive candidates but without excluding the rest of the *APLEC* genes as responsible for the underlying effect during EAE. On the other hand, Mcl and Mincle have recently drawn a lot of attention due to their recognition as sensors of TDM, a molecule of the Mycobacterium Tuberculosis cell wall, and their implication in a number of diseases such as EAE, TBI and cerebral ischemia.

mRNA expression levels of *APLEC* genes in *in vitro* generated bone marrow (BM) APCs revealed more drastic differences in *Mcl* and *Mincle* expression between DA and APLEC strains (data not shown). Luckily, in our laboratory there is another congenic strain (R11R6) available, harboring PVG alleles in only three of the *APLEC* genes (*Dcar1*, *Mcl* and *Mincle*), on the susceptible DA background. R11R6 congenic rats were drastically protected upon MOG immunization, designating any of *Dcar1*, *Mcl* and/or *Mincle* as risk gene/s. Therefore, we decided to focus our further studies on how genes in the R11R6 congenic strain modulate EAE. Studies on R11R6, harboring three instead of the seven genes in the APLEC congenic, may easier identify the risk gene(s) and the underlying molecular pathways that lead to protection during EAE, though we do not imply that *Dcir* genes have no effect in disease development.

4.3.3 C-type lectin receptors (Mcl and Mincle) modulate EAE

In the last study of the thesis we utilize R11R6 congenic strain to study the role of Mcl and Mincle receptors during EAE development. Besides *Mcl* and *Mincle* genes, R11R6 congenic harbors *Dcar1* as well. There is no known natural ligand for Dcar1 to date, thus it is not easy to study the type of response it would induce in our system. Importantly, besides TDM binding, Mincle has also been recognized to sense SAP130 [177], released from dead cells, thus acting as DAMP, suggesting a prominent role of the receptor in host immune homeostasis. Furthermore, we observed more striking differences in *Mcl* and *Mincle* than *Dcar1* mRNA expression between R11R6 congenic and DA control rats. Therefore, we decided to focus our studies on how Mcl and Mincle may modulate EAE, though we do not

ignore the possibility that Dcar1 may also have an effect in disease development and we are planning to test Dcar1 effect as well.

We observed lower *Mcl* and *Mincle* mRNA expression in a number of myeloid cells such as *in vitro* G4-bone marrow dendritic cells (BMDC), generated with GM-CSF/IL-4 resembling monocytes (MO), (MO)-derived inflammatory DCs (MO-DCs), BM macrophages (BMMa), generated with M-CSF, and in brain-derived microglia in the R11R6 compared to DA rats. Additionally, R11R6 blood MOs and granulocytes displayed decreased Mcl and Mincle expression compared to DA control rats. Furthermore, upon ligand (TDM or its synthetic analog trehalose dibehenate (TDB)) stimulation, myeloid cells from the two strains exhibited differential response in a number of cytokines, such as IL-6, IL-23, IL-12/23p40 (implicated in Th17 pathway), TNF and iNOs, and chemokines such as CXCL1, CCL2 and CCL3 (involved in neutrophil, monocyte and macrophage chemoattraction respectively). *In vitro* studies revealed that supernatant from R11R6 myeloid cells promoted T cells to produce more IL-10 and less IFNγ and IL-17. Upon MOG protein immunization without mycobacteria, R11R6 rats were drastically protected and displayed lower demyelination and inflammatory cell infiltration in the CNS compared to DA rats. In a kinetics experiment, we did not observe any qualitative differences in the inguinal LNs, draining the site of injection, at different time points after MOG immunization between the two strains. At day 13 during EAE, the CNS of R11R6 rats displayed diminished numbers of infiltrating cells such as lymphocytes, Ma/MO and granulocytes and infiltrating cells were also less activated. Additionally, CNS infiltrating T cells in R11R6 congenic rats had reduced capacity to polarize into Th17 cells, shown by lower levels of IL-17 cytokine, while Th1 was not affected, indicated by similar IFNγ response between the two strains (Figure 16).

We demonstrate here that Mcl and Mincle signaling skew T cell development towards Th17 subsets, whose prominent role during EAE has already been recognized. The diminished IL-6 and IL-23 mRNA expression observed in R11R6 versus DA myeloid cells (Figure 16) further support this notion, as Th-17 cells and IL-17 production can be amplified by the presence of IL-6 and IL-23 [281].

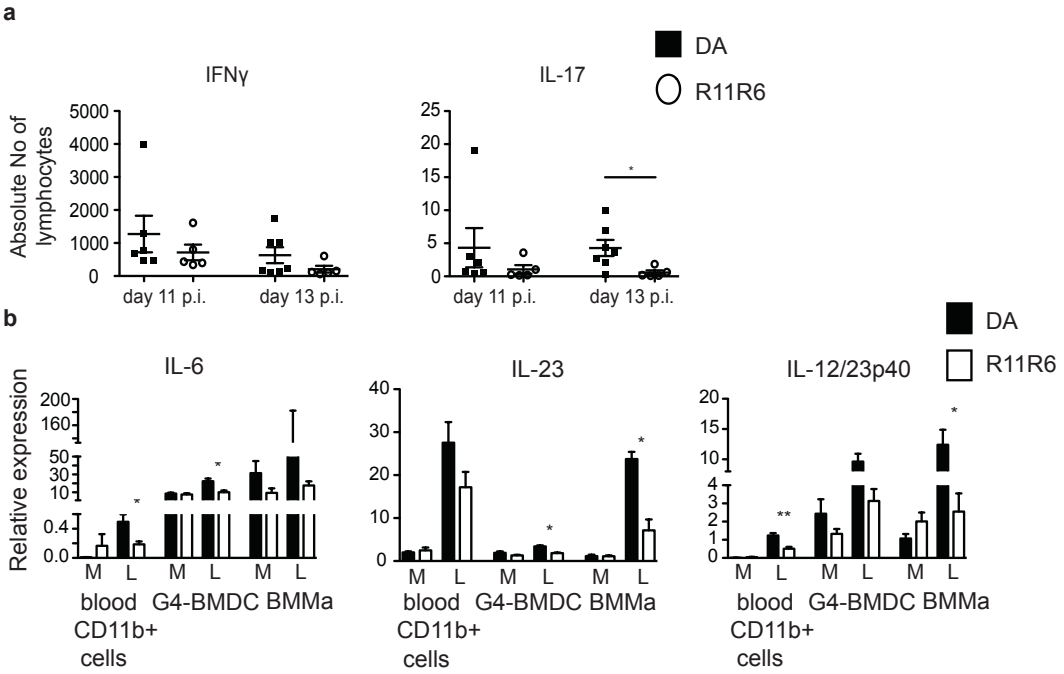

Figure 16. C-type lectin receptors skew T cells towards Th17 differentiation. a) R11R6 congenic rats displayed diminished CNS infiltrating IL-17-producing lymphocytes compared to DA rats while IFNγ production was similar 13 days p.i.. b) mRNA expression of IL-6, IL-23 and IL12/p40, cytokines associated to Th17 lineage, was reduced in *in vitro* generated myeloid cells upon TDM and/or TDB stimulation in R11R6 compared to DA rats. G4-BMCD, Bone marrow dendritic cells generated with GM-CSF/IL-4; BMMa, Bone marrow macrophages.

Interestingly, we did not observe any qualitative differences in the inguinal LNs, where T cell priming occurs, indicating that C-type lectin receptors (CLRs) do not influence initial T cell activation in the peripheral compartment during EAE, in our system. We could detect SAP130 in the CSF of DA rats both in naïve state and during EAE. Thus, we hypothesize (Figure 17) that upon MOG immunization, perivascular macrophages and meningeal microglia, playing essential role in primary reactivation of auto-reactive T cells in the CNS [282], may bind SAP130 through Mincle and subsequently re-activate T cells. Although we observed diminished *Mcl* and *Mincle* mRNA expression in *in vitro* generated myeloid cells of R11R6 congenics, we did not determine the exact cell-type that mediates Mcl and Mincle signaling. We also observed reduced numbers of CNS infiltrating cells and diminished levels of TNF and CXCL1, CCL2 and CCL3, important for cell infiltration and attraction into the CNS, in the R11R6 rats. It has been shown that signaling through Mincle results in increased TNF, CXCL1 and CXCL2 [185]. Decreased production of TNF and IL-17 observed in R11R6 myeloid cells may account for the diminished levels of chemokines resulting in lower number of infiltrating cells into the brain [177,283,284].

IL-17 will further induce endothelial cells to produce G-CSF and IL-6 and these cytokines will in turn induce bone marrow to produce neutrophils, a process known as emergency granulopoiesis [285,286]. R11R6 congenic rats displayed diminished granulocyte numbers both in the CNS and in the peripheral blood (data not shown) 13 days p.i. compared to DA rats, suggesting that R11R6 may display lower emergency granulopoiesis.

Importantly, knockdown of Mcl *plus* Mincle receptors resulted in slightly lower EAE severity in the DA strain in a pilot study. Although we did not observe significant differences between DA rats treated with Mcl *plus* Mincle and scramble siRNA, and the conditions of the experiment need to be optimized, the results were promising.

Albeit *Dcar1* has a nonsense mutation in the DA rat [287] and Dcar1 receptor signals through ITAM binding, implying activating function in the R11R6 congenic, it may very well have regulatory functions as well. For example, BDCA2, the human homologous to Dcar1 receptor, has been shown to play an inhibitory role in the homeostatic regulation of plasmacytoid DCs (pDCs) and type I IFNs production during SLE development [168] and pDC secretion of TNF-related apoptosis-inducing ligand (TRAIL) is inhibited by BDCA signaling [288]. Thus, *Dcar1* may very well confer protection in R11R6 congenic rats by inhibiting autoimmune response, while DA rats may be susceptible due to the nonfunctional mutation they carry in *Dcar1*. Though, the limited available tools to study Dcar1 prompted us to focus on Mcl and Mincle for the time being but with the intention to investigate potential involvement of Dcar1 in EAE pathogenesis in our future studies.

Exactly how CLRs are involved in MS pathogenesis is not clear and needs further investigation. Molecular mimicry is one of the proposed mechanisms for disease initiation. Upon CLR binding of microbial antigens, cross-reacting with endogenous danger molecules such as SAP130, may activate DCs and prime Th17 cells that in turn become encephalitogenic after their reactivation within the CNS by DCs that recognize cross-reacting alarm signals (Figure 17).

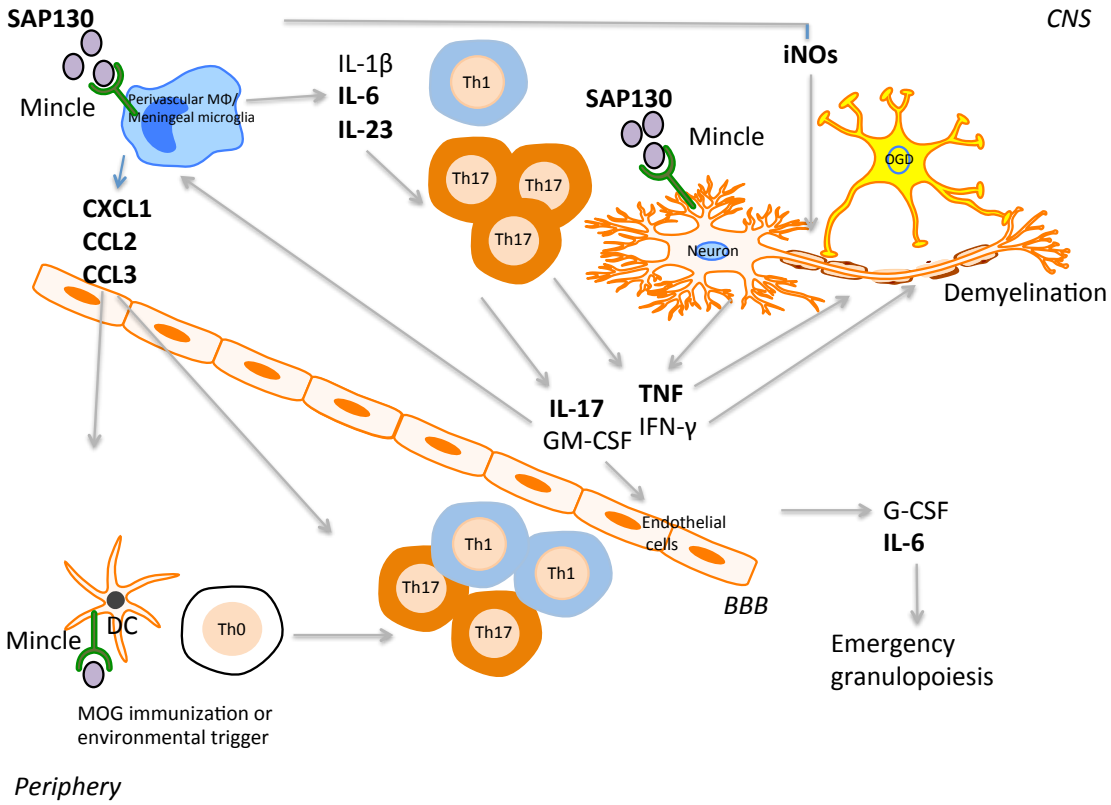

Figure 17. Proposed hypothesis on how C-type lectin receptors may confer risk in EAE/MS.

5 CONCLUDING REMARKS AND FUTURE PERSPECTIVES

MS is a complex disease with increased heterogeneity in regards to clinical symptoms/signs/course, immunopathology and response to disease modifying treatments. Complex genetic factors interacting with environmental triggers and epigenetic changes may account for disease heterogeneity, leading to a number of pathophysiological mechanisms and sub-phenotypes. To date MS cause has not been elucidated and the available therapeutics do not substantially halt the disease, but they rather limit its impact by reducing disease relapses and provide symptoms' relief. MS is a devastating disease, affecting mainly young adults, decreasing quality of life and life expectancy. Thus, it seems imperative to elucidate gene variants and underlying molecular pathways conferring risk for disease development. MS is a disease difficult to study, due to limited access and availability of the target organ, the CNS. Thus, EAE is a great tool facilitating investigation of disease genetic and molecular mechanisms and validation of therapeutic compounds.

5.1 POSITIONAL CLONING

In this thesis following an unbiased approach we utilized EAE to study the genetic control and involvement of anti-MOG and anti-neurofascin Abs in disease. We determined that anti-MOG Abs are under polygenic control and *APLEC* genes are the major regulators of their response. For our genetic dissection, we made use of a number of different rat populations, that enabled us to identify both MHC and non-MHC alleles. BC populations were used for the initial genetic mapping of immune phenotypes, identifying broad QTLs harboring many genes. An AIL G10 population fine-mapped anti-MOG Abs-regulating loci, and congenic strains, consisting of PVG *APLEC* alleles in the susceptible DA background, were utilized to further validate identified Ab-controlling QTL and enabled functional studies to investigate risk gene(s) and the underlying molecular mechanisms. Thus, we concluded that forward genetics approach (Figure 18), enabling the identification of naturally occurring polymorphisms, is a powerful tool for genetic dissection of phenotypes and detection of genes and molecular pathways relevant to disease Although in this thesis we did not reach to the point to test our findings on human material, which is

planned for future studies, there are examples that discoveries made in experimental models have been successfully translated to human disease. Among others, *MHC2TA* polymorphism associated to RA, MS and myocardial infarction was initially identified in rodents [232]. Nonetheless not all genetic findings can be directly translated to humans, although molecular pathways controlled by identified genes may operate similarly between species. The paradigm of the positional cloning of *Ncf1* in experimental arthritis [289] that led to the investigation of a novel pathway in humans and the discovery of *NCF4* association to RA [290] reflects this notion.

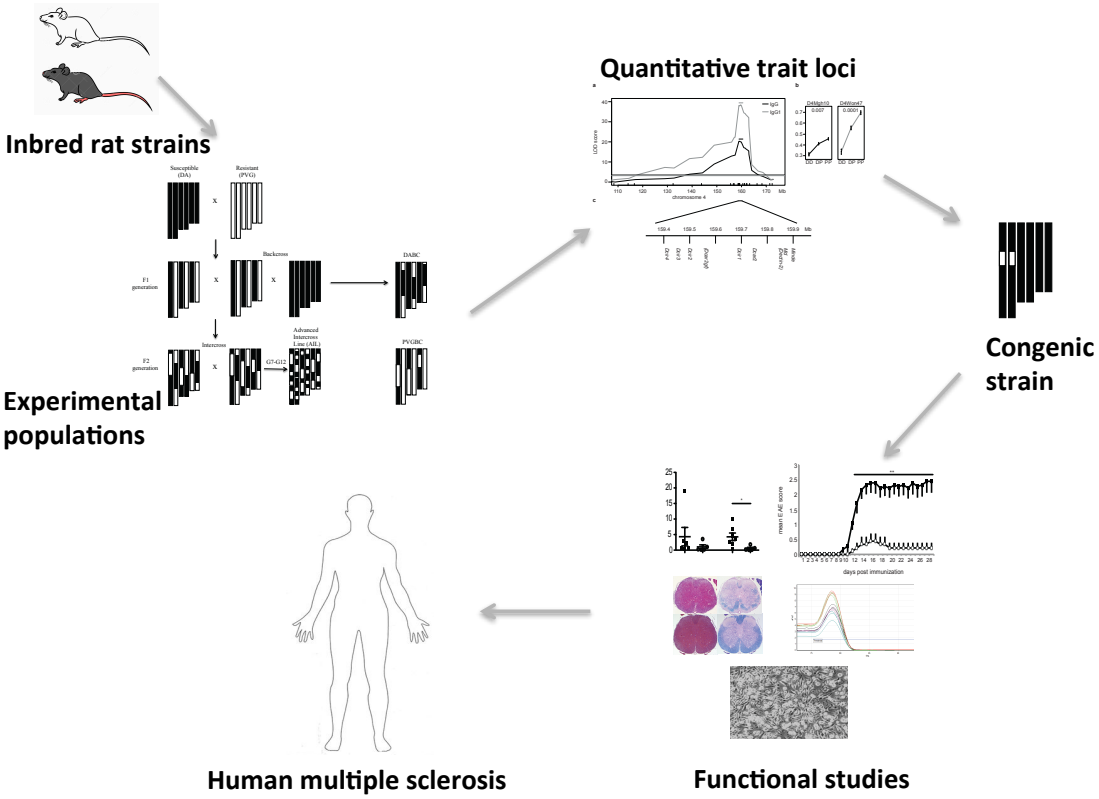

Figure 18. Summary of our translational approach. Quantitative trait loci (QTLs)-regulating disease phenotypes (e.g. Abs response) were initially identified in experimental rat populations (e.g. backcrosses) and subsequently fine-mapped in advanced intercross lines. Congenic lines were used to validate QTLs effect and functionally characterize the region and pinpoint risk genes. Candidate genes or underlying molecular pathways can be further tested for association in human cohorts.

In this thesis we also made use of a unique population, the rat HS that attempts to resemble a human outbred population. The rat HS is a powerful genetic mapping tool that enabled detection of QTLs spanning narrow regions and comprising fewer genes than those identified in other populations used. Additionally, genome-wide association mapping in HS dissected both MHC and non-MHC regions and pinpointed risk genes. MHC dissection in HS rats, not possible in any other populations used in this thesis, is particularly important, as MHC alleles exert the highest impact on MS susceptibility and unraveling molecular pathways underlying MHC control may give insight in disease mechanisms.

HS rats have the advantage of the known genomic sequences of their founder strains. Thus, SNP associations can be traced back to their original strains and that enables the development of congenic rat strains to further validate SNPs effect. A number of other immune phenotypes of HS rats such as pro- and anti-inflammatory cytokines, and tissues and organs such as spleen and spinal cord, that we have available, will facilitate further investigation of the identified regions and will be used for follow-up studies of defined risk genes. Additionally, identified candidate genes may be tested for genetic association in large MS case-control cohorts.

5.2 ANTIBODIES IN MULTIPLE SCLEROSIS

Anti-MOG Abs correlated with disease susceptibility and severity. Anti-neurofascin Abs correlated also with disease severity and their response was genetically regulated, though whether the identified genomic regions are specific for anti-neurofascin response or they control general antibody responses is not clear. The finding that anti-neurofascin Abs response is genetically regulated is particularly important in the view of current development of MS genetics, encouraging further genetic studies in relation to severity and intermediate sub-phenotypes. Autoantibodies have been implicated in MS pathogenesis for many years. While there is convincing evidence for the clinical significance of anti-aquaporin-4 Abs in neuromyelitis optica, anti-acetylcholine receptor Abs in myasthenia gravis and anti-citrulline Abs in RA, autoantibodies contribution to MS pathogenesis is still under debate. Identification of autoantibodies genetic regulation and underlying molecular pathways will facilitate answering the question whether autoantibodies are essential in disease pathogenesis or whether they reflect an ongoing activation of the B cell compartment as APCs that might mediate further activation and antigen spreading. And if eventually autoantibodies are shown to be non-pathogenic, they can still serve as biomarkers for diagnosis, prognosis, treatment responses and/or disease sub-categorization.

We identified a protective effect of Th2-related anti-MOG IgG1 isotype compared to Th1-driven isotypes IgG2b and IgG2c. One way to investigate their relative importance in vivo would be to inject EAE rats with affinity purified anti-MOG IgG1 or IgG2b Abs and determine if they protect or promote disease respectively. Though this procedure of affinity purification of specific Abs (anti-MOG), certain subclass (IgG) and certain isotypes (IgG1 and IgG2b) is quite laborious requiring multiple purification steps and thus a substantial amount of serum.

Our findings of anti-MOG IgG1/IgG2b protective role in EAE suggest that shifting the balance towards Th2 response may be beneficial during disease. For example, GA, a first line treatment for MS has been shown to exert its therapeutic efficacy by Th2-polarized immune response as well [291]. Admittedly, shifting the balance towards Th2 predominance might not be without side effects as Th2 immune response is associated also to hypersensitivity reactions.

5.3 C-TYPE LECTIN RECEPTORS IN MULTIPLE SCLEROSIS

We have also tried to pinpoint the risk gene(s) among the ones in *APLEC* gene locus and the underlying molecular mechanisms contributing to disease during EAE. An older genome scan in MS affected families revealed a peak, although not replicated in future studies, which covered *APLEC* locus, implicating *APLEC* genes in disease pathogenesis [292]. Additionally, *APLEC* alleles have also been associated to human RA [293] and were linked to experimental models of arthritis and T1D [242]. Taken together, these data indicate that one or more of the CLRs encoded by *APLEC* genes may be involved in the pathogenesis of several autoimmune diseases and suggest that conserved mechanisms apply between species. Furthermore, these data provide evidence for the usefulness of positional cloning on experimental animal models to identify genes and pathways relevant to human disease.

CLRs that function as PRRs are important components in host defense against pathogens. The recognition of SAP130, released by damaged cells, as Mincle endogenous ligand, pinpointed the potential role of CLRs in host immune homeostasis. According to the danger model proposed by Pauly Matzinger, some autoimmune diseases may be caused by abnormal physiological death and defective clearance processes, or by environmental pathogens or toxins that cause cellular stress or death [294]. In concordance, exacerbated inflammatory responses to dying cells have been associated to autoimmune diseases such as SLE, and HMGB1, a non-histone nuclear protein, has been recognized as a trigger of sterile inflammation in autoimmune diseases [295]. SAP130 may also turn out to provoke

autoimmune responses through CLR binding and currently the type of inflammatory response that SAP130 induces in APCs and APC:T cell co-cultures is under investigation in our laboratory. Additionally, Mass spectrometry analysis of CSF of MS patients may reveal SAP130, implying its potential role as alarm signal in disease pathogenesis.

CLRs have been implicated in a number of autoimmune diseases. Evidently the role of CLRs can be both pathogenic and inhibitory regardless of their association with ITIM or ITAM, thus it is not easy to determine the risk gene(s) in our model. Therefore, we focused on the role of Mcl and Mincle during EAE, though how *APLEC* variants confer risk is not entirely clear. BM chimera experiments will facilitate answering the question of whether blood or CNS resident myeloid cells are the critical components on Mcl and Mincle signaling. Conditional deletion of *Mcl* and *Mincle* on certain immune cell types will also contribute to the elucidation of cellular mediators of Mcl and Mincle receptors and will pinpoint either of the genes (if not both) as candidates. In our laboratory we have available $Mcl^{Flox/Flox}$ and $Mincle^{Flox/Flox}$ mice and a number of mouse Cre-*LoxP* lines expressing recombinase Cre in certain cell types such as immune cells (*Rosa26-Cre*), microglia (*Cx3cr1-Cre*) and myeloid cells (*LysM-Cre*). Additionally, R11R6 congenic strain and conditional knockdown mice will be tested in other experimental models, such as TBI and stroke, and that may provide further evidence for a general implication of *APLEC* variants in CNS disorders and may facilitate unraveling molecular mechanisms.

Whether both Mcl and Mincle or only one of the receptors account for the effect during EAE is not clear. Findings showing that Mcl positively facilitates Mincle expression [182] and signaling and that co-expression of Mcl and Mincle leads to a dramatic increase of Mincle phagocytic activity [180] imply that receptors interact in a synergistic manner and maybe both are essential to confer risk in our system.

Upon ligand binding Mcl and Mincle recruit Syk that induces Bcl10-Malt1-Card9 signaling cascade resulting in NF-κB activation and inflammatory cytokines induction. *MALT1* and *BCL10* were found to be strongly associated to human MS in GWAS [41,193]. A search for MS associated SNPs in either *MCL* or *MINCLE* or in any of the signaling cascade molecules might provide further hints for the implication of the receptors or their downstream targets in disease pathogenesis. Additionally, we have access to human peripheral blood mononuclear cells (PBMCs) and the corresponding genotypes from healthy donors and MS patients. CyTOF (Cytometry by Time-Of-Flight) analysis, a new technology providing multiparameter single-cell analysis, of PBMCs upon TDM or TDB

stimulation may reveal differential response of individuals with certain genotypes in particular SNPs thus providing clues for predisposing genes in Mcl/Mincle pathway and may offer valuable insight into pathophysiological mechanisms.

Taken together, CLRs are essential contributors in host immune homeostatic control and may play prominent role in development of autoimmunity. Thus, similar to TLR agonists and antagonists that have already been approved or are currently under clinical trials for the treatment of autoimmune diseases such as SLE, RA and MS [296,297] CLRs may also represent candidate targets for the designing of new therapeutic agents. Likewise, SAP130 may also serve as a potential therapeutic target, as has been shown for HMGB1 neutralization by mAbs that leads to down-regulation of inflammatory cascade in a number of experimental models including EAE [298,299].

In conclusion, in this thesis genetic and immunological studies were combined to pinpoint risk genes and unravel disease pathogenic mechanisms. Our findings enhance MS knowledge and may facilitate the development of effective prognostic, diagnostic or therapeutic tools/agents (Figure 19).

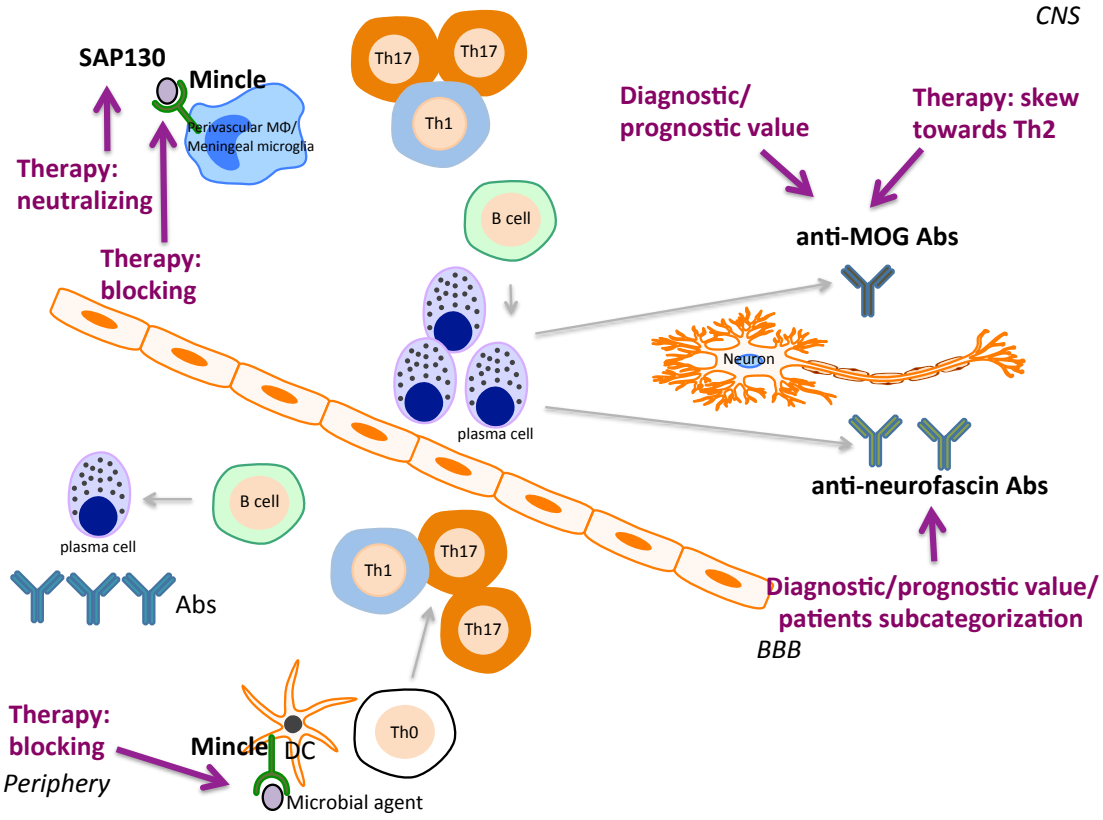

Figure 19. Summary of thesis findings and their potential implication in design diagnostic and prognostic tools or therapeutic agents for multiple sclerosis.

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"You have your brush, you have your colors, you paint paradise, then in you go!"

Nikos Kazantzakis, 1883-1957

"Έχεις τα πινέλα, έχεις τα χρώματα, ζωγράφισε τον παράδεισο και μπες μέσα!"

Νίκος Καζαντζάκης, 1883-1957

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