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GENETIC AND IMMUNOLOGICAL MECHANISMS REGULATING NEUROINFLAMMATION

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To my wonderful family and friends
John and Jeff – missing you always

If you really want something in this life, you have to work for it.
Now quiet! They're about to announce the lottery numbers.

-Homer Simpson

ABSTRACT

Multiple Sclerosis (MS) is the most common neurological disorder in young adults and imposes both health and socioeconomic burdens on society. The cause and aetiology of MS are incompletely understood and current treatments are inadequate. Pathologically, prolonged chronic inflammation and widespread demyelination in the central nervous system leads to atrophy and progressive worsening of disease. This thesis combined use of *in vivo* animal models, *in vitro* cellular assays and *in silico* computational methods to characterise pathogenic mechanisms and translate findings from models to human disease.

The animal model of MS, experimental autoimmune encephalomyelitis (EAE), was evaluated in light of novel findings in MS aetiology and further analyzed to explore differences in strain susceptibility. Susceptible rats had increased interleukin 7 receptor (*Il7r*) and *Il2ra* expression as well as altered isoform signatures in naïve lymphoid tissue, setting the stage for T cell differentiation towards pathogenic T helper 1 (T_H1) and T_H17 subtypes. Moreover, increased *Il18r1* expression described in susceptible rats was explored in MS. Dysregulation of this receptor can mediate disease initiation through T cell differentiation as well as T cell and macrophage activation. IL18R1 levels were increased in peripheral immune and central nervous tissues in MS. Inflammatory molecules that are dysregulated in EAE likely represent true pathogenic mechanisms in humans.

Multiple approaches were used to define tumour necrosis factor (TNF) regulation of disease severity. A region on chromosome 4 in the rat regulated TNF production in macrophages following innate inflammatory stimulation. Additional inflammatory molecules were also genetically regulated, modifying the cellular phenotype and severity of multiple diseases. This specific inflammatory control provides insight into disease pathogenesis and future treatment options.

The approach of combining genetic and immunological approaches in both models and human samples will continue to improve disease understanding and provide novel therapeutics through identification of key regulators and general immune and non-immune pathways.

LIST OF PUBLICATIONS

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

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

II. Alan Gillett, Klio Maratou, Chris Fewings, Robert A. Harris, Maja Jagodic, Tim Aitman and Tomas Olsson.
Alternative splicing and transcriptome profiling of experimental autoimmune encephalomyelitis using genome-wide exon arrays.
PLoS One. 2009;4(11):e7773.

III. Alan Gillett, Monica Marta, Tao Jin, Jonatan Tuncel, Patrick Leclerc, Rita Nohra, Stefan Lange, Rikard Holmdahl, Tomas Olsson, Robert A. Harris and Maja Jagodic.
TNF production in macrophages is genetically determined and regulates inflammatory disease in rats.
Journal of Immunology. 2010;185(1):442-50.

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

*These authors contributed equally to the work

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LIST OF ABBREVIATIONS

AIL	Advanced Intercross Line
APC	Antigen-Presenting Cell
BBB	Blood Brain Barrier
CIA	Collagen-Induced Arthritis
CIS	Clinically Isolated Syndrome
CIITA	MHC Class II Transactivator
CNS	Central Nervous System
CpG	Cytosine-Guanine
CSF	Cerebrospinal Fluid
CTL	Cytotoxic T Lymphocyte
DA	Dark Agouti
DNA	Deoxyribonucleic Acid
EAE	Experimental Autoimmune Encephalomyelitis
EBV	Epstein-Barr Virus
EIMS	Epidemiologic Investigation of Multiple Sclerosis
ELISA	Enzyme-Linked Immunosorbent Assay
F2	Intercross Generation 2
G12	Generation 12
GWAS	Genome-Wide Association Study
HLA	Human Leukocyte Antigen
ICE	IL1 Cleavage Enzyme (Caspase I)
IFN	Interferon
IL	Interleukin
IL18R	Interleukin 18 Receptor
LOD	Logarithm of Odds
LPS	Lipopolysaccharide
MHC	Major Histocompatibility Complex
MiDAS	Microarray Detection of Alternative Splicing
MOG	Myelin Oligodendrocyte Glycoprotein
MRI	Magnetic Resonance Image
MS	Multiple Sclerosis
MyD88	Myeloid Differentiation Primary Response Gene 88

mRNA	Messenger Ribonucleic Acid
NFκB	Nuclear Factor kappa-light-chain-enhancer of activated B cells
NK	Natural Killer
OND	Other Neurological Disorders
PBMC	Peripheral Blood Mononuclear Cell
PLP	Proteolipid Protein
p.i.	Post-Immunization
PIA	Pristane-Induced Arthritis
PP	Primary Progressive
PVG	Piebald Virol Glaxo
qPCR	Quantitative Polymerase Chain Reaction
QTL	Quantitative Trait Locus
RA	Rheumatoid Arthritis
RGMA	Repulsive Guidance Molecule A
rMOG	Recombinant MOG (amino acids 1-125)
RMA	Robust Multi-Array Analysis
RNA	Ribonucleic Acid
RR	Relapsing-Remitting
SNP	Single Nucleotide Polymorphism
SP	Secondary Progressive
T_{FH}	Follicular Helper T cell
TGF	Transforming Growth Factor
T_H	T Helper Cell
TIR	Toll/IL1 Receptor
TLDA	TaqMan Low Density Array
TLR	Toll-like Receptor
TNF	Tumour Necrosis Factor
TRAF	TNF Receptor-Associated Factor
T_{REG}	T Regulatory Cell
VitD	Vitamin D

1 MULTIPLE SCLEROSIS

This thesis focuses on multiple sclerosis (MS) and its animal model, experimental autoimmune encephalomyelitis (EAE). *In vivo* animal models, *in vitro* immuno-cellular assays and *in silico* computational approaches have been applied resulting in four studies aimed at delineating pathogenic mechanisms and translating findings for human relevance.

1.1 Characteristics

Multiple sclerosis is a chronic inflammatory disease of the central nervous system (CNS) that was originally described by Jean-Martin Charcot in 1868.[1] It is the most common neurological disorder in young adults of the Western world. Approximately two million individuals are directly affected by this disease with countless others suffering economic and social burdens. Patients present with neurological defects, including disturbances in vision, sensation, motor function or autonomic problems, dependent on the location of large, multifocal, demyelinated plaques in the CNS.[2] Extensive remyelination occurs in a subset of patients but with reduced density and thin myelin sheets.[3] MS preferentially affects females and encompasses a spectrum of disease that depends on several pathogenic mechanisms mediated through immune cells, antibodies, apoptosis and degeneration.[4, 5] CNS pathology includes blood-brain barrier (BBB) integrity breakdown and accompanying changes in both white and grey matter with a reduction in brain volume and axonal loss over time.[6-10]

1.2 Clinical Course

MS patients are defined by clinical appearance of multiple criteria, including oligoclonal bands in cerebrospinal fluid (CSF), lesions on magnetic resonance imaging (MRI) scans and bouts of neurological symptoms.[11, 12] The disease courses encompass a multitude of neurological defects and can be classified into four sub-types: relapsing-remitting MS (RRMS), primary progressive MS (PPMS), secondary progressive MS (SPMS) and progressive relapsing MS (Figure 1). The majority of patients present with RRMS but often progress to a

SPMS sub-type, which is likely due to continued axonal loss.[10] Current therapies target relapses but fail to slow progression in SPMS, a state without effective treatment possibilities.[2]

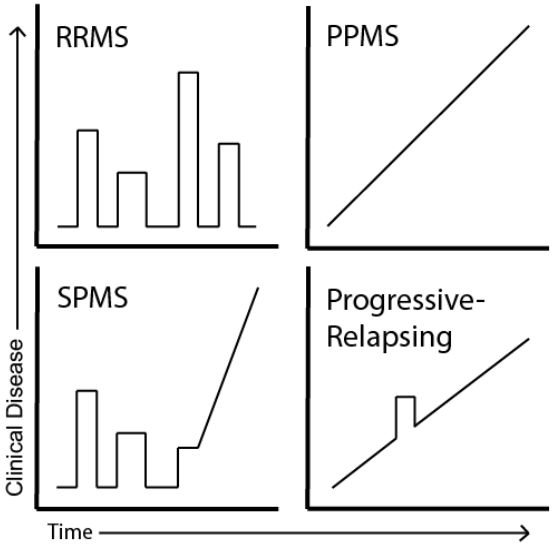


Figure 1: Multiple sclerosis is a spectrum of disease courses resulting in clinical disability. Patients commonly experience relapsing-remitting MS (RRMS) and the majority of cases become secondary progressive (SPMS). However, some patients present with aggressive primary progressive (PPMS) or a progressive-relapsing form of the disease.

1.3 Autoimmunity

Autoimmunity is defined as relating to, or caused by autoantibodies or T cells that attack molecules, cells, or tissues of the organism producing them. A large body of evidence suggests that MS is an autoimmune disease targeting the CNS, although some controversy surrounds this topic. MS fulfils many of the postulates of autoimmunity including the appearance of autoantibodies and autoreactive immune cells, the ability to induce an animal model with specific autoantigen and the possibility to transfer disease (Box 1).[2, 13, 14]

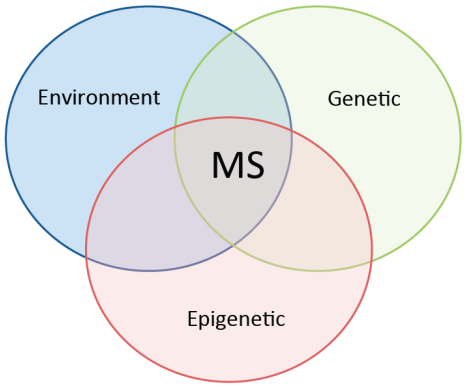
Box 1: Autoimmunity postulates fulfilled by MS.

Postulates	Multiple Sclerosis
Autoantibodies	Myelin-specific Autoantibodies
Specific Autoantigen	T cells and Antibodies to MOG and other myelin antigens
Animal Model Induced with Autoantigen	Experimental Autoimmune Encephalomyelitis (EAE)
Transfer Disease in Animals	Adoptive transfer of T cells (Passive EAE)
Transfer Disease in Humans	Accidently induced disease during Rabies vaccination

In further support of the autoimmune theory, the largest examination of autopsy brains revealed inflammation related to all demyelinating events.[15] However, opponents state that formal proof, the transfer of disease to healthy individuals, has not been demonstrated. Additionally, some believe that primary defects or insults to the nervous system underlie MS.[16] The resolution of this debate will help determine causative pathogenic mechanisms.

1.4 Triggers

The cause and relapse triggers of MS are currently undefined. Many hypotheses have been generated but causal evidence continues to elude investigators. Viral infections have long been associated with MS and several distinct viruses have been implicated in pathogenesis, including measles, Epstein-Barr virus (EBV), human herpes virus 6 and Torque teno virus, through mechanisms of molecular mimicry, inflammatory response and bystander activation with epitope spreading or direct demyelination.[17-25] Accordingly, MS-like disease can be induced in animals using viruses.[26-29] Alternatively, autoreactive T cells may escape negative selection during development and cause disease; however, this theory would suggest that healthy individuals do not have these self reactive T cells, but this is not the case.[30-32] Nonetheless, autoreactive T cells from patients have increased inflammatory capacity and autoreactive T cell transfer can passively induce disease in animals.[33-35] Additionally, bacterial infections may promote disease through molecular mimicry and degenerate T cell receptors.[36]



1.5 Risk Factors

Epidemiological and familial studies have helped frame our understanding of disease aetiology. MS is a complex disease with overlapping influences from genetics, the environment and epigenetic signatures (Figure 2).

Figure 2: Environmental, genetic and epigenetic factors interact and contribute to MS susceptibility.

1.5.1 Environmental Factors

MS prevalence increases with distance from the equator, indicating a strong environmental influence.[2] Additionally, studies of habitation highlight an increased risk when moving between high and low risk geographical areas before adolescence.[37] But what factors in the temperate regions increase susceptibility to disease? Several explanations have been proposed including sun exposure, trauma, toxins, education (related to hygiene), hormones as well as infection type and timing.[38, 39] Additionally, increases in weight and obesity are linked to MS susceptibility.[40] Extensive studies of Vitamin D (VitD) have explored connections between sun exposure, genetics, diet, immunity and disease susceptibility; VitD seems to have protective effects against MS.[41] Finally, there is a strong link between environmental triggers such as EBV infection and MS induction and relapses. However, environmental exposure alone does not explain the distribution of MS.

1.5.2 Genetic Factors

MS is regarded as a polygenic disease in which multiple genes convey susceptibility. Our first understanding of genetic susceptibility to MS was guided by familial aggregation evidence.[42, 43] Additionally, twin studies demonstrated increased risk in monozygotic (300-fold) compared to dizygotic (30-fold) twins.[44] Exploration into the causative genetic component of MS identified the human leukocyte antigen (HLA) region.[45, 46] For many years this was the single reproducible susceptibility locus but technological advancements refined our understanding; complex interactions involving Class I and Class II loci within the HLA are now evident. The highest risk alleles are the Class I *HLA-A*0301* and Class II *HLA-DRB1*1501* alleles.[47-50] However, *HLA-A*0201* and *HLA-C*05* contribute protective effects.[51, 52] Recent gene-centred and genome-wide association scans (GWAS) have provided further insights into the pathogenesis of MS. Numerous candidate genes outside of the HLA region have been replicated (Table 1).[53-69] Additional genes continue to be identified and validated. However, the combined influence of the identified genetic factors of complex diseases explains only a small portion of the heritability. For example, the variants in the interleukin 7 receptor (*IL7R)* and

IL2 receptor alpha (*IL2RA)* genes explain only 0.2% of the variance in the risk of developing of MS.[56] Further analyses of variants across the genome demonstrate only a 3% explanation of disease variance.[70] This 'missing heritability' may be explained by a combination of several theories:[71, 72]

1) Over-estimated heritable influence
2) The locus-attributable risk is actually higher because variants are only proxies for true causal mutations
3) The number of risk loci is much higher than currently identified
4) Unidentified rare polymorphisms explain most of the genetic variance
5) Other variants such as copy number variation contribute substantially
6) Over-simplified models of genotype-phenotype correlation
7) Lack of gene-gene and gene-environment (epistasis) analysis

In support of some of these theories, the incorporation of more single nucleotide polymorphisms (SNPs) to explain genetic variance has increased power in new analysis, probably due to the true effect of thousands of SNPs on common diseases.[73] Furthermore, the combination of environmental exposure and genetic inheritance can explain increased variance. For example, VitD binding to its receptor activates MS-associated HLA-DRB1*1501 gene transcription.[74] Additionally, smoking and HLA genotypes synergistically regulate disease susceptibility (personal comm. T.Olsson).

Table 1: MS-associated genes.

Study	Type	Newly Associated Genes
Sawcer et al., 2005	Genome Linkage	HLA
Swanberg et al., 2005	Gene-Centered	CIITA
Lundmark et al., 2007 and Gregory et al., 2007	Gene-Centered	IL7R
Hafler et al., 2007	Genome-wide	IL2R
Rubio et al., 2008	Gene-Centered	RPL5, CLEC16A
Aulchenko et al., 2008	Gene-Centered	KIF1B
Hoppenbrouwers et al., 2008	Gene-Centered	EV15
De Jager et al., 2009	Gene-Centered	CD58
De Jager et al., 2009	Meta-Analysis	CD6, IRF8, TNFRSF1A
Hafler et al., 2009	Gene-Centered	CD226
Jagodic et al., 2009	Gene-Centered	VAV1
Jakkula et al., 2010	Gene-Centered	STAT3
Sanna et al., 2010	Gene-Centered	CBLB
IMSGC, 2010	Replication	KIF21B, TMEM39A
IMSGC, 2010	Meta-Analysis	IL12A, CDK2AP1, RGS1

1.5.3 Epigenetic Factors

Epigenetics refers to the inherited changes in phenotype caused by mechanisms other than changes in deoxyribonucleic acid (DNA) sequence, including marks on nucleotides and histone proteins.[75] Epigenetic modifications are referred to as the 'epigenetic code' and collectively regulate gene expression and may partly explain the 'missing heritability' in complex diseases. Epigenetic changes result from both extracellular and intracellular signalling thereby providing a link between environment, genetics and disease.[76] Monozygotic twins with identical genomes are often discordant for disease, a phenomenon that can be partially explained by the epigenetic code controlling gene expression and disease susceptibility.[77, 78] Further evidence comes from allelic parent-of-origin (i.e. the inheritance of alleles from the mother or father) effects in MS patients.[79, 80] Additionally, chronic inflammation, as occurs in MS, mediates epigenetic changes to control disease processes. A specific example of DNA hypomethylation at a cytosine-guanine (CpG) island in the promoter region of the peptidyl argininedeiminase type II gene has been demonstrated in MS patients.[81]

1.5.4 Alternative Splicing

Alternative splicing is an evolutionary mechanism to create increased complexity without the need to expand the genome. A single gene can encode messenger ribonucleic acid (mRNA) molecules that can be processed in multiple ways to create distinct mRNAs encoding proteins with novel functions (Figure 3). Alternative splicing involves the inclusion of different exons and occurs widely across tissues and time to control important biological processes.[82, 83] Natural alternative splicing is dependent on cell type, genetics and epigenetic signatures. Dysregulated alternative splicing can result in disease.[84, 85] Several MS-associated genes control susceptibility through alternative splicing; soluble versus membrane bound IL2RA and IL7R are examples.[54, 86] However, exploration of alternative splicing and disease regulation has only been examined in a few select cases to date.

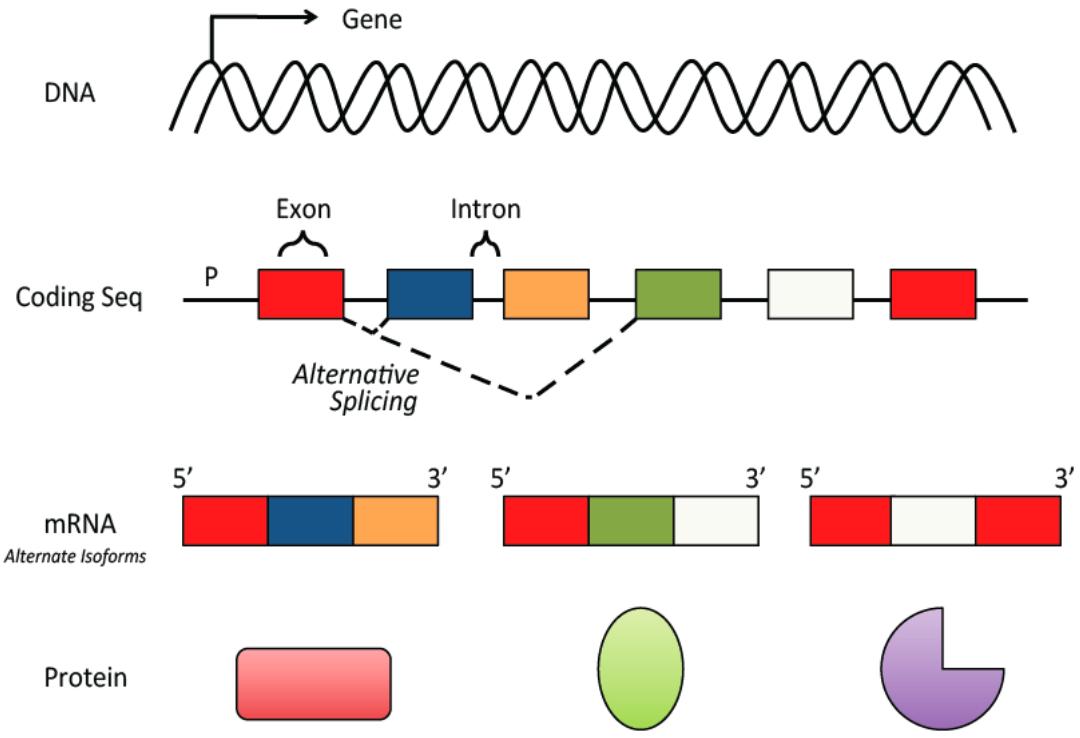


Figure 3: Genes are encoded by coding sequences in DNA. Exons code for amino acids while introns are spliced out of processed messenger ribonucleic acid (mRNA). Alternative splicing results in different mRNA and subsequently altered proteins.

2 EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS

The animal model for MS, namely experimental autoimmune encephalomyelitis (EAE), was described in the 1930s when the complications of Rabies vaccination were investigated in monkeys.[87] Since this time EAE has been widely used to study pathogenic mechanisms with the assumption that there are conserved mechanisms leading to neuroinflammation between species. Most of our fundamental understanding of MS is derived from EAE studies.

2.1 Models

Many models of EAE have been developed to mimic different aspects of human MS. EAE can be actively induced in a number of species including mice, rats, guinea pigs, rabbits and monkeys using an injection of brain, myelin or myelin proteins together with adjuvant.[88] The most commonly used adjuvant is Freund's adjuvant containing mineral oil together with *Mycobacterium tuberculosis*. Injections of pertussis toxin are also necessary for several models. Following immunization local antigen presenting cells (APCs) take up emulsion and migrate to secondary lymph nodes where they activate autoagressive T cells. These primed T cells migrate to the CNS and cross the BBB using adhesion molecules.[89] A cascade of immune activation follows reactivation of these T cells by resident APCs, including the release of tumour necrosis factor (TNF) and interferon-γ (IFNγ), activating resident cells and attracting further effector cells such as macrophages that primarily destroy myelin.[88, 90, 91]

Both progressive and relapsing-remitting disease models have been generated, reflecting the variability in the human disease. For example, immunization with the proteolipid protein $(PLP)_{139-151}$ peptide in inbred SJL mice induces a relapsing-remitting disease while myelin oligodendrocyte glycoprotein $(MOG)_{35-55}$ peptide induces a chronic form of EAE in inbred C57BL/6 mice.[92, 93] Alternatively, passive EAE can be induced through transfer of myelin-specific T cells.[33] EAE does not naturally occur in nature but spontaneous models have been created using transgenic mice harbouring myelin-specific T and B cells.[94-97] Furthermore, non-myelin autoantigens, such

as amyloid precursor protein and glial fibrillary acidic protein, can induce EAE, and degenerate MOG-specific T cell receptors may cross-react with other CNS antigens to initiate disease.[98-100]

This thesis focuses on the widely relevant relapsing-remitting model of MOG-induced EAE (MOG-EAE) in dark agouti (DA) inbred rats using incomplete Freund's adjuvant [101] (further explained in *Section 5.1*). Clinical signs begin 10-12 days after disease induction and are associated with infiltration and demyelination of the CNS.[101, 102] DA rats demonstrate a progressive worsening of disease while piebald virol glaxol (PVG) inbred rats are resistant to the same induction protocol (Figure 4).

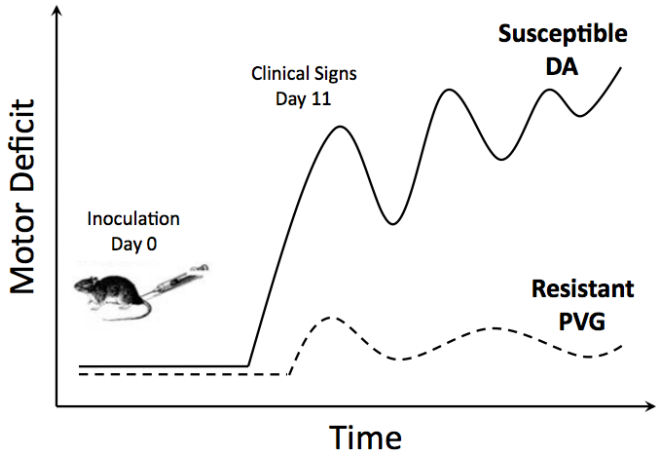


Figure 4: DA rats are susceptible to EAE induction using MOG and develop a relapsing-remitting disease with bouts of increased demyelinaiton and motor deficit.

2.2 Genetic Control

Susceptibility to EAE is governed by both genetic and environmental influences. Both EAE and MS are polygenic diseases. Whole-genome scans in mice and rats have identified at least 50 quantitative trait loci (QTLs) regulating disease.[103] These QTLs likely represent an even larger number of genes regulating disease because some loci with minimal effects will be missed in linkage studies and identified QTLs may harbour numerous genes.[104-106] Genetic susceptibility to MS and EAE appears to be very similar. The major histocompatibility complex (MHC), homologous to the HLA locus in humans, is the major genetic disease risk factor.[107, 108] There is also significant overlap of non-MHC genes between EAE and MS.[109]

The identification of genes underlying the non-MHC QTLs is a focus of our lab. The ultimate goal being the identification of pathogenic mechanisms for

better prognosis and therapeutic development. We use crosses between EAE-susceptible DA and EAE-resistant but MHC-identical PVG.AV1 rats to define QTLs across the genome regulating disease (explained in more detail in *Section 5.2*). Continued work in rats has provided insight into the regulation of EAE and MS including the identification of the MHC Class II transactivator (*Ciita*),[53, 110] β-chemokines,[104, 111] repulsive guidance molecule A *(Rgma)* and *Il21r*[112] as well as the oncogene *Vav1.*[65] Other genetic determinants are also being elucidated.

2.3 Environmental and Epigenetic Influences

EAE is a predictable model under strict environmental control that permits the study of the effects of modifying parameters on the induction and later disease stages. Several environmental influences have been identified to regulate disease including the use of pertussis toxin, age, seasonal variation and the physical structure of the inoculation emulsion.[113-117]

Inheritance of disease susceptibility is complicated by parent-of-origin influences. Maternal and paternal transmission of alleles can be affected by mechanisms of genomic imprinting through epigenetic marks, mitochondrial inheritance and the Y chromosome, all of which have been implicated in EAE.[102, 118, 119] Our current studies elucidating specific genetic loci in rats under parent-of-origin influences have demonstrated an increased power to explain genetic inheritance of disease susceptibility, thus contributing to the discovery of the 'missing heritability'. EAE and MS are therefore similar in their incorporation of genetic, environmental and epigenetic influences on disease susceptibility.

3 THE IMMUNE SYSTEM

Several lines of evidence have demonstrated that MS and EAE are immune-mediated diseases. Firstly, the pathology of these diseases includes plaques of demyelination in the CNS with accompanied inflammation.[15, 101] Secondly, autoreactive T cells and autoantibodies are commonly detected in the blood and CSF of patients as well as animal models.[2, 13, 14, 120, 121] Thirdly, the genetic causes of MS and EAE are primarily components of the immune system (Table 1). Finally, immunomodulating therapies are effective in ameliorating disease.[2, 122, 123]

3.1 Immune Cells

Most immune cell subsets of both the innate and adaptive immune systems have been described in EAE, some with protective influences and others with detrimental effects. However, the reports are often contradictory and new theories have emerged in the past decade to further explain the complexities of disease. This section will concentrate on the balance of cell types, plasticity in the immune system and the multiple roles each cell type can perform, with a focus on T cells and macrophages.

EAE and MS are described as T cell-mediated diseases because adoptive transfer of T cells can induce disease and T cell infiltrates are evident in EAE and MS lesions.[33,124-127] $CD8^+$ cytotoxic T lymphocytes (CTLs) can directly kill myelinating oligodendrocytes and neurons, causing disease, and are the predominant T cell infiltrate in MS lesions.[128,129] However, $CD4^+$ T helper (T_H) cells are the major T cells studied in EAE. Accordingly, HLA Class I and II genes are implicated in MS, while MHC Class II genes are critical for EAE.[47-52,108]

The $CD4^+$ T_H lineage was a dichotomy between T_H1 and T_H2 commitment until recently when the discovery of IL23 revolutionized our understanding of the complexity of T cell subsets.[130] IL23 shares a common p40 subunit with the T_H1-driving IL12 cytokine but has a distinct p19 subunit.[131] IL23 is critical in EAE because it drives induction of IL17-producing pathogenic T_H17 cells.[132-134] Although both T_H1 and T_H17 cells can induce disease, the combination and

relative quantities of T_H1 and T_H17 cells controls the distribution of CNS infiltration and disease regulation.[135, 136] Thus the idea of shifting the balance away from T_H1 and IFNγ towards a T_H2 response is no longer a simple option. Additionally, new T_H phenotypes and lineages have been described, including IL9/IL10 producing T cells (T_H9), follicular helper T cells (T_{FH}) and regulatory T cells (T_{REGS}), each phenotype being determined by stimulatory and costimulatory molecules on APCs, such as dendritic cells and B cells, as well as the local cytokine milieu (Figure 5).[137-142] The specific transcription factors, effector molecules and cellular functions of specific T_H cell subtypes are being investigated in normal and disease states.

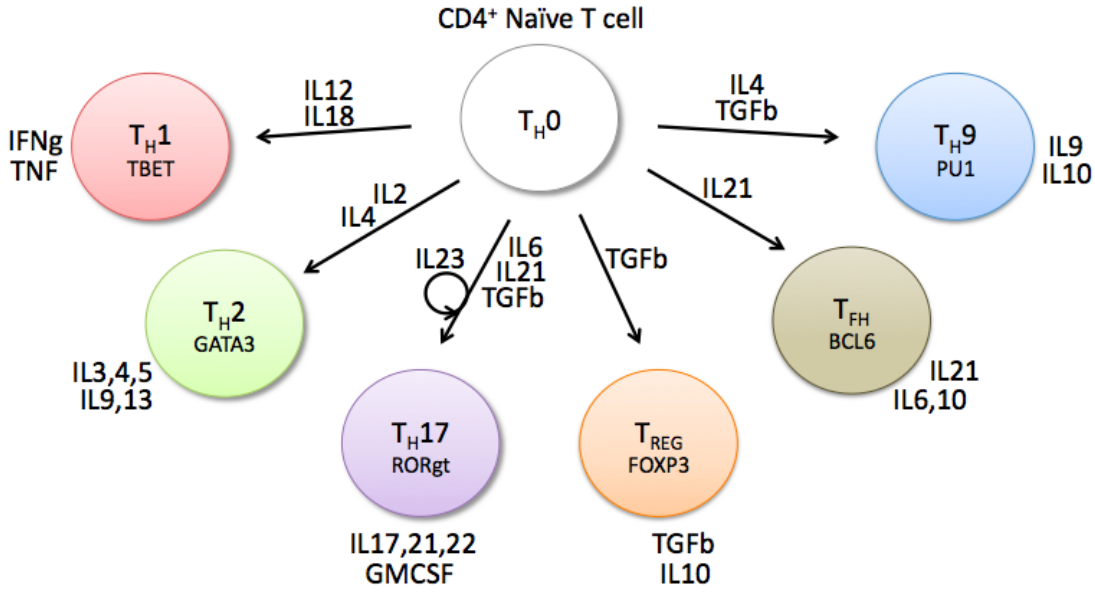


Figure 5: Naïve $CD4^+$ T helper (T_H0) cells can become an array of effector cells depending on the local cytokine environment. Each lineage is dependent on major transcription factors and results in a distinct pattern of cytokine production. The cell types have specialized functions including immunoregulation (T_{REGS}), B cell activation (T_{FH}) and protection (T_H1, 2 and 17). Adapted figure.[143]

To add to the complexity, the lineage choices are not definitive but instead demonstrate plasticity, with certain cytokines promoting more than one lineage (Figure 6). A unimodal program does not exist in T cell differentiation, as demonstrated by T_H17 cells that can convert to T_H1 cells *in vivo* and the ability of cells to secrete cytokines characteristic of multiple lineages.[144, 145] Additionally, T_{REGS} are not stable without proper epigenetic control over specific genetic loci and may convert to pathogenic cells.[146, 147] The quantity and effectiveness of T cell subsets ultimately manage a balance between immunosuppression and aggression.

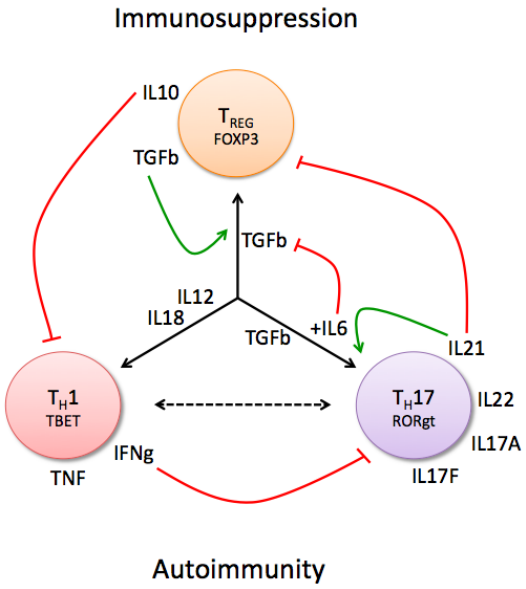


Figure 6: $CD4^+$ T helper (T_H) cells can become immunosuppressive T_{REGS} or autoreactive T_H1 and T_H17 cells depending on the local cytokine milieu. The cytokines produced by each distinct lineage can inhibit other lineages while promoting positive feedback for its own commitment. Additionally, T_H cells can switch between lineages under certain conditions. Adapted figure.[148]

Intrinsic differences in T cells determine susceptibility to MS. Genetic differences in *IL7R* and *IL2RA* directly affect T cell function through regulation of T cell survival and development as well as T_{REG} capacity.[149, 150] Additionally, autoaggressive T cells from MS patients are more reactive.[34, 35] T_{REGS} would normally control these processes but their function is impaired in MS patients.[150-152] Furthermore, priming of T cells expressing dual TCRs by pathogens in the periphery may induce disease during normal surveillance in the CNS.[153]

Macrophages are myeloid derived cells that are present in all tissues.[154] They protect against invading pathogens and clean up debris to permit biological turnover and function.[155] Macrophages are recruited to sites of injury or immune stress and produce inflammatory mediators in response.[156,157] In MS the effector cells in the CNS are infiltrating macrophages and activated microglia, which strip neurons of myelin to create plaques.[156, 158] Accordingly, depletion of macrophages improves EAE.[159] However, macrophages display a spectrum of phenotypes involved not only in active inflammation, but also in wound healing and resolution of immune reactions [160] (Figure 7). Subsets of macrophages can therefore perform different roles during disease and the introduction of immunomodulatory macrophages can ameliorate disease.[161, 162]

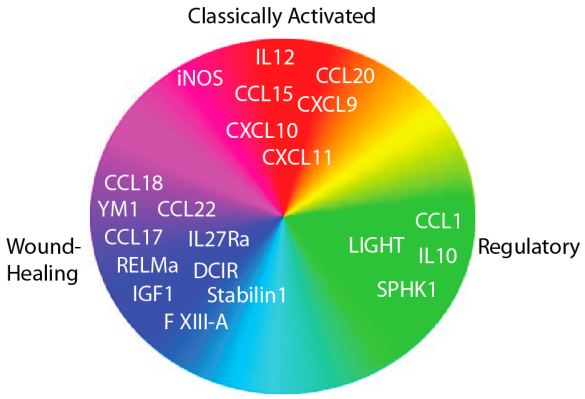


Figure 7: Macrophages respond to environmental cues creating a plastic spectrum of activation states characterized by expression of receptors and production of enzymes and cytokines. Adapted figure.[160]

3.2 Cytokines

Cytokines are small molecules that transfer signals between cells to elicit cellular changes in phenotype or migratory behaviour. Cytokines are often dichotomized into pro- and anti-inflammatory types, although the true plethora of their actions depends on the entire milieu, receptor expression and the intracellular signalling cascades. This thesis focuses on three cytokine pathways: IFNγ, IL18 and TNF.

Interferons are classified into type I (IFNα and IFNβ) or type II (IFNγ) and modulate the immune system. The type I IFNs are produced by host cells in response to viral infection in order to elicit antiviral action.[163] Conversely, IFNγ is primarily produced by activated T_H1 cells and natural killer (NK) cells.[164] IFNγ regulates MHC expression as well as mononuclear cell activation.[165-167] Furthermore, IFNγ regulates immunoglobulin isotype switching of B cells and controls T cell differentiation.[168, 169] IFNγ is associated with T_H1 responses and was originally considered as an evil cytokine in MS; however, IFNγ deficient mice develop more severe EAE.[170]

Interleukin 18 is a member of the IL1 superfamily and promotes inflammatory responses of T cells, macrophages and NK cells.[171, 172] IL18 was originally described as an IFNγ-inducing factor for NK cells and signals through the IL18 receptor to cause nuclear factor κ-light-chain-enhancer of activated B cells (NFκB) translocation and activation [171, 173] (Figure 8). Alternatively, IL18 can be neutralized by the IL18 binding protein to limit its potential.[174] Further effects in promoting T_H1 cell differentiation and activating $CD8^+$ T cells have been credited to this pleiotropic molecule.[175, 176] MS patients have increased IL18 levels compared to healthy controls indicating a biomarker and a pathogenic

mechanism.[177-180] IL18 may play a role in EAE but it is not critical for disease induction.[172, 181]

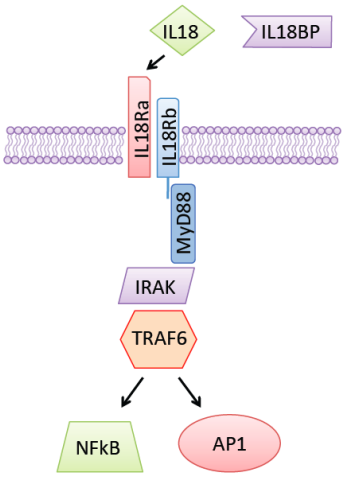


Figure 8: IL18 binds to the high-affinity IL18Rα and signals through the IL18Rβ. An intracellular signalling cascade results in activation of transcription factors. IL18BP (binding protein) can sequester free IL18 and control activity. Adapted figure.[182]

Tumour necrosis factor was originally described as a tumour killing agent and a mediator of cachexia, a wasting disease.[183, 184] After decades of intense research TNF is now known to regulate a range of biological processes including apoptosis, inflammatory activation and immune resolution as well as cellular differentiation and proliferation.[185] TNF can play multiple roles in the immune system through action on different receptors, altered forms of the protein, selective localization and synergy with other molecules.[186] TNF is a central immune mediator and control over its production and signalling is vital to biology; excessive TNF is associated with rheumatoid arthritis (RA), MS and septic shock while reduced TNF causes liver disease and opportunistic infection.[187-190] TNF therapy has been extremely successful in treating arthritis as well as Crohn's disease and ankylosing spondylitis.[191] However, although TNF blockade is effective in EAE it had the opposing effect in MS, with patients developing elevated symptoms.[192-194] Additionally, some RA patients receiving TNF treatment have developed MS-like symptoms.[195] These findings point to the dual role TNF can have in disease regulation, both causing immune activation and resolving an inflammatory response.[196, 197]

3.3 Receptors

For cytokines to exert effects on target cells receptors must be expressed on their surfaces. IL18 signals through the IL18 receptor heterodimer, a part of the

Toll/IL1 receptor (TIR) superfamily consisting of an IL18-specific IL18Rα (encoded by the *IL18R1* gene) and a common signalling subunit, IL18Rβ (Figure 8). The receptor is expressed on astrocytes, macrophages, NK, T and dendritic cells.[176] Activation of the receptor induces differentiation of T_H1 and T_H17 cells and activation of NK cells.[171, 172] IL18R1 was recently described to regulate EAE through IL18-dependent and –independent actions.[172]

Invading pathogens need to be recognized and destroyed by the immune system. One mechanism for this defence is the activation of the innate toll-like receptor (TLR) family, which are pattern recognition receptors that bind to common microbial components to detect non-self antigens such as bacterial and fungal components.[198] Binding of these antigens causes cross-linking of TLRs to induce intracellular signalling cascades.[199] One member of this family, TLR4, binds to lipopolysaccharide (LPS) to induce immune responses and the release of TNF.[200] TLR4 signals through the common myeloid differentiation primary response gene 88 (MyD88) as well as TNF receptor-associated factor (TRAF) pathways.[199] The result is activation of sets of genes involved in antimicrobial responses and inflammatory induction.[201] Several EAE models depend on TLR activation.[202]

3.4 Therapeutics

Successful treatments for MS to date have focused on the immune system. Many have been developed with the aid of EAE. However, several findings in animal models have not translated into human applications for MS, including IFNγ and anti-TNF treatments.[88] The currently approved therapeutics include IFNβ, glatiramer acid, anti-α4β1 integrin antibody, intravenous immunoglobulin, mitoxantrone and corticosteroids.[2] Additionally, two oral therapies, commercially known as Cladribine and Fingolimod, are undergoing approval (Box 2).[203-205] These therapies target a variety of pathogenic mechanisms that ultimately modulate the immune system or cellular entry into the CNS. Additionally, treatments specifically targeting B cells and antibodies have been successful. The addition of autoreactive antibodies exacerbates disease.[206, 207] Accordingly, plasma exchange has been effective in some patients and clinical trials of Rituximab, a monoclonal anti-CD20 antibody that reduces B cell

numbers, gave promising results.[208, 209] However, current treatments do not halt disease and remyelination after acute and chronic inflammation does not effectively revert disease progression.[2] Additionally, immunosuppression can lead to infectious complications. Specifically relating to MS, several cases of progressive multifocal leukoencephalopathy were observed following Tysabri treatment.[210-213]

Box 2: MS therapies and modes of action.

Therapeutic	Brand Name	Mode of Action
IFNβ	Avonex, Rebif, Betaferon	Anti-viral with immune modulation properties
Glatiramer Acid	Copaxone	Alternative activation of macrophages
αVLA4	Tysabri/Natalizumab	Blocks lymphocyte trafficking to CNS
IVIG	e.g. KIOVIG	Immune suppression
Mitoxantrone	Novantrone	Topoisomerase inhibitor causes DNA damage and cell death in dividing cells
Corticosteroids	e.g. Medrol	Suppress immune response
FTY720	Fingolimod	S1P1R inhibitor blocks egression from secondary lymphoid tissue
Leustatin	Cladribine	Nucleoside analogue causes leukocyte cell death

4 THESIS AIMS

This thesis work set out to achieve the following scientific goals:

1) *To delineate pathogenic mechanisms in models of inflammation.*

2) *To define genetic susceptibility loci regulating inflammation.*

3) *To extrapolate findings from models to human disease using a translational approach.*

5 METHODOLOGICAL CONSIDERATIONS

This thesis comprises a variety of *in vivo* models, *in vitro* systems and *in silico* analyses. Some of the methodology is reviewed in the following section with a focus on advantages, disadvantages and technological advancements.

5.1 Choice of Model

To study MS we have employed an animal model of the human disease, namely MOG-EAE in DA and PVG rats. DA rats are susceptible and display a range of immunological and neurological deficits over time while PVG rats are relatively resistant to the same induction protocol. Typically, a relapsing-remitting phenotype of ascending paralysis occurs in susceptible rats, although a spectrum of disease phenotypes can be observed.[101, 214] The clinical symptoms (paralysis of the tail, wobbling gait, etc.) are the result of cellular infiltration in the CNS and subsequent demyelination of nerve axons. Importantly, MOG is the only protein known to induce a demyelinating autoantibody response in EAE.[88] MOG-EAE thus mimics the human disease in a number of aspects, especially relating to the common relapsing-remitting subtype. Beyond its clinical aspects the MOG-EAE model has many similarities with regards to genetic regulation of disease susceptibility. Accordingly, this model is optimal for understanding pathogenic mechanisms and testing new therapeutics. Benefits over mouse models include the large animal size and milder induction protocol without the requirement of *Mycobacterium Tuberculosis* or pertussis toxin. It is also interesting to consider MOG-EAE as a model of pure inflammation; the recall system, whereby autoreactive cells are withdrawn from lymphoid tissue and restimulated with autoantigen, offers a model of autoimmunity and inflammatory response.

Using an animal model has many advantages over human studies, especially with regard to MS because the CNS is difficult to sample (Box 3). CSF is routinely collected but it is questionable how the component cells, proteins and fluid accurately reflect the composition of the brain and spinal cord. Furthermore, it is difficult to correlate changes in demyelination or clinical disease activity to alterations in CSF or peripheral tissues such as blood. There

are currently no reliable relapse markers or indicators of time to the onset of the common secondary progressive phase. An additional difficulty of studying the human disease is the long time frame; disease develops over years and sampling is often not performed during active stages.

Box 3: Benefits of EAE

Benefits of MOG-EAE
Large Sample Sizes
Access to Tissues
Controlled/Modifiable Genotype
Similar Physiological and Pathological Mechanisms
Monitored Environment
Overlapping Genetic Loci

Although MOG-EAE has many similarities with MS, some differences are important to consider. MOG-EAE is an induced model; the cause of MS is unknown and the triggers only vaguely described, but induced disease is practical and predictable. Additionally, EAE is a $CD4^+$ T helper cell-driven disease whereas patients have predominant $CD8^+$ CTL infiltration in demyelinated plaques.[33, 124, 129] IFNγ has been implicated in both EAE and MS [215, 216]; however, treatment with IFNγ was effective in EAE but exacerbated MS.[217] Furthermore, the combination and balance of T_H1 and T_H17 cells may be critical in EAE although the effect on disease pathogenesis of MS is unknown.[135] Additionally, EAE has a short duration, with experiments often finishing within 30 days, thus limiting its potential extrapolation to neurodegeneration, a key component of MS. Nonetheless, multiple models of EAE, inflammation and neurodegeneration may together encompass most of the variability and complexity of MS.

5.2 Linkage Studies and Congenic Strains

Susceptibility to EAE is a quantitative trait whereby multiple loci in the genome regulate disease. To define these disease-regulating QTLs we use crosses of inbred strains that differ in susceptibility to EAE, followed by unbiased linkage analysis. In linkage analysis, disease phenotypes are linked to genetic inheritance of regions of the genome through consequent generations. The

crosses used in this study include F2 and advanced intercross line (AIL) (Figure 9). AIL mapping offers higher resolution of disease-regulating loci compared to F2 analysis but requires additional breeding.[218] We identify different inherited alleles from the two parental strains using markers such as microsatellite markers, which are di- or tri-nucleotide repeats flanked by non-repetitive and identifiable sequences. Markers that correlate with a disease phenotype lie close to the causative loci. The level of confidence for linkage is measured as a logarithm of odds (LOD) score; higher LOD scores mean that we are more certain that a particular genetic region regulates susceptibility.

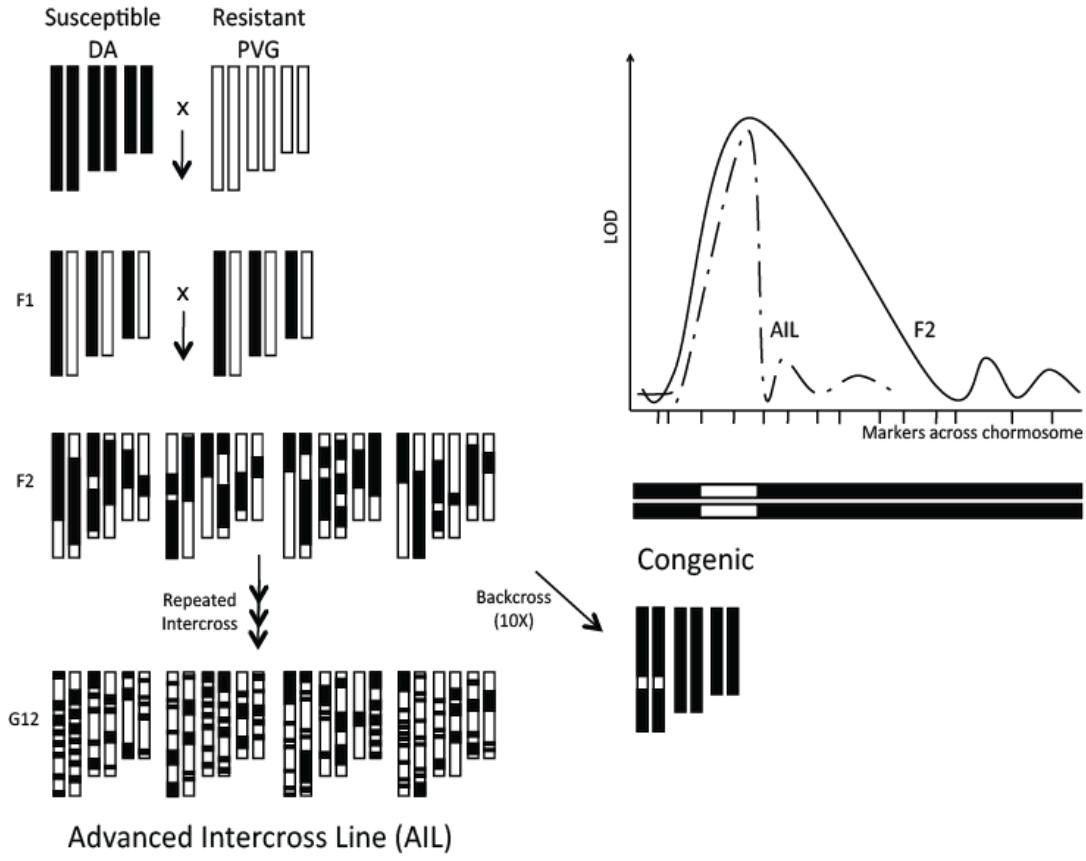


Figure 9: EAE-susceptible DA and EAE-resistant PVG rats can be crossed to produce different populations of offspring (F1, F2, G12 for example), which can be tested for linkage between phenotypes (EAE susceptibility for instance) and genetic loci. The resulting quantitative trait loci (QTLs) can be refined with further breeding (G12) and AIL mapping. Congenic rats, which harbour protective alleles at a QTL can be bred through backcrossing, are useful for functional experimentation.

Once QTLs are identified an in-depth dissection of the genetic and functional processes must be undertaken. We have used congenic breeding to isolate

small regions of protective PVG alleles on susceptible DA backgrounds (Figure 9). Thus a single genetic region controls the resulting effects on disease and pathogenic mechanisms. Some advantages of this approach include a short generation time using the speed congenic approach,[219] the comparison of different naturally occurring alleles (instead of presence/absence as in knockout strategies) and the unlimited number of genetically identical rats. Recent successful attempts to resolve MS genes using congenics include *VAV1*, *CIITA*, *IL21R* and *RGMA.*[53, 65, 110, 112]

An alternative approach to congenics is the use of genetically modified knockout and transgenic mice; the effect of a single gene on homeostasis and disease can be determined through systematic phenotyping. Although these models are outside the scope of this thesis, new methodology is being made available for rat geneticists that will offer alternative approaches. These techniques, including embryo culture, transposon-mediated mutagenesis and zinc-finger gene targeting, enable the generation of genetically modified knockout and transgenic rats.[220-222]

5.3 mRNA and Protein Identification

The identification and determination of effector molecules is a major obstacle in scientific studies. We have examined mRNA and protein levels in hopes of defining mechanisms regulating disease. We used two methods to define mRNA levels: real-time quantitative polymerase chain reaction (qPCR) and Affymetrix Arrays. qPCR utilizes fluorescent signals upon detection of target mRNA while Affymetrix chips use hybridization technology.[223-225] The benefits of using mRNA as a surrogate marker for activation and response include the ease of tissue handling, reproducibility, sensitivity and mass data acquisition. These advantages are important when analyzing large numbers of precious human samples with reduced access to tissues such as the CNS in MS patients. However, not all mRNA is transcribed into protein, the true effector molecule.

To measure protein levels we used enzyme-linked immunosorbent assays (ELISAs), which utilize specific antibodies to quantify target protein in samples.[226] ELISA is a reliable, reproducible and efficient method making it

suitable for our rat studies. We used a commercial TNF ELISA kit to determine protein levels in supernatants from whole blood stimulated with LPS from 463 genetically unique rats. This simple phenotype of TLR4 activation still required logistical planning such as careful handling of samples to ensure minimal degradation of protein and optimal stimulation time. The comparative use of several methods to define effector molecules will provide the greatest level of knowledge about the model system and pathogenesis.

5.4 Technological Advancements

Several technological advancements during the past decades have led to the rapid identification of candidate genes and targets that regulate disease. Sequencing technology has been at the forefront of change and has revolutionized genetic and functional studies. The human genome was first published in 2001 by two collaborative projects, a private venture using a whole-genome shot-gun methodology and a public initiative using bacterial artificial chromosome cloning methods.[227, 228] The subsequent HapMap project followed in 2003 and gave insight into population structure and variation between individuals and ancestral groups.[229] Next-generation sequencing continues to reduce costs and sequencing time; the sequences of several species' genomes are now publically available, including the rat.[230] New technology is also being developed with the capability to read single strands of DNA.[231]

In addition to genotyping, advances in mRNA detection using expression array chips and plates have been made. We used Affymetrix Exon Arrays and Applied Biosystems TaqMan Low Density Arrays (TLDAs) in our studies. The Exon Arrays test expression of all exons across the genome using hybridization technology; alternative splicing and gene-level expression can then be studied using statistical methods.[232] The TLDAs offer an alternative approach using qPCR methodology but only explore expression across user-selected genes.[233] TLDAs are an intermediate level between individual target mRNA quantification and genome-wide levels in a quick, easy-to-use format. Both systems generate large data sets that highlight the need for computational and statistical improvement.

The TLDA analysis is based on change of expression for each target compared to reference housekeeping genes.[234] We used three housekeeping genes in our studies to reduce noise and experimental variance between samples, as could occur if housekeeping genes are regulated by stimulation. However, no standard method or statistical program has been developed for this purpose. The Exon Arrays were normalized using Robust Multi-Array Analysis (RMA), which accounts for whole-chip expression levels.[235] To subsequently explore alternative splicing we applied several methods to reduce false-positives, although the strict approach likely increases false-negative results:

1) Microarray Detection of Alternative Splicing (MiDAS; Alternative Transcript Analysis Methods for Exon Arrays; Affymetrix-White-Paper).
2) Rank Product of Splice Index.[236]
3) Alternative Splice Analysis of Variance model (Partek)
4) Visual Discrimination of exon level expression (Partek)

Future detection and statistical methods will likely reduce the complexity of this method making Exon Arrays an attractive alternative for mRNA determination. Alternatively, RNA sequencing offers a superior quantitative method but is currently hampered by high costs.

5.5 Case-Control Cohorts

Technical advancements and reduced pricing eventually gave rise to the possibility of genotyping large groups of diseased and control individuals. The frequencies of genetic variants, such as SNPs, between affected and unaffected individuals in a population can be tested in GWAS. This approach has been applied to a range of diseases in multicentre ventures and has identified hundreds of candidate genes (catalogued by the National Human Genome Research Institute; http://www.genome.gov/gwastudies/). GWAS is based on the common disease common variant hypothesis; common diseases are attributable to common allelic variants (>1% of the population) that combine to manifest a disease.[237-240] The major benefits of association studies in humans include the ability to test genetic variants in unrelated individuals, the greater statistical power to detect common genetic variants that confer a modest risk and the fine localization of disease-causing variation.[241] However,

there are also limitations: identified SNPs are often surrogate markers; very large cohorts are needed to detect variants with low odds ratios; strong effect but rare alleles are not included in the studies; population sub-structure and heterogeneity can affect results; and no adequate gene/gene or gene/environment interaction analysis is currently available.[71, 242]

Several GWAS studies have been completed in MS research and a number of genes are associated with disease; however, a major portion of the disease heritability is still as yet unaccounted for (see *Section 1.5.2*). Nonetheless, this effort has identified genes that provide insight into pathogenic mechanisms and future increased case-control cohorts will increase power to detect variants with even lower odds ratios. Accordingly, there is currently an effort by the Wellcome Trust and a variety of institutions around the world, including Karolinska Institutet, to collect approximately 11,000 cases and 17,000 controls for an extensive MS GWAS. However, this initiative raises several questions about the analysis: how will the allele frequencies in different populations be accounted for?; how will different environmental influences be taken into consideration since MS is only partly hereditary?; and how will different diagnoses of patients reflect the spectrum and variability of MS?

Some of the problems suggested above have been accounted for in Swedish association studies, including our research on *IL18R1* in MS. Swedish biobanks offer a multitude of information for each individual through their personal identification number (*personnummer*). Knowledge about health and hospital visits (vaccinations, other diseases, etc.), economic status as well as regional living has already been gathered. The biobank also contains samples from blood and CSF for many patients and controls, permitting us to test the correlation between genotype and phenotypes, such as mRNA expression. Furthermore, MS patients are part of a registry that tracks the use of medications, relapse occurrence and disability progression. All this information can be taken into account for epidemiological and experimental studies. Additionally, a group of Swedish MS patients have been directly matched with controls having the same ancestry, residential area, sex and age as part of the Epidemiologic Investigation of Multiple Sclerosis (EIMS) study. Swedish cohorts thus offer a unique advantage for clinical studies on diseases such as MS.

6 RESULTS AND DISCUSSION

The four scientific papers included in this thesis (I - IV) are the result of several lines of investigation including the delineation of pathogenic mechanisms, the unbiased definition of disease-regulating genetic loci and the transition of findings from models to disease.

6.1 Delineating Pathogenic Mechanisms of Inflammation

We used EAE as a model of inflammation in the context of susceptible and resistant genetic background to assess MS candidate genes and known pathogenic mechanisms (I and II), alternative splicing (II) and the regulation of TNF (III) during disease. Genes regulating MS susceptibility have been identified in several GWAS and gene-centred association and linkage studies (Table 1). However, the assessment of biological relevance requires further functional experimentation. We tested the expression of several MS candidate genes in our MOG-EAE model in lymph nodes, the site of T cell activation and immune initiation, during early disease. Two of the most reproduced MS-associated genes, *IL7R* and *IL2RA*, were upregulated in susceptible DA rat lymph nodes compared to resistant PVG rat lymph nodes (I) in accordance with other studies.[243, 244] Furthermore, our genome-wide expression study reproduced these findings and also identified upregulation of *Ev15* and *Irf8* (II). However, *Kif1b*, *Rpl5*, *Irf5*, *Cd226*, *Cd6* and *Tnfrsf1a* were all equally expressed in day 7 lymph nodes.

We also examined pathogenic pathways during disease initiation. In mouse EAE T_H1 and T_H17 pathways are critical for disease but their contribution to our rat model system had not been specifically addressed. We determined that the lineage-specific effector molecules IFNγ, IL17 and IL22 were upregulated in the susceptible DA strain following restimulation with MOG autoantigen (I). The data support a model of T_H1 and T_H17 differentiation in the periphery followed by infiltration into the CNS and activation of effector responses. We also tested genome-wide pathway regulation and found enrichment for genes involved in glycosylation, apoptosis and cellular differentiation during early disease stages as well as T cell activation and apoptosis after restimulation (II). In concert with

these findings, autoreactive DA lymphocytes had a higher proliferative capacity (I). These results provide evidence that MOG-EAE is a representative model for human MS and provides tools to study contribution of these molecules and mechanisms to disease.

Alternative splicing has recently been associated to disease whereby alternate isoforms of soluble and membrane bound interleukin receptors distinguish MS patients.[54, 86] The involvement of alternative splicing in EAE disease susceptibility was examined using Affymetrix Exon Arrays and analytical methods. We studied genome-wide alternative splicing with the hypothesis that altered isoforms of mRNA and eventually proteins control disease states. We identified alternative splicing in genes involved in general signalling and transcriptional regulatory mechanisms (II). *Nab1*, *Cpsf3l*, *Btbd10* and *Usf1* modulate transcription while *Rasa1* and *Rock1* control cell motility, proliferation and differentiation.[245-250] Furthermore, five genes were constitutively spliced between the strains indicating important disease-regulating isoforms.

A determinant of disease severity is the regulation of key central inflammatory mediators. This has been directly and indirectly demonstrated with therapies in autoimmune diseases. TNF blockade ameliorates RA, while blocking CNS entry with Tysabri and other approved MS therapies stops pro-inflammatory mediators from reaching their target, thus preventing disease.[251, 252] We specifically examined the connection between TNF production and disease severity. Control of macrophage TNF production was associated with severity of EAE, sepsis, and pristine-induced arthritis (PIA) (III). These data demonstrate that central innate mechanisms are important in multiple diseases. However, no effect was evident in collagen-induced arthritis (CIA) indicating differences in models of RA, an important consideration when evaluating results. A combined approach with multiple models is therefore recommended. These results provide further insight into the regulation of inflammation; TNF production was part of a plethora of phenotypic changes occurring after TLR stimulation. The entire phenotype of the macrophage may therefore be critical in determining severity of disease through direct and subsequent indirect effects.

In summary, the results from Papers I-III contribute substantial knowledge to mechanisms regulating inflammation. Candidate genes, T cell differentiation,

alternative splicing and central immune mediators control an inflammatory response. Susceptible DA rats had increased *Il7r* and *Il2ra* expression as well as altered isoform levels in naïve lymphoid tissue, setting the stage for T cell differentiation towards pathogenic T_H1 and T_H17 subtypes. The subsequent B cell activation mounted a humoral and adaptive response against CNS myelin. The migration of all these cell types caused inflammatory influx in the CNS leading to demyelination and EAE (Figure 10).

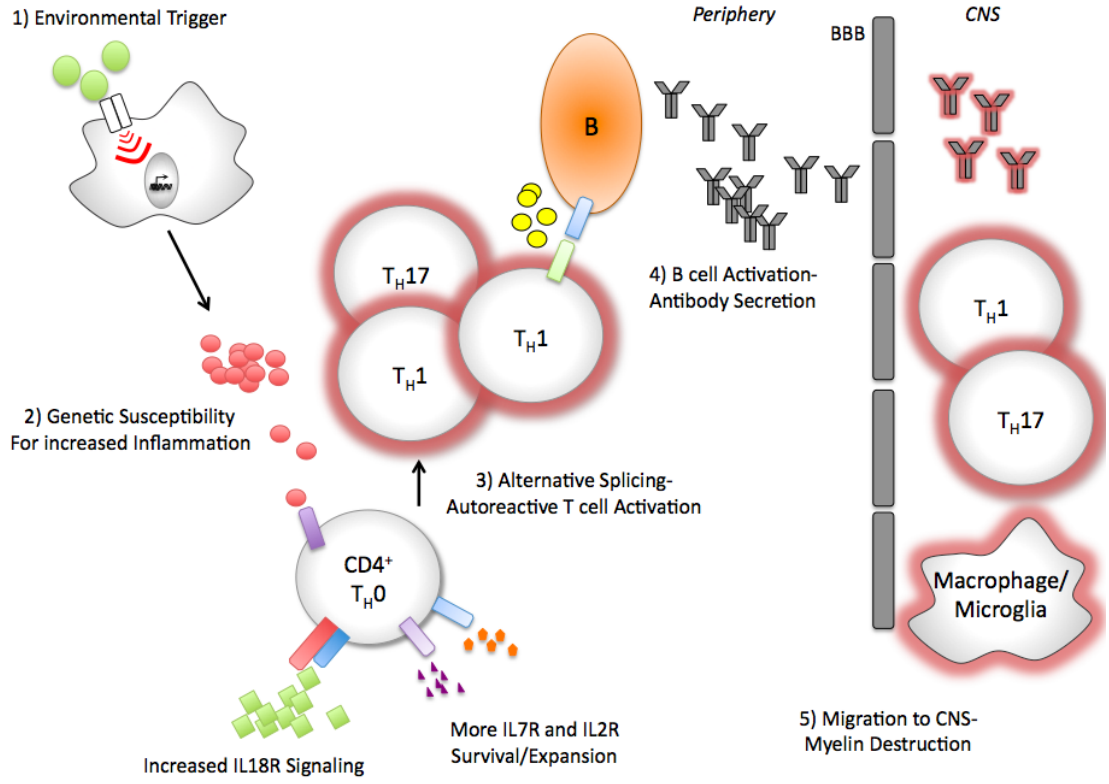


Figure 10: Sequential events occur in EAE/MS to induce an immune response that is misdirected towards self-tissue and results in myelin destruction and disease symptoms.

6.2 Defining Loci Regulating Inflammation

We applied an unbiased approach of linkage analysis followed by congenic breeding and phenotyping to define a mechanism whereby a QTL regulated TNF production (III). Mapping a single trait, TNF production after TLR4 stimulation with LPS, had a high probability of success because TNF is a central cytokine in inflammation that is critical in several diseases with susceptibility QTLs in our region of interest, including EAE and experimental models of neuritis and arthritis.[193, 253-255] Additionally, our group previously demonstrated non-MHC genetic control of TNF production following LPS stimulation in an F2 cross, although the loci had not been refined.[256]

This project helped elucidate the mechanisms of TNF regulation and subsequent control of disease severity (Figure 11). The results give insight into varying patient responses to TNF blockade therapy. Macrophages destroy tissue through TNF-mediated mechanisms but some patients have low levels of TNF in inflamed joints and do not respond to TNF blockade.[257-259] In the human disease no single genetic locus is attributed to this effect.[260] However, using animal models may be beneficial because they circumvent genetic heterogeneity. Additionally, MS patients may develop worse disease and RA patients can gain MS pathologies following TNF therapy.[192, 194] A focused treatment in macrophages could potentially ameliorate both diseases more effectively.

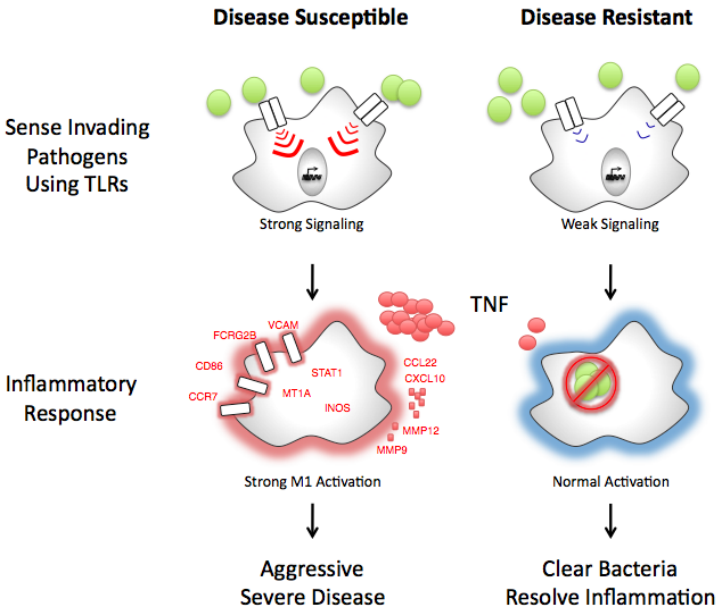


Figure 11: Macrophages from susceptible and resistant strains respond differently to external innate stimuli. Susceptible macrophages initiate a strong inflammatory response causing severe disease.

The identification of a locus regulating TNF is interesting; however, defining the causative allele is important for our understanding of both immune activation and dysregulation. By combining several methods we increase our capacity to define the causal gene and mechanism (Figure 12). Multiple pathways of macrophage activation led to same phenotype, which gives clues as to the responsible gene. We speculate that the effect is due to a transcription factor or chromatin modifier because of the extensive changes in genes encoding receptors, enzymes and cytokines. Additionally, the use of expression analysis can be useful to define candidates, as has been demonstrated for models of neuroinflammation,[53] glomerulonephritis,[261, 262] cancer,[263] left ventrical mass,[264] and heart failure.[265] Associated genotypes often control expression of their own

transcripts, as is the case for *IL2RA* and *IL7R*.[55, 86] Alternatively, genes including *RGMA*, *IL21R* and *VAV1 control* effector molecules, such as IFNγ or TNF, as a mechanism for disease susceptibility.[65, 112]

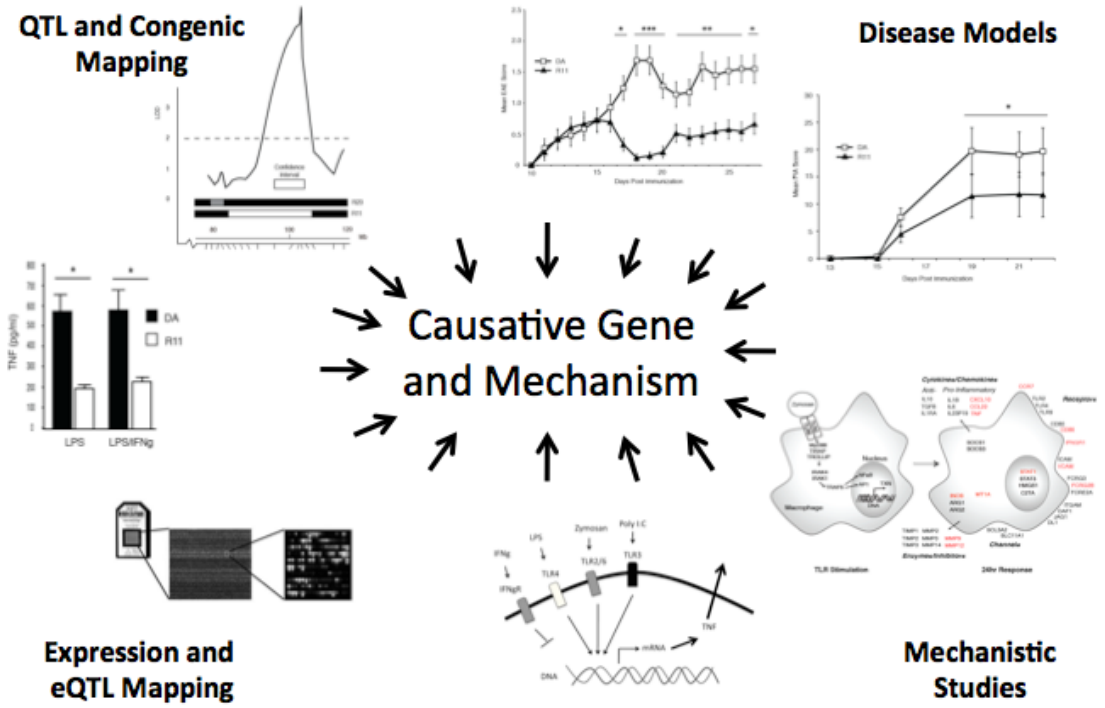


Figure 12: Multiple approaches can be used to determine causative genes and mechanisms regulating disease susceptibility and severity.

In conclusion, multiple approaches were used to define a region and mechanism of TNF regulation and disease severity. However, further experimentation is required to elucidate the responsible gene. New technology, including knockout and transgenic rats or small interfering RNA, could rapidly progress this project. Additionally, the simple phenotype of whole blood stimulation with LPS could be used for sub-congenic breeding and the definition of a smaller genetic region regulating TNF, ultimately 'positional cloning' the gene. The benefits of more specific therapies will need to be evaluated, keeping in mind the dual roles of inflammatory mediators; TNF can initiate and potentiate responses, but it is also critical for resolution.[197, 266]

6.3 Translational Approach

MS is difficult to study: it is complex; it develops over a very long timespan; disease encompasses a spectrum of symptoms and courses; tissues are difficult to obtain; the causes and pathogenesis are poorly characterized; and we lack diagnostic tools. However, a combined and translational approach provides a variety of tools to develop a deep insight into disease and treatment. We not only utilize *in vitro* and *in vivo* models but also have amassed a large biobank of human DNA as well as peripheral blood mononuclear cells (PBMCs) and CSF cells.

A translational approach has been applied to understand the contribution of a cytokine receptor, IL18R1, in neuroinflammation and MS. Although IL18 has been studied in several populations and autoimmune diseases, little is known about the receptors' influence. IL18 is upregulated in MS but is not critical for disease in experimental models.[172, 177-180] However, IL18R1 is necessary for EAE through the influence of both IL18-dependent and -independent mechanisms.[172] We first examined the levels of IL18R1 in susceptible and resistant rat strains; *Il18r1* was upregulated in DA during disease initiation, indicating an important role in EAE pathogenesis (I). This dysregulation was then confirmed (II) and warranted further investigation of the receptor in patients. *IL18R1* was increased in CSF cells and PBMCs compared to controls with other neurological disorders (OND) (IV and Figure 13). Interestingly, the receptor levels were also increased in clinically isolated syndrome (CIS) patients, most of whom will eventually develop MS,[267] indicating IL18R1 as an early disease biomarker. However, receptor levels were not empirically regulated during disease.

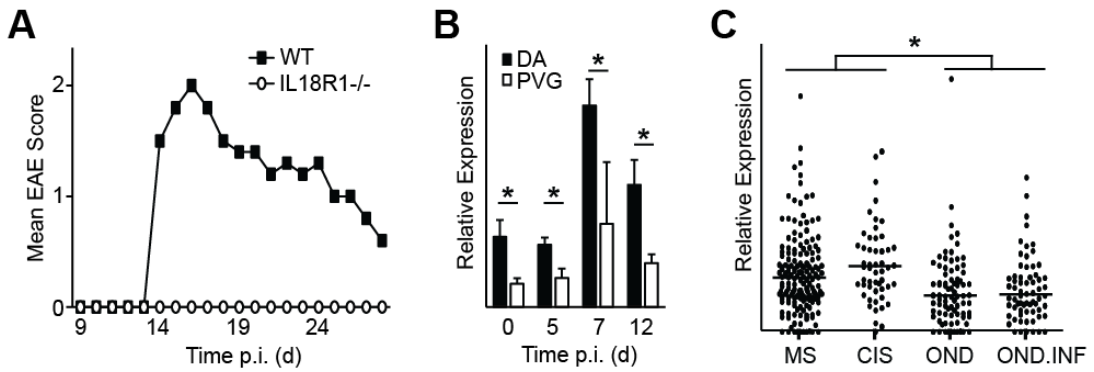


Figure 13: **A)** IL18R1 is critical for EAE susceptibility (Adapted figure[172]). Both EAE-susceptible DA rats (**B**) and MS patients (**C**) have increased IL18R1 expression during disease. p.i.; post-immunization.

We next tested if polymorphisms within the *IL18R1* gene associated with MS in a large case-control study but determined no evidence for association (IV). Furthermore, we investigated if polymorphisms in rat regulate *Il18r1* levels in spleens although no *cis*-expression-QTL, or direct genotype-phenotype correlation at the *Il18r1* gene, was determined. These results infer that other loci in the genome determine IL18R1 expression and EAE/MS susceptibility.

Therapeutics relating to the IL18 pathway, including IL18 binding protein administration and IL1 cleavage enzyme (ICE; Caspase I) inhibitors, have been explored but off-target or weak therapeutic effects hamper their development.[268, 269] However, monocloanal antibodies to IL18Rα have a strong effect on disease.[172] Other antibody therapies are effective in MS (Tysabri and Rituximab for example) and other inflammatory diseases providing hope for anti-IL18Rα treatment.[270]

We then tested if *IL18R1* expression could be a biomarker for treatment evaluation. *IL18R1* levels were determined before and after Tysabri treatment, which blocks lymphocyte trafficking to the CNS and ameliorates EAE/MS,[251, 271] but recorded no difference. This result may be due to the method of qPCR which measures expression on a per cell basis, because there are less infiltrating cells after treatment, although the same cell types and inflammatory mediators persist, especially in the periphery.[272] Nonetheless, increased expression was defined in susceptible DA rats and CIS patients, indicating that IL18R1 may be a biomarker of disease initiation in EAE and MS.

This project gives promise for translating MOG-EAE results to human MS; dysregulated inflammatory molecules in EAE likely represent true pathogenic mechanisms. The results also demonstrate immune activation in both the periphery and CNS of patients, providing a mechanism of IL18 and IL18R1 dysregulation mediating disease initiation through T cell differentiation as well as macrophage and T cell activation (Figure 14). However, IL18R1 is not a biomarker for disease progression or treatment evaluation. It is interesting that the IL18R1 has been largely ignored in other inflammatory diseases with a vast amount of information linking IL18 to disease.

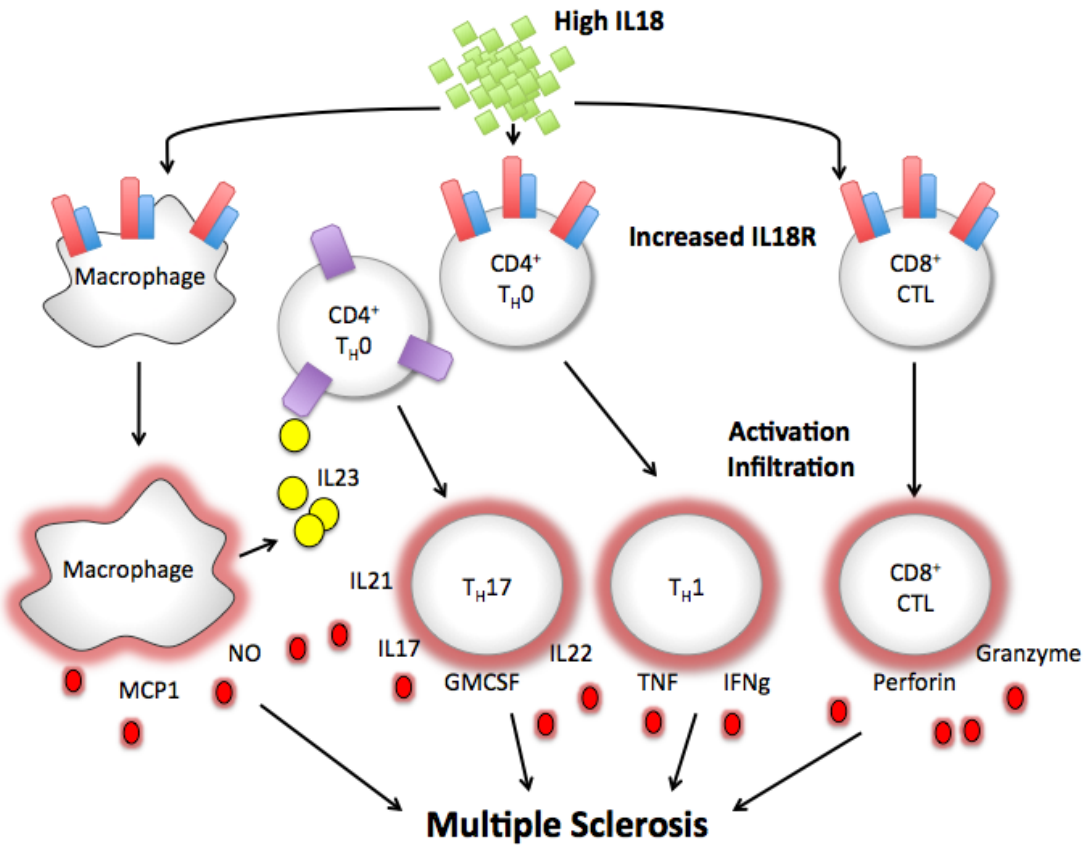


Figure 14: The dysregulated IL18/IL18R pathway in MS leads to activation of macrophages and T cells with preferential enrichment of pathogenic T_H1 and T_H17 $CD4^+$ as well as cytotoxic $CD8^+$ cells.

7 POINTS OF PERSPECTIVE

Inflammation and inflammatory diseases are difficult to study because of complex interactions of networks of molecules and factors, many of which are currently unknown or misunderstood. MS is a common complex disease with an incompletely defined aetiology; the causes, triggers and pathogenic mechanisms differ between patients and are not fully penetrant. Furthermore, the available diagnostics and treatments require refinement and continued development. This thesis aimed to understand the mechanisms underlying disease through hypothesis-driven research, unbiased screening and translational approaches. The four papers encompass a wide variety of methodologies and models that provide new insights, but the extrapolation and evaluation of the findings in the context of inflammation, autoimmunity and disease is vital to future studies.

Evaluation and re-evaluation of models is a critical and an ongoing procedure. We tested the role of MS-associated genes in MOG-EAE in DA rats, an important model for therapeutic drug testing. We determined that several candidate genes, including *Il7r, Il2ra, Irf8,* and *Ev15*, were regulated at the level of expression during disease. Our results also demonstrated that similar mechanisms occur during MS and EAE, with T_H1 and T_H17 responses being the focus of our study. Our model thus represents a range of aspects of the human disease it mimics. Additional disease-genes and pathways that will be discovered in the future should also be evaluated in models of inflammation.

EAE is a model of inflammation and MS but it is likely representative of a number of diseases. MS is an autoimmune disease, as are type 1 diabetes, Crohn's disease, Hashimoto's thyroiditis, lupus and RA. Taken together autoimmune diseases represent a major burden to society with approximately 5% of the population being directly affected. These diseases share common mechanisms as reflected by common genetic susceptibility loci.[53, 273-275] Additionally, the CNS demyelination of MS is a key component of other conditions such as optic neuritis, progressive multifocal leukoencephalopathy and leukodystrophies. Insights into the pathology of MS and treatments aimed at blocking demyelination and promoting remyelination will likely be effective for a range of patients. Neurodegeneration is a large component of progressive

MS and shares similarities with Parkinson's and Alzheimer's diseases. However, the MOG-EAE model we use has a short timeline so it may not be optimal for studies of neurodegeneration. Our results demonstrated that alleles from susceptible and resistant strains can regulate central inflammatory mediators and are critical for severity in several diseases. Accordingly, therapeutics often have some effect in several inflammatory or autoimmune diseases: anti-CD20 therapy that targets B cells is effective in RA and has also shown promise in MS.[209, 276] Furthermore, another approach could be the use of therapeutics to understand disease. We can study neuroinflammation, for instance, by applying effective therapies and scrutinizing the effects to better understand the underlying pathogenic mechanisms.

We performed the first genome-wide alternative splicing analysis in EAE. A likely next step will be a follow-up in human MS. However, this may be difficult at a genome level because of a lack of statistical methods and comprehensive knowledge of mRNA processing in different tissues. Therefore, focus could be given to associated and known mechanisms; *IL7R* and *IL2RA* are associated with MS and regulate disease through alternative soluble and membrane-bound isoforms.[54, 86] Alternatively, rapidly-advancing sequencing technology could provide a new approach to define genetic, epigenetic and transcriptional control in patients.[77, 78] Additionally, alternative splicing is a widespread phenomenon and likely contributes to natural and disease variation between individuals.[82-85] Investigation across tissues and time-points in MS and other diseases may provide additional pathogenic insights. Furthermore, the mechanisms controlling alternative splicing, including polymorphisms, splicing complexes and promoter affinities are being elucidated and could be applied to disease susceptibility.[277]

A translational approach has traditionally been successful in identifying new therapeutic targets and is deemed necessary for drug development. Several new MS therapeutics have recently been approved or are undergoing approval procedures, including Tysabri, as well as the first two oral medications Fingolimod and Cladribine. Additionally, the anti-CD20 antibody therapy Rituximab is effective against several autoimmune diseases including RA, type 1 diabetes and MS.[209, 278-280] However, first-line treatment retains 'tried and true' methods such as IFNβ injections. A continued mass effort aiming to

understand causes, define pathogenic mechanisms and develop treatment or even a cure will continue to dominate medical advances, although the complexity and heterogeneity of disease may make the road a difficult one. Combining genetic and immunological approaches will improve disease understanding and provide novel therapeutics through identification of key regulators and pathogenic pathways.

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