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DEFINITION OF GENETIC AND PATHOGENIC MECHANISMS REGULATING NEUROINFLAMMATION

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To my dear family

LIST OF PUBLICATIONS

I. **Amennai Daniel Beyeen,** Milena Z. Adzemovic, Johan Öckinger, Pernilla Stridh, Kristina Becanovic, Hannes Laaksonen, Hans Lassmann, Robert A. Harris, Jan Hillert, Lars Alfredsson, Elisabeth G. Celius, Hanne F. Harbo, Ingrid Kockum, Maja Jagodic and Tomas Olsson. ***IL22RA2* associates with multiple sclerosis and macrophage effector mechanisms in experimental neuroinflammation.**
In Press, Journal of Immunology.

II. Maja Jagodic, Celine Colacios, Rita Nohra, Anne S. Dejean, **Amennai Daniel Beyeen,** Mohsen Khademi, Audrey Casemayou, Lucille Lamouroux, Christine Duthoit, Olivier Papapietro, Louise Sjöholm, Isabelle Bernard, Dominique Lagrange, Ingrid Dahlman, Frida Lundmark, Annette B. Oturai, Helle B. Soendergaard, Anu Kemppinen, Janna Saarela, Pentti J. Tienari, Hanne F. Harbo, Anne Spurkland, Sreeram V. Ramagopalan, Dessa A. Sadovnick, George C. Ebers, Maria Seddighzadeh, Lars Klareskog, Lars Alfredsson, Leonid Padyukov, Jan Hillert, Michel Clanet, Gilles Edan, Bertrand Fontaine, Gilbert J. Fournié, Ingrid Kockum1, Abdelhadi Saoudi and Tomas Olsson. **A role for VAV1 in experimental autoimmune encephalomyelitis and multiple sclerosis.**
Science Translational Medicine. 2009 9 Dec: 1(10):10ra21

III. **Amennai Daniel Beyeen**, Alan Gillett, Melanie Thessen Hedreul, Steffen Möller, Tomas Olsson and Maja Jagodic. **Epistasis between genetic variants in *Raet1l* and *Klrk1* determines NK cell regulation of experimental neuroinflammation.**
Manuscript.

IV. Alexander Huberle***** and **Amennai Daniel Beyeen***, Johan Öckinger, Miriam Ayturan, Maja Jagodic, Katrien L. de Graaf, Nicolas Fissolo, Monica Marta, Peter Olofsson, Malin Hultqvist, Rikard Holmdahl, Tomas Olsson and Robert Weissert. **Advanced Intercross Line Mapping Suggests That N*cf1* (*Ean6*) Regulates Severity in an Animal Model of Guillain-Barré Syndrome.**
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***** These authors contributed equally to this study

ABSTRACT

Although complex inflammatory diseases affect 5% of the population we still do not understand fully the underlying disease triggers and mechanisms. This partly explains why current treatments are not curative but only modify disease. These diseases arise from combined genetic, environmental and unknown effects.

In this thesis, I have focused on identifying the genetic factors that regulate complex disease in experimental models. The rationale is that these genetic determinants will provide insight into the conserved mechanisms also important for human disease. These mechanisms can then be targeted therapeutically. I have worked with the neuroinflammatory diseases multiple sclerosis (MS) and Guillain-Barré syndrome (GBS) and their respective animal models, experimental autoimmune encephalomyelitis (EAE) and experimental autoimmune neuritis (EAN).

To identify risk genes in an unbiased phenotype-driven manner, we established intercrosses and recombinant lines between rat strains with opposing susceptibilities to EAE and EAN. Linkage analyses and functional studies in rat lines then successfully positioned five genes that regulate experimental neuroinflammation, namely *Il22ra2*, *Vav1*, *Raet1*, *Klrk1* and *Ncf1*. *IL22RA2* and *VAV1* were also translated as risk genes in MS cohorts.

More importantly, however, the five genes targeted immune mechanisms and events that correlated well with disease. In our hands, Il22ra2 regulated macrophage activation, Vav1 controlled effector T cell activity and regulatory T cell proportions, *Raet1* and *Klrk1* displayed a gene-gene interaction that modified NK cell activity and Ncf1 controlled oxidative burst from mononuclear cells. All these mechanisms also have described roles in both MS and GBS.

By finding genetic determinants of distinct pathogenic mechanisms we may both discover novel targets for treatment and also more accurately define which current therapies are more suitable for different patients.

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LIST OF ABBREVIATIONS

AD	Autoimmune disease
AIDP	Acute inflammatory demyelinating polyradiculoneuropathy
AIL	Advanced intercross line
APC	Antigen presenting cell
CD	Crohn's disease
CDCV	Common disease - common variant
CFA	Complete Freund's adjuvant
CFTR	Cystic fibrosis transmembrane conductance regulator
CI	Confidence interval
CSF	Cerebrospinal fluid
CNV	Copy number variation
DA	Dark Agouti
DC	Dendritic cell
EA	Experimental arthritis
EAE	Experimental autoimmune encephalomyelitis
EAN	Experimental autoimmune neuritis
EBV	Epstein-Barr virus
eQTL	Expression quantitative trait locus
GBS	Guillain-Barré syndrome
GM-CSF	Granulocyte-macrophage colony stimulating factor
GWAS	Genome-wide association scans
HH6V	Human herpes virus 6
HLA	Human leukocyte antigen
IFNβ	Interferon beta
Ig	Immunoglobulin
IL22RA2	Interleukin 22 receptor alpha 2

IL7R	Interleukin 7 receptor
LD	Linkage disequilibrium
LPS	Lipopolysaccharide
MBP	Myelin basic protein
MHC	Major histocompatiblity complex
MOG	Myelin oligodendrocyte glycoprotein
mRNA	Messenger RNA
MS	Multiple sclerosis
NCF1	Neutrophil cytosolic factor 1
NOXC	NADPH oxidase complex
OR	Odds ratio
PVG	Piebald Viral Glaxo
QTL	Quantitative trait locus
RA	Rheumatoid arthritis
RAET1	Retinoic acid early transcripts 1
RNO	Rat chromosome
SLE	Systemic lupus erythematosus
SNP	Single nucleotide polymorphism
T1D	Type 1 diabetes
TGF-β	Tumor growth factor beta
$T_{H}1$	T helper 1
TNF	Tumor necrosis factor
T_{REG}	Regulatory T cell
VLA4	Very late antigen 4
WSC	Whole spinal cord homogenate
ZFN	Zinc finger nuclease

1. GENETIC BACKGROUND

Most, if not all, diseases have a hereditary component. This has primarily been established in family studies, in which an increased disease risk is observed in relatives of affected individuals. For monogenic diseases this familial aggregation is caused by mutations in a single gene. Most genes underlying monogenic diseases have been identified as a result of human genetic studies (1). This includes diseases such as cystic fibrosis, caused by mutations in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene, and Huntington's disease, driven by mutations in the *huntingtin* gene. Although monogenic diseases can often be severe they are typically rare in the general population and the global prevalence of all known single-gene diseases is approximately 1% (2).

The majority of diseases are polygenic in nature, meaning that multiple genes regulate disease. These genes, or alleles, will often exert a small effect to increase the overall disease susceptibility. The combined effect of these genes on disease can be additive, meaning that each gene's effect is independent of the other, or it can be multiplicative as a result of gene-gene interactions (3).

Polygenic diseases also arise from environmental and as yet undefined factors, often interacting with genetic factors. Most polygenic diseases are therefore also referred to as *complex diseases*. Examples of complex diseases are most of the disorders we see around us, ranging from depression, anxiety and metabolic disorders to chronic inflammatory conditions such as Crohn's disease (CD), rheumatoid arthritis (RA), type 1 diabetes (T1D) and multiple sclerosis (MS). The environmental factors are difficult to assess properly and can strongly confound human genetic studies (4). The complexity of the interplay between subtle genetic and environmental risk factors likely explains why a given risk allele combination will not always produce the same phenotype (disease symptoms) in every individual. This variation so often observed in complex diseases is termed phenotypic heterogeneity. In addition, different gene combinations can cause the same disease phenotype and this is termed genetic heterogeneity. The combined risk from genetic and environmental factors will shift an individual's risk towards the disease threshold (5, 6) (Figure 1).

Disease etiology is often poorly understood for complex disorders. By studying their genetic basis we have a better chance to understand the events that drive disease in some individuals but not in others.

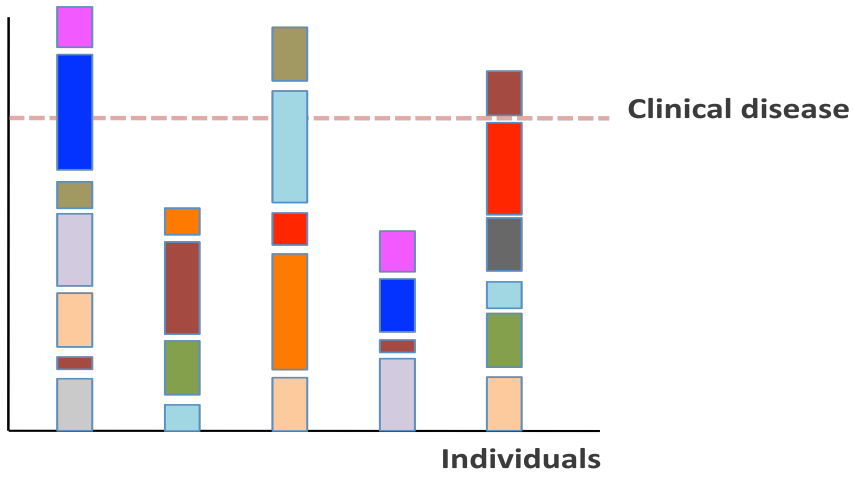

Figure 1. A schematic threshold model for genetic susceptibility to complex diseases. The combination of risk alleles (represented by filled blocks), either alone or in interaction, determines the overall risk of developing disease. The effect of one gene can vary from individual to individual, depending on interactions with other genes/environment, as demonstrated by the bars of different sizes with the same color. This susceptibility is further affected by environmental and stochastic factors.

This thesis explores the genetic regulation of complex inflammatory disorders, by identifying risk genes and defining their roles in disease pathogenesis. I focus on the neuroinflammatory diseases MS and Guillain-Barré syndrome (GBS) and their respective animal models.

2. MS AND NEUROINFLAMMATION

MS is a chronic inflammatory disease of the central nervous system (CNS). It affects over 2 million people worldwide with a prevalence in Sweden of 0.1 - 0.2% (7). Like most autoimmune disorders, it is more common in women than in men with an approximate ratio of 2.5:1 (8, 9). MS results from an attack of the immune system on the oligodendrocytes, which produce the myelin sheaths that surround the axons in the CNS. As the disease progresses, or possibly in parallel to the demyelination, there is also axonal damage (10). The symptoms of MS vary widely depending on which signals are interrupted. They include changes in sensation, visual problems, muscle weakness, coordination and speech difficulties, severe fatigue, cognitive impairment, balance disturbance and pain. In more severe cases, MS causes impaired mobility and disability.

MS can exhibit several different forms of progression with symptoms either occurring in discrete attacks or slowly becoming more severe over time. These symptoms sometimes resolve completely between attacks but permanent neurological problems often persist, especially as the disease advances (Figure 2).

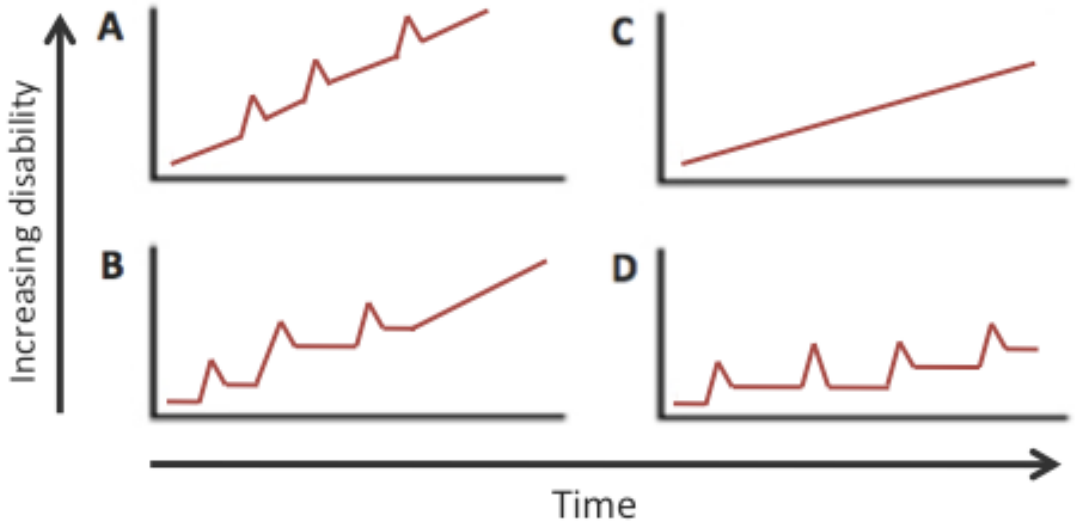

Figure 2. Different disease courses of MS. A) Progressive-relapsing MS; steady decline with superimposed attacks. B) Secondary-progressive MS; initially relapse-remitting MS, but then begins to decline without remission. C) Primary progressive MS; steady decline without remission. D) Relapse-remitting MS; unpredictable bouts which sometimes cause permanent damage, followed by periods of remission.

Relapsing-remitting MS is the most common form, affecting 85% of patients. A majority of these patients eventually progress to the secondary-progressive phase. Onset of MS occurs between the ages 20-40 years and 50% of MS patients are unable to work 10 years after diagnosis and are thus excluded from the workforce. The magnitude of the socio-economical impact is reflected in that MS, constitutes the same overall economic burden on society as RA, which is five times more prevalent than MS (Kristina Gottberg, personal communication).

2.1. HISTOPATHOLOGY OF MS

The main histopathological hallmark of MS is focal demyelinated lesions. The detection of different patterns of demyelination suggests heterogeneity in the mechanisms involved in lesion development. Different types of lesions, or plaques, can be present in the same patient at various stages (11). The most common pattern is characterized by perivascular inflammation, infiltrating T lymphocytes, macrophages with increased complement at sites of active myelin breakdown. In addition, deposition of immunoglobulin Gs (IgGs) can be detected around lesions. Alternatively, lesions can display patterns of prominent signs of oligodendrocyte dystrophy without IgG or complement deposition, suggestive of primary oligodendrocyte damage (11).

2.2. GENETICS OF MS

The genetic component in MS is well established. There is a clear increase in recurrence risk in families of affected individuals compared to the general population (12, 13), whereas adoptee studies reveal no increased risk for disease compared to the general population (14). Twin studies have consistently proven that MS concordance is increased among monozygotic twins compared to dizygotic twins (15-17). As one moves from siblings to more distant relatives, the risk decreases while still being higher than the general population (18).

The relevance of the major histocompatibility complex (MHC) locus, referred to as the human leukocyte antigen (HLA) complex in humans, in MS has been well documented. It unambiguously associates with disease in virtually all genetic studies (19-23). During the past years the effects within the HLA have been refined, with Class II HLA-DRB1*1501 alleles being the major single genetic risk factor (24, 25). Conversely, HLA-A2*0201 alleles confer the strongest protective influence (26). Moreover, it is clear that interactions between HLA haplotypes exert even stronger effects (27-29). More recent large studies have now also

identified non-MHC risk genes, such as interleukin 7 receptor (IL7R), IL2RA, CD58, CLEC16A, KIF5A, IRF8 and CD226 (24, 30-36). Collectively, these studies demonstrate a heavy bias towards immune-related risk genes and thus likely importance of immune-related mechanisms in pathogenesis of MS.

2.3. ENVIRONMENTAL FACTORS IN MS

There is a distinct geographical distribution of MS with the highest prevalence being observed in temperate latitudes and in the western hemisphere (Figure 3). Individuals who migrate from low- to high prevalence regions before adolescence acquire the elevated risk but not if they migrate later in life (37-39). Accordingly, there is also a clear association between MS and lack of sun exposure. This fits well with the observed protective association of high vitamin D levels with onset of MS (40). Vitamin D is now known to have several immunomodulatory properties (41).

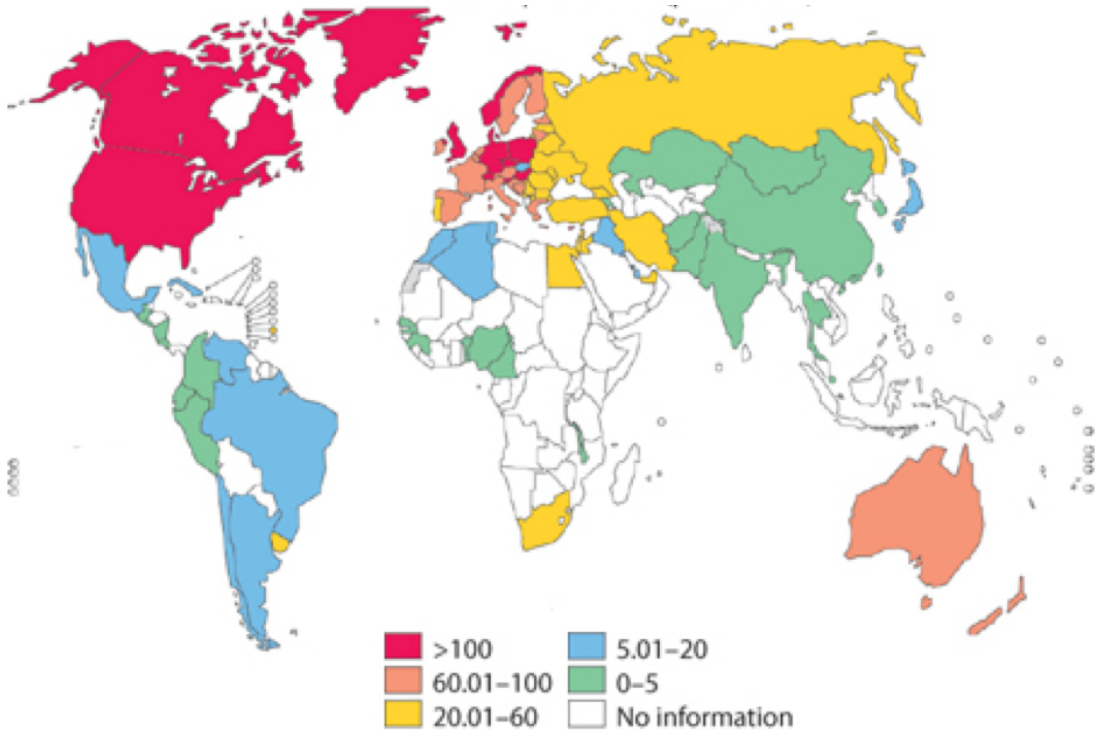

Figure 3. Worldwide prevalence of MS per 100,000 inhabitants. Adapted from the World Multiple Sclerosis Resource Center, http://www.msrc.co.uk.

Moreover, a number of infectious agents have been associated with MS, in particular Epstein-Barr virus (EBV) and human herpes virus 6 (HHV6) (42, 43). They

have been reported to affect the development and clinical course of MS (44), but a clear association with a particular viral pathogen has not been determined. The role of infectious and viral agents in MS etiology and pathogenesis is thus still poorly understood.

Epidemiological studies also establish smoking as a risk factor for MS, especially when interaction with the HLA locus is taken into account (45). Interestingly, smoking also appears to enhance the association between MS and antibody titers to EBV antigens, highlighting how environmental factors can interact to affect disease (46).

2.4. TREATMENT OF MS

The current treatments for MS are only disease-modifying and do not cure disease, reflecting our relatively poor understanding of the disease pathogenesis. There are currently two first-line treatment regimens, recombinant interferon-β (IFNβ) and the polypeptide glatiramer acetate (47). In addition, treatment with steroids can temporarily reduce ongoing symptoms (48). A humanized monoclonal antibody, Natalizumab, directed against the α4-integrin of the adhesion molecule very late activating antigen (VLA)-4 on leukocytes has more recently emerged as a potent disease-ameliorating drug (49, 50). However, Natalizumab has also been associated with more severe adverse effects (47, 51). Novel MS drugs are evaluated continuously and some of these have significantly reduced disease in large clinical trials (52-54).

2.5. GUILLAIN-BARRÉ SYNDROME

Guillain-Barré syndrome (GBS) is an inflammatory disease of the peripheral nervous system encompassing different subtypes, with acute inflammatory demyelinating polyradiculoneuropathy (AIDP) being the most prevalent subtype (55). In AIDP, macrophages invade the myelin sheaths of the peripheral nervous system through a mechanism likely involving activated T cells and antibodies. AIDP therefore shares many characteristics with MS. Symptoms of GBS are diverse, depending on whether sensory, motor or autonomous nerves are affected, but typically peak four weeks after onset. Disease symptoms then resolve but 20% of patients are left with persistent and significant disability (55).

3. DISEASE MODELS

3.1. EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS

The most common animal model of MS is experimental autoimmune encephalomyelitis (EAE) and has been established in several species including rats, mice, guinea pigs, marmosets, rabbits and primates (56-61). EAE can be induced by subcutaneous injection of recombinant or purified CNS antigens, synthetic peptides, whole CNS tissue or infection with encephalitogenic viruses (62, 63). Depending on the antigen and genetic background, these models recapitulate distinct features of human MS, both regarding disease course and pathogenic mechanisms. There are numerous CNS antigens that induce EAE, and three of them have been used in my thesis. The most extensively used model in my studies is the myelin oligodendrocyte glycoprotein (MOG)-induced EAE model in rats, which appears to accurately reflect the distinct disease courses of MS. MOG-EAE is characterized by a disease onset at 10-14 days post-immunization resulting in an ascending paralysis with periods of remission (64) (Figure 4). Another model, whole spinal cord homogenate (WSC)-induced EAE, displays similar relapsing disease characteristics (63, 65). In contrast to these models, the myelin basic protein (MBP)-induced EAE model is acute and resolves after the first disease bout (66). This classical model requires *Mycobacterium tuberculosis* for its induction, and the rapid disease course is typically devoid of demyelination.

EAE can also be induced passively. In this alternative induction protocol, autoreactive T cells are injected intravenously or intraperitonaelly and give rise to a transient demyelination and motor impairment (67). Spontaneous EAE mice models also exist, but require use of genetically engineered strains in which T cell receptors have been restricted to recognize CNS antigens (68, 69). Spontaneous disease can also be exacerbated by introduction of myelin-specific B cell receptors (70).

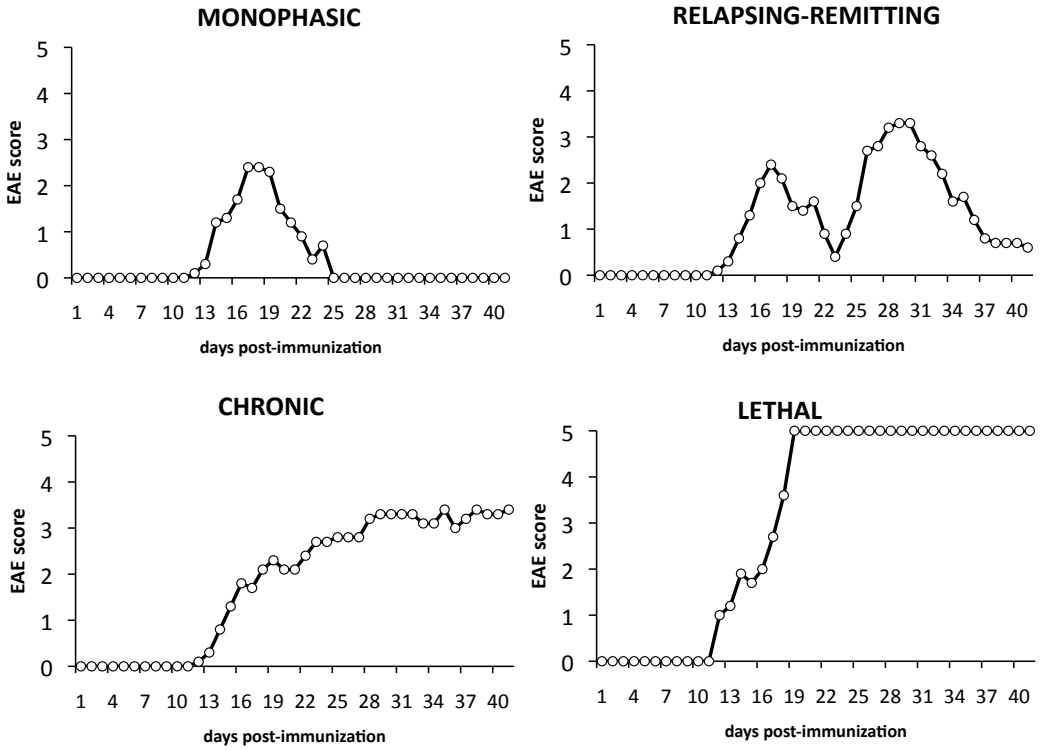

Figure 4. Representative graphs of the various EAE courses observed in the rat MOG-EAE model. The y-axis represents days post-immunization (p.i.). The x-axis represent EAE severity, where 0 = no clinical symptoms, 1 = limp tail, 2 = disturbed gait, 3 = complete hind leg paralysis, 4 = tetraplegy or moribund, 5 = dead.

Most immune cells have been attributed roles in EAE pathogenesis, which can explain why EAE has been extensively used for general studies of inflammation (71). For this reason EAE is occasionally criticized for not truly representing MS, with concerns that some therapeutic approaches in EAE have been unsuccessful in MS (72). However, a positive outcome to a given treatment has depended on the EAE model employed. Indeed, most approved MS therapies used today have first been characterized in various EAE models (73).

3.2. GENETICS OF EAE

The genetic effect in EAE is clearly established as different strains display great variation in disease susceptibility under the same environmental conditions (74, 75). As in MS, the MHC locus is the major genetic determinant of disease (76-79). This effect is fairly profound and some strains carry MHC haplotypes that are only susceptible to certain CNS antigens (79). However, there is a substantial non-MHC gene contribution and to date, at least 50 genetic regions are known to regulate

EAE in rodents (75, 80-88). Successful examples of identifying the responsible gene have emerged, both for EAE and other experimental disease models (89-92). Collectively, these findings indicate great similarities in genetic regulation between EAE and MS. Given the controlled environment and tissue availability in experimental studies, candidate gene investigation in EAE serves as a powerful complement to analogous human efforts. EAE risk genes can be translated to MS risk genes and can provide valuable insight into the origin of disease mechanisms. Gene-gene and gene-environment interactions can also more readily be examined due to the reduced variation. The methods employed in our studies to identify disease genes in rodents will be discussed later in this thesis.

3.3. EXPERIMENTAL AUTOIMMUNE NEURITIS

Experimental autoimmune neuritis (EAN) is a model of GBS and its pathology closely resembles that of the AIDP subtype (55). EAN is induced in a similar fashion to EAE, by immunization of peripheral myelin antigen. The disease is characterized by a CD4+ T cell mediated response specific for the myelin proteins P2, P0, or PMP22 expressed on Schwann cells, the myelinating cells of the peripheral nervous system (93-95). Demyelination is macrophage-mediated but most immune cells are likely to be important in disease, as seen for EAE. The disease onset is usually around 10-12 days p.i. and manifests as an ascending paralysis with pronounced gait disturbances.

4. THE IMMUNE SYSTEM

We do not yet fully understand the pathogenic mechanisms that regulate MS. What is clear is that several immune cells and pathways are essential in disease development, a notion that is also supported by genetic studies. Moreover, investigation of EAE pathogenesis has provided important insights into the mechanisms underlying MS (71). There is no formal proof that MS, and many other autoimmune diseases (ADs) are initiated by an autoimmune event. The term ADs will therefore refer to the downstream pathogenic mechanisms whereby self-tissue or antigens are destroyed, irrespective of the trigger.

Adaptive immune responses have for a long time been in focus in MS and EAE research, with the notion that disease ensues upon breakage of tolerance to self-antigens. Concordant with this thinking, the major genetic risk factor in both MS and EAE is the HLA/MHC locus and the thus far identified non-MHC risk genes indicate a role for adaptive immunity and, in particular, T cells (34). Additionally, transfer of T cells specific for myelin antigens is sufficient to induce EAE (96). For these reasons, the importance of T cells in neuroinflammation has been intensively explored and many studies have suggested that T helper 1 (T_H1) polarized CD4+ T cells are important in MS initiation (97). This is supported by an increased number of T_H1 cells producing IFNγ in the blood, cerebrospinal fluid (CSF) and around lesions of patients (98-100). IFNγ is the T_H1 hallmark cytokine with many pro-inflammatory effects, including activation of macrophages (101). However, during the last few years the relative importance of T_H1 cells has been debated. Instead, the T_H17 subset has emerged as an important mediator of disease. T_H17 cells differ from T_H1 cells in their cytokine profile; and rather secrete IL17, IL21, IL22 and granulocyte-macrophage colony stimulating factor (GM-CSF) (102, 103). It is noteworthy that adoptive transfer of either subset will generate EAE, albeit with distinct characteristics (96, 104).

$CD8^+$ T cells have been relatively ignored in the pathogenesis of MS. This is in part due to the stronger genetic association of MHC class II alleles to MS compared to MHC class I alleles, and the dominance of CD4+ T cells in most EAE models (105). Conversely, CD8+ T cells outnumber CD4+ T cells around MS lesions (106), and MS therapies that target CD4+ T cells have been unsuccessful whereas whole T cell-depleting therapies reduce disease considerably (105).

B cells also contribute to disease through antigen presentation and autoantibody production (107, 108). These autoantibodies appear to recognize large conformational epitopes rather than short peptides (109).

Adaptive immune responses can also be disease-protective. In particular, regulatory T cells (T_{REGS}) which are often $CD4^+CD25^+Foxp3^+$, maintain peripheral tolerance by suppressing autoreactive T cells through mechanisms involving cell-cell contact and cytokines such as IL10 and TGF-β (71). Under normal steady-state conditions T_{REGS} potently inhibit excessive inflammation. Conversely, failure of T_{REGS} to curtail inflammation has been described for MS as T_{REGS} from patients have defective suppressive capacities compared to those of healthy controls (110, 111). $CD8^+$ T cells have also been attributed a protective role in some studies (112-114). Similarly, other reports suggest an additional influence from a disease-ameliorating B cell subset (70, 115, 116).

The mechanisms mentioned so far primarily relate to antigen-specific events, but innate immune cells are also essential in disease development and progression. These innate mechanisms include antigen presentation to lymphocytes and various responses through activation of their predetermined surface receptors.

For example, dendritic cells (DCs), which accumulate in the CNS during inflammation, are professional antigen presenting cells (APC) and potent producers of both pro- and anti-inflammatory cytokines. As such, DCs play a major role in determining the activation and differentiation of naïve T cells into effector or suppressive cells (117, 118).

Microglia and macrophages likely contribute to disease through similar mechanisms. The two cell types share many features, including phagocytosis of surrounding cells and tissue, cytokine production and antigen presentation, but they differ in their tissue distribution (119). Microglia are CNS-resident cells, where they are the most common immune cells. Macrophages infiltrate the CNS from the periphery. During MS, phagocytosing microglia and macrophages are primarily responsible for the CNS tissue destruction (120).

Another innate cell mechanism involves natural killer (NK) cells which appear to be mainly disease-protective in MS and EAE (121, 122). Decreased numbers of NK cells have been detected in MS patients and depletion of NK cells in EAE leads to increased severity (71).

Many more cell types are implicated in the pathogenesis of MS and EAE, including mast cells (123), γδ T cells (124) and NKT cells (125). The interactions between these cells are poorly defined and future studies will hopefully further our understanding of the molecular events that trigger and sustain disease.

4.1. CYTOKINES AND RECEPTORS

Cytokines and receptors, which comprise several heterogeneous subgroups, have frequently been studied in my thesis. In particular, the cytokines tumor necrosis factor (TNF), IFNγ and IL22 and their receptors have been of interest. During disease initiation in the MOG-EAE model, all three cytokines are upregulated in the susceptible Dark Agouti (DA) rat strain compared to the resistant Piebald Viral Glaxo (PVG) strain which may indicate an enhanced differention of T_H1 and T_H17 effector cells (126). In the WSC-EAE model, IFNγ and TNF expression in CNS infiltrating cells correlates with disease symptoms (127, 128), and this expression is MHC-haplotype dependent (77). This section will illustrate examples of the pluripotent and sometimes opposing roles these secreted molecules can play in shaping the immune response.

Tumor necrosis factor (TNF) is rapidly produced upon infection to confer immunity to the host. TNF regulates several biological processes including inflammation, apoptosis and cellular expansion (129), Both soluble and membrane-bound forms of the cytokine exist with the ability to bind several distinct receptors (130-133). Elevated TNF expression is observed in inflammatory disorders such as MS, RA and septic shock (134-136), whereas reduced TNF levels associate with increased risk of infections (137).

Moreover, TNF-blocking agents potently reduce severity of several ADs (138, 139). By contrast, similar TNF-depletion in MS patients worsens disease symptoms demonstrating how blocking TNF is not always beneficial (140). The disparate functions of TNF may be partly attributed to the diverse functions of its receptors (141-143).

IFNγ, mainly produced by T cells and NK cells (144), is another cytokine displaying opposing roles in autoimmunity. On one hand, IFNγ drives inflammation by increasing T_H1 differentiation and enhancing MHC expression and activation of innate immune cells (144-147). Accordingly, treatment of MS with IFNγ worsened disease symptoms (148). On the other hand, IFNγ and IFNγ receptor knockout strains convert EAE-resistant mice to a susceptible phenotype (149-151).

IL22 is a cytokine that associates with CD and psoriasis (152, 153). Different subsets of immune cells excrete IL22, with T_H17 cells being the primary producers (154-157). In addition, one report suggests that monocytes can also produce IL22 in presence of IL23 and lipopolysaccharide (LPS) (152). Its surface receptor, IL22R1, is expressed on a variety of epithelial tissues (158). The IL22 system also consists of a

soluble receptor, IL22R alpha 2 (IL22RA2), with relatively unknown function in biology (159, 160).

These examples illustrate that in order to fully understand cytokines' impact on disease one must determine their cellular source as well as temporal and spatial expression of the cytokine and the corresponding receptors.

4.2. SHARED IMMUNE MECHANISMS

ADs encompass disorders such as MS, GBS, CD, T1D and rheumatic diseases such as RA and systemic lupus erythematosus (SLE). They all have in common that the immune system attacks self tissues or antigens. It is also likely that their disease pathways converge in part into key endpoint mechanisms. If such mechanisms were genetically determined, we would expect ADs to cluster in families at higher risk. Although some debate surrounds this hypothesis, larger population-based surveys demonstrated that families with members affected by RA or MS were more likely to also manifest other ADs (161, 162). The frequency of AD aggregation was higher in families containing multiple members with MS compared to families with a single member affected by MS (162). Several genetic studies support this notion, with existing reports on shared risk genes between ADs, including *IL2RA* and *CLEC16A* for MS and T1D and *PTPN22* for RA, T1D and SLE (163, 164).

While strongly support a partially common basis for ADs these findings suggest that distinct alleles and possibly mechanisms control distinct ADs, with little overlap in between. In this thesis, shared genetic regulation has been investigated with the aim of identifying shared pathways. Shared pathways may implicate for example T_{REG} activity or elevated pro-inflammatory cytokine levels such as TNF and IFNγ. In experimental models of ADs, several shared genetic regions have been reported, making them highly suitable for this purpose (165).

When successful, this approach will facilitate treatment against pathways controlling distinct diseases, irrespective of the exact polymorphisms regulating them. For example, TRAF1C-5 and TNFSF15, which both implicate a role in disease for TNF, are risk genes for RA and CD, respectively (166, 167). In analogy with this, TNF-blockade is a successful treatment of both diseases (138, 139).

5. AIMS OF MY THESIS

This thesis focuses on dissecting and understanding genetic regulation of complex inflammatory diseases. I used an unbiased approach to identify disease-regulating genes in experimental models of two human diseases: MS and GBS. The specific aims of my thesis are categorized as follows:

1) Position disease risk genes in experimental neuroinflammation and translate findings to human disease.

2) Define the pathogenic mechanisms controlled by these genes.

3) Identify shared mechanisms across autoimmune diseases.

6. METHODOLOGY

6.1. FINDING RISK GENES IN HUMANS

The identification of disease-regulating gene variants has proven difficult. The relatively small effect exerted by the risk genes has been one issue. Another obstacle is the failure to reach statistical power capable of addressing the genetic heterogeneity and confounding factors, including environment, that are inherent to complex human disorders. Traditionally, linkage mapping - where we follow the inheritance of a disease with a marker linked to the risk gene - has been used to identify disease genes. Similar studies in complex diseases can offer an advantage in identifying rare risk variants that aggregate in some families, but thus far linkage studies have mostly proven fruitful for monogenic diseases. In MS, several linkage analyses have failed to accurately detect any non-HLA risk loci (22, 168-174). The obstacles include too weak or inconsistent statistical signals, and poor resolution due to an inadequate number of families being included (22). For these reasons, the only region unequivocally linked to MS was for long the HLA, due to its major genetic effect represented by an increased relative disease risk, or odds ratio (OR) of approximately 3 (18, 19, 28, 175).

Instead, technical advantages in genotyping and the sequencing of the human genome have enabled use of genome-wide association scans (GWAS) of complex diseases. In an association study, a vast amount of genetic markers (into the millions) located throughout the genome are tested in a case-control cohort for association to disease. The association is then performed by comparing frequency of each marker, or allele, between cases and controls. The markers are typically single nucleotide polymorphisms (SNPs), but more recent GWAS also include larger variations, such as copy number variations (CNVs) (176). When adequately powered, GWAS have identified several risk genes (30, 34, 163, 166, 167, 177, 178). The number of required cases and controls in GWAS depends largely on the expected effects and frequency of the risk genes (14). So far, most identified common disease risk alleles display weak ORs, increasing the relative risk with a factor of less than 1.5 (179). Typically, several thousands of cases and controls are necessary for a reliable investigation (14, 177). On the other hand, this means that even larger GWAS hold the potential to discover genes with very small effects.

One assumption in the design of association studies is that a large part of the genetic variation controlling disease resides in common gene variants. This is referred to as the *common-disease common-variant (CDCV) hypothesis* (2), whereby several common alleles each contribute to disease with weak effects. A

common allele is typically defined as having a minor allele frequency>1% in the population (180). This rationale has been essential for the International Hapmap project, in which several reference individuals in search for common variations, or single nucleotide polymorphisms (SNPs) have been mapped across the genome (180). SNPs that are in high linkage disequilibrium (LD) are then selected. LD refers to the non-random co-occurance of two loci during recombination (181). A high degree of LD between two SNPs therefore indicates that they are likely to co-segregrate. By selecting such SNPs, also called tagging SNPs, most of the genetic variation can be captured with a limited number of markers.

An opposing hypothesis to CDCV is that disease is instead regulated by rare mutations conferring relatively strong effects. By default, such variations would be difficult to identify relying on conventional association studies. It is likely that common disorders arise by a combination of these two scenarios (182-184). The GWAS of MS performed to date have provided the power and resolution to identify some non-MHC risk genes (30, 34). Future studies with even larger cohorts are likely to identify more variants.

Another problem is why the identified risk genes typically combine to only explain fractions of the genetic variance. One reason for this may be the largely undiscovered epistatic interactions between loci across the genome. Epistasis in this context refers to the effect of one gene being dependent on another (or several other) genes (185). Studying epistasis in complex diseases has proven difficult with few successful examples, all providing limited functional insights (186-188). Remaining obstacles include the need for better understanding the functional relevance of these polymorphisms. This is largely due to tissue inaccessibility, although some disease-relevant material can be collected. Examples of this are peripheral blood and cerebrospinal fluid (CSF) in which transcript levels of risk genes can be correlated with specific genotypes.

6.2. FINDING RISK GENES IN RODENTS

Genetic studies in experimental models of MS hold the advantage of controlling environmental factors, minimal heterogeneity by use of inbred strains and large study cohorts providing sufficient power to identify low-risk alleles (75). The tissue availability and possibility to generate recombinant strains offer powerful tools that can confirm the discovered genetic influences.

The typical approach is to perform linkage studies in rodents with opposing disease susceptibilities, in our case EAE in rats (81, 84, 189). By crossing EAE-

susceptible and resistant strains we can obtain genetically unique individuals and the disease pre-disposition for each individual will depend on which alleles were inherited. This is first performed in an intercross (F_2 or backcross (N_2), where genetic regions, or quantitative trait loci (QTLs) and their relative effects can be determined across the genome (Figure 5).

Although practical in initial identification of broad QTLs, F_2 crosses have their limitations. The QTLs identified in F_2 crosses typically comprise hundreds to thousands of genes and this hampers candidate gene investigation. Moreover, large QTLs can in fact harbor several QTLs that may or may not interact with each other (190). To address these issues one can generate an advanced intercross line (AIL) between susceptible and resistant strains by randomly intercrossing the F_2 progeny (avoiding sibling mating) for several generations (Figure 5)(191).

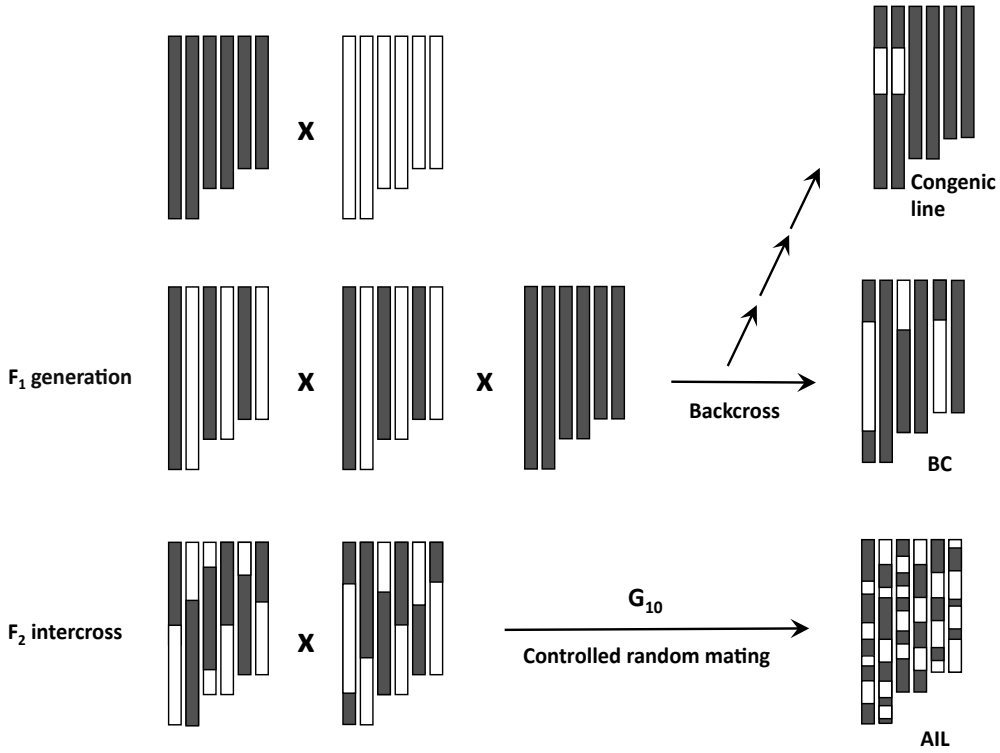

Figure 5. Various rodent crossing strategies. An F_1 progeny is first generated from EAE-susceptible and EAE-resistant parental strains. By crossing F_1 individuals we then obtain the F_2 intercross. Alternatively, a backcross (N_2) is generated by crossing F_1 individuals with either of the parental strains. Congenic lines are created similarly, by backcrossing and selecting for the region of interest. AILs are produced by intercrossing F_2 individuals for several generations.

The greater number of recombination events in an AIL enables fine-mapping of QTLs originally detected in F_2 crosses (Figure 6). Compared with an F_2, an G_{10}

yields a 5-fold reduction of the QTL interval (191). This provides high mapping resolution, identification of clustered QTLs within a broad QTL and better resolution of possible interactions between QTLs.

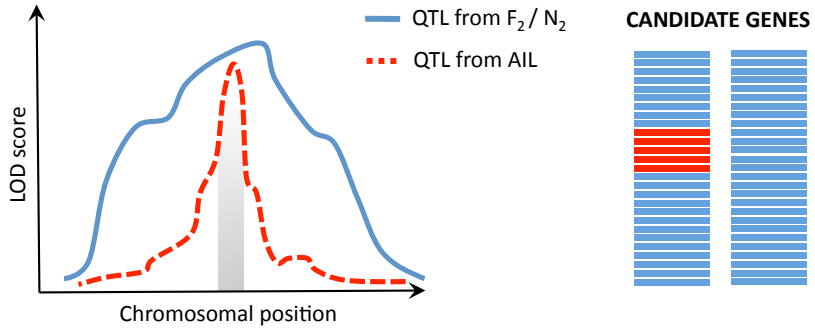

Figure 6. Refinement of QTL and candidate gene lists by using AIL compared to F_2 intercrosses or backcrosses. The number of candidate genes within the QTL is reduced using this strategy. Gray bar represents the narrowed confidence interval

Mapping in rodent intercrosses provide us with statistical linkage of a candidate gene region, which in a best-case scenario contains 10-20 genes. However, to confirm this linkage biologically and to investigate the functional role of the genes within the QTL, congenic lines are often created (Figure 5). In these, the alleles from the resistant strain comprising the QTL are introgressed onto the genetic background of the susceptible strain, or *vice versa* (192). We can here perform functional investigation of the QTL in isolation, providing a permanent tool for future experiments. Congenic and AIL breeding can successfully be conducted in parallel, based on previous reports in F_2 intercrosses or backcrosses. Accompanying AIL analysis will more rapidly guide the congenic breeding towards the specific region of relevance. Through guided functional experiments in congenic lines the importance of each candidate gene can then be assessed. When an EAE risk gene is finally identified it can be tested for association in MS cohorts (Figure 7).

6.3. LINKAGE ANALYSIS

With linkage analysis we aim to link our trait (often disease) with the genotype (DNA marker). We typically use interval mapping, which includes the genotype effect both at and between the markers analyzed. It is a powerful method for estimating the QTL location, as the underlying variation can reside far from the genotyped DNA marker. In interval mapping, multiple markers are analyzed

simultaneously and the genotypes between are predicted depending on the recombination frequency (193, 194).

The significance of the linkage is displayed as the logarithm of odds (LOD). A LOD score describes the likelihood of QTL presence given the data available compared to the likelihood of no QTL presence, given as log value with the base of 10. To illustrate this, a QTL displaying linkage to disease with a LOD score of 4.0 is 10,000 times more likely to be truly linked than not (at the given marker). The linkage studies in Papers I, III and IV were conducted using the R/qtl software (195).

We establish confidence intervals (CI) in order to estimate the likely location of a QTL. A 95-percent CI means that there is a 0.95 probability that the true location of the QTL is within the boundaries of the CI. In papers I, III and IV, the CI was determined by first using a LOD drop of 1.8 (196), and then using the flanking marker as boundaries.

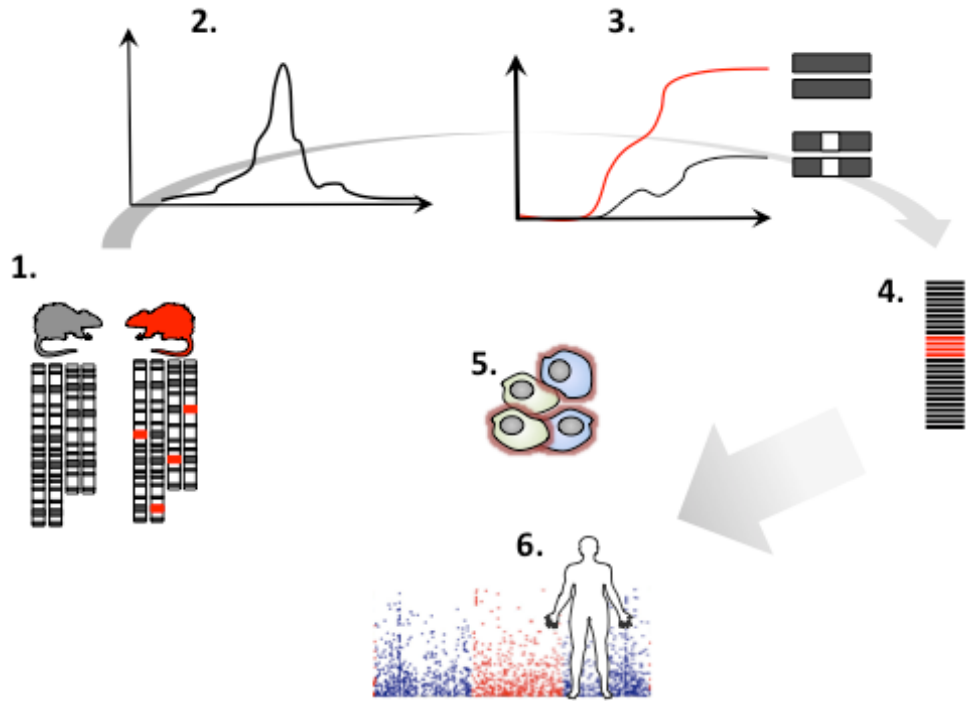

Figure 7. Summary of our translational forward genetics approach. Disease-regulating QTLs are first dissected in intercrosses (1,2) and confirmed in congenic lines (3). Through sub-congenization and guided experiments, a few candidate genes can be selected (4). These candidates can be further studied functionally in the congenic lines (5), and also be translated to MS risk genes in multiple cohorts (6).

6.4. IMPORTANT VARIABLES

The positional cloning of a gene underlying a disease trait in experimental models is often a time-consuming process, especially when using an unbiased, forward genetics approach. The timeline for the initial intercross mapping is rather constant, although advances in SNP identification in rats will offer large arrays as alternatives to conventional microsatellite genotyping (197).

It is rather in the fine-mapping of the candidate gene where most time is spent. Narrowing-down a QTL typically involves sub-congenization, aiming at isolating the disease-regulating gene. The timeline of this depends on several factors, including:

- The variation of the trait
- Strength, or dominance, of the gene's influence on the trait
- Gene density within the QTL
- Clustering of related genes within the QTL
- Rate of recombination in the region

The disease variation in EAE is substantial, as for most models of complex disease. This is largely due to environmental, technical factors and the numerous redundant immune mechanisms that can potentially override the influence from a particular candidate gene. As a consequence, the effect of the candidate gene (i.e. the genetic variation regulating disease) must be strong enough to surpass this 'noise threshold'.

Another important consideration is the genetic architecture of the QTL. Gene-dense regions are more difficult to study, as minimal congenics (<1 Megabase) are required to fully exclude non-disease regulating genes. In addition, clustering of functionally related genes, e.g. cytokines, chemokines and receptors, makes it difficult to tailor assays to target a specific gene, as the phenotypic read-out will likely lay downstream of several genes within the QTL. For all these reasons, true positional cloning, meaning the isolation of the disease-regulating polymorphism, is extremely difficult and time-consuming. However, detailed characterization in the congenic line can identify the disease mechanism without knowing the exact underlying genetic determinant.

6.5. WHAT IS THE RELEVANT PHENOTYPE?

When conducting linkage studies in experimental models of complex diseases, the penetrance of the gene of interest is important. This is also true for the follow-up functional studies in congenic lines. For example, if the phenotype is EAE severity, the underlying genetic variation has to be strong enough to cause significant differences in EAE severity between individuals who are carriers of different alleles in order to be identified. Sometimes this is not the case as other genes might regulate disease at various levels and 'override' the genetic variant we are studying. In this scenario we would not observe linkage, as the effect is too weak to detect. One way to overcome this potential obstacle is to have a sufficient number of experimental animals, well powered enough to handle this complexity of the genetic regulation.

However, it is also possible to use other phenotypes that lie more proximal to the genetic variation. If clinical disease represents the ultimate end-point read-out, measurement of protein levels or mRNA transcript levels would constitute earlier events. This can for example be secretion of an inflammatory cytokine by T cells or macrophages. The advantage here is that the phenotype is less complex resulting in a stronger correlation between the genotype and the phenotype. One must of course bear in mind that such phenotypes can merely be associated with disease and may not actually be disease-causing. We can minimize this risk by only studying disease-relevant tissues and time points that correlate with clinical disease. In the studies included in this thesis we have used clinical phenotypes such as disease severity and susceptibility. Simpler cellular phenotypes include lymphocyte activation upon receptor stimulation and CNS infiltration. This will also logically affect protein and mRNA levels such as autoantibody titers, cytokine secretion or transcript levels of inflammatory genes. An interesting mechanism that is receiving increasing attention is the epigenetic regulation of complex diseases. Epigenetic mechanisms can involve DNA methylation, histone modifications, non-coding RNAs such as micro RNAs (miRNA). All these mechanisms will affect all the above-mentioned events (198-202). Our studied phenotypes are summarized in Figure 8, also illustrating the complexity of identifying natural gene variants that regulate clinical disease.

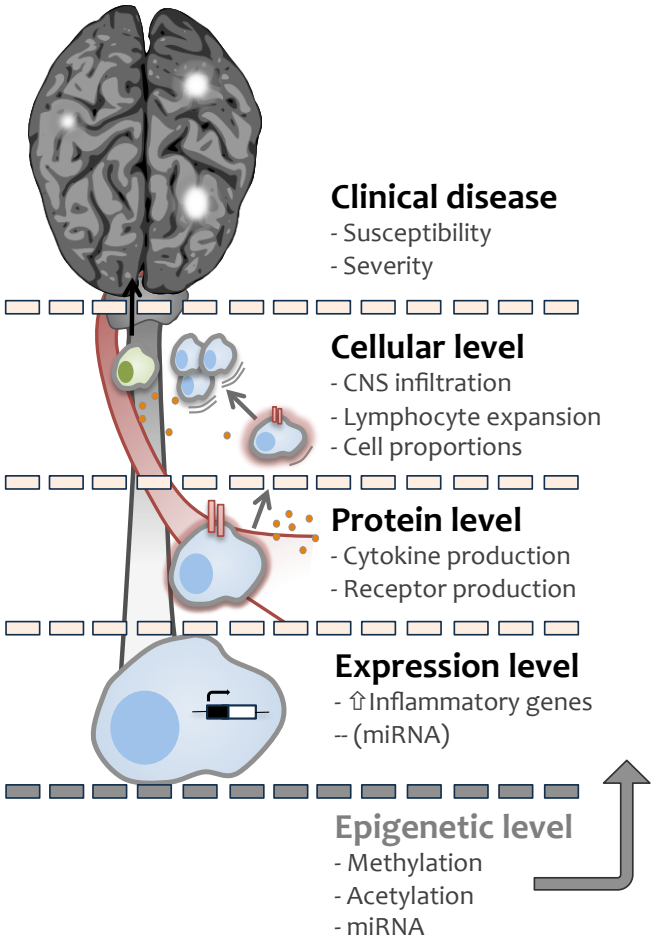

Figure 8. Schematic overview of levels of phenotypes studied in this thesis, ranging from clinical symptoms to transcript expression.

6.6. THE -OMICS ERA

Microarray technologies are increasingly being employed in applications of complex diseases. For genotyping it has been essential in designing GWAS studies and will be crucial in analogous experimental efforts (203, 204). At the messenger RNA (mRNA) level microarrays enable studies of whole networks of transcription, including correlation of disease-relevant genes and enrichment for transcripts regulating distinct disease mechanisms (205, 206). This regulation of expression can be characterized by expression QTL (eQTL) mapping, where genome-wide expression levels are tested for linkage to genetic loci. The approach has successfully facilitated identification of several genes and pathways important for complex disease (207-209). Similar profiling of the proteome and epigenome has also been conducted (210, 211). When such studies are combined with conventional linkage or association mapping, we will be able to study whole

disease pathways, not confining to only studying candidate genes in isolation. This will likely shed new light on the complex pathogenesis underlying MS and other diseases.

6.7. WHY STUDY MS USING RATS?

The laboratory rat (*Rattus norvegicus*) has long been employed as a tool for establishing models of human disease (197, 204). Reasons for this include facilitated analysis at organ and cellular levels because of the rat's larger size, and generally strong similarity with human disease. Accordingly, EAE is a model that displays several disease characteristics more similar to MS compared to the mouse. There is also a high degree of genetic similarity between MS and rat EAE, with several MS risk genes being differentially expressed between EAE-susceptible and resistant EAE rat strains (126). Another strong advantage in the context of EAE is the milder disease induction protocol for most rat strains. It does not require use of Complete Freunds Adjuvant (CFA) or pertussis toxin, which is common for many mouse EAE protocols. This is of high significance for linkage studies of inflammatory diseases, as introduction of mycobacteria or toxins will themselves generate specific responses that are genetically regulated. This can generate statistically significant linkage peaks that are not 'true' peaks arising from the autoantigen (212).

Conversely, genetic studies in the rat have suffered from shortage of tools and the genetically engineered strains that are available in mice. This is, however, rapidly changing as antibodies and other reagents are increasingly becoming available for rats. The previous inability of embryonic stem cell (ES) targeting in rats has also led to development of other efficient strategies e.g. zinc finger nuclease (ZFN) technology and transposone-mediated mutagenesis (213, 214). Moreover, the first rat knock-out strains demonstrate that recombinant technology targeting disease candidate genes will soon be standard practice in rat genetic studies of complex diseases (215). Importantly, numerous inbred rat strains exist in various laboratories, each bearing its own susceptibility to EAE. These differences in EAE mostly originate from the genetic background, where some strains carry disease-driving variants of risk genes and others carry disease-protective variants (Figure 9). We can exploit these genetic differences between rodent strains to identify the disease risk genes, as also more and more rat strains are being whole-genome sequenced (216).

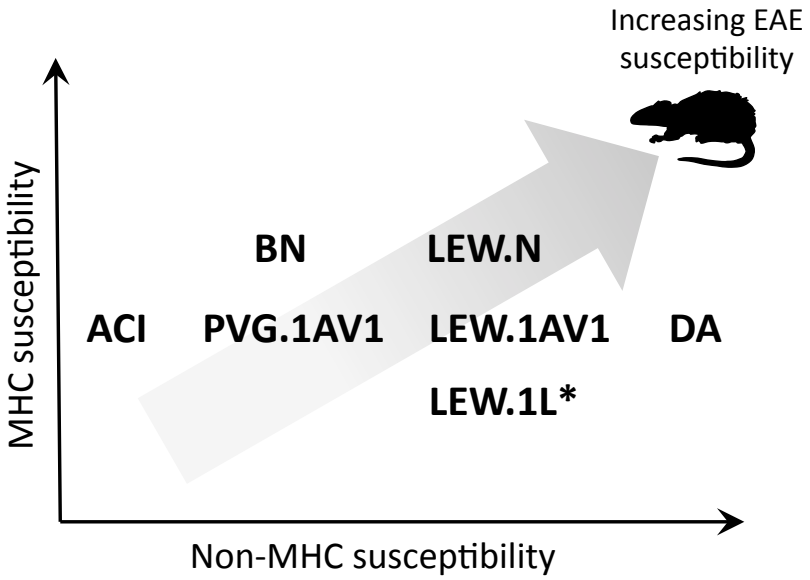

Figure 9. Varying MHC-dependent and non-dependent susceptibilities to EAE in rat strains relevant for this thesis. *LEW.1L susceptibility is model-dependent; it is resistant to MOG-EAE but susceptible to MBP-EAE.

7. RESULTS AND DISCUSSION

As mentioned earlier, my objective has been to identify genes in our experimental models that can be translated as risk genes in human disease. I also wish to exploit the animal models to better understand the pathogenic mechanisms conferred by the risk genes. In total, my four papers identified five candidates risk genes in neuroinflammation. The identification of these genes is hereby shortly summarized:

7.1. POSITIONAL CLONING

7.1.1. Translating EAE risk genes to MS

In the first two studies of this thesis (Paper I and II), we successfully conducted a translational three-step approach to identify two candidate genes, interleukin 22 receptor alpha (*Il22ra2*) and *Vav1*. We first defined the disease-regulating regions by linkage mapping in the rat. We then established congenic lines allowing us to confirm the biological effect and refine the underlying pathogenic mechanisms, which led us to the two candidate genes. Finally, both genes (and adjacent candidates) could then be tested as MS risk genes in several cohorts. In Paper I we could demonstrate the power of using an AIL for linkage studies with a 4-fold reduction of the *Eae29* interval, compared to the initial F2 intercross (217). The *Vav1* study (Paper II) specifically demonstrated a near-formal proof of disease regulation by *Vav1* in the rat, and influence of *VAV1* was subsequently confirmed in six MS cohorts.

We confirmed the biological importance of *Eae29* and *Eae4* by creating disease-protective congenic lines (Figure 10). The follow-up functional studies in these congenic lines then tested for differential activity of different cell types between congenics and parental strains. In a stepwise manner we could establish a role for *Il22ra* in macrophage function and *Vav1* in T cell activity.

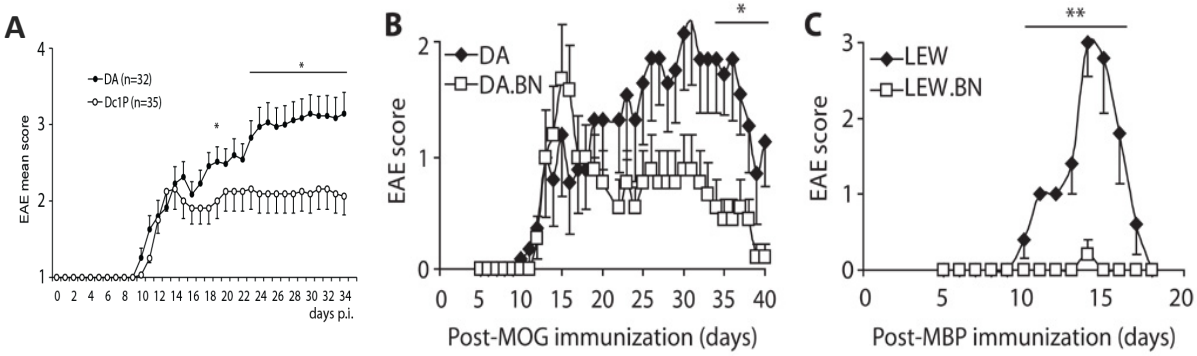

Figure 10. A DA.PVG congenic line carrying resistant *Eae29* alleles (A), and DA.BN and LEW.BN congenic lines carrying resistant *Eae4* alleles (B, C) all reduce disease compared to the susceptible parental strains.

The subsequent MS association studies indicated the need for including multiple cohorts. The observed ORs were, as expected, rather low, with 1.26 for IL22RA2 and 1.18 for VAV1, respectively (Figure 11). It was therefore necessary to include several thousands of cases and controls for statistical significance. All disease-associated SNPs were intronic, which is fairly common for association studies. Whether these are true disease-regulating SNPs or just lie in proximity to causative SNPs remains to be determined. Replication studies by performing deep sequencing of the genetic regions surrounding the associated SNPs are likely to more accurately position the causative polymorphisms.

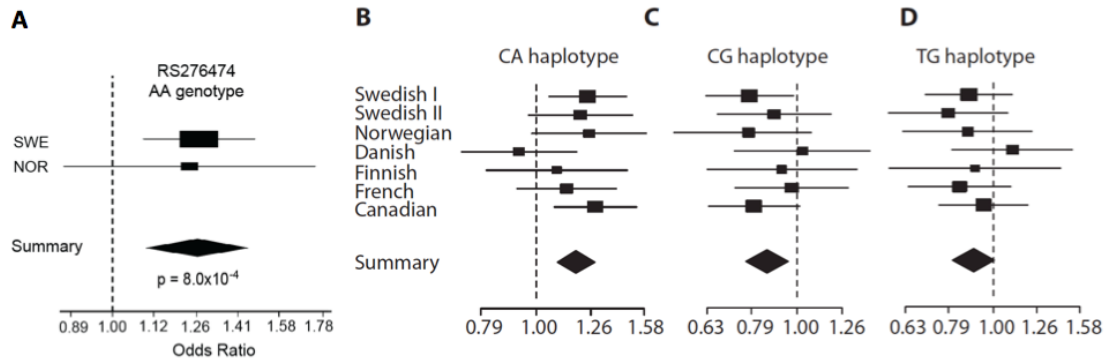

Figure 11. A polymorphism in *IL22RA2* (A) and a haplotype in *VAV1* (B-D) both associate with MS in combined MS cohorts.

7.1.2. Epistasis in EAE

Papers I and II identified risk genes with ORs in the range of 1.18 - 1.26. This reinforces the observation that common disease risk genes on their own only exert small effects, which do not combine to completely explain the genetic variance. The discrepancy might be accounted for by unidentified gene-gene interactions, which are difficult to study in humans.

In Paper III we used an eQTL mapping combined with pathway analysis and classical QTL mapping, to dissect the first epistatic interaction on a molecular/gene level in EAE. We identified two eQTLs regulating expression of the NK cell receptor *Klrk1,* and its ligand cluster, retinoic acid early transcripts (*Raet1*), respectively (218, 219)(Figure 12). *Raet1* served as a modulator of *Klrk1* expression that ultimately regulated NK cell activity and EAE severity.

The eQTLs were identified in a backcross originating from DA and PVG strains. The linkage analysis revealed that both NK cell receptor and ligand expression were *cis*-regulated, meaning that the eQTLs map to the physical location of the regulated transcripts. Higher mRNA-levels were conferred by PVG alleles for both transcripts. By utilizing the D1cP congenic line that carries PVG alleles in *Raet1* we could confirm the functional importance of the interaction on NK cell function and abundance. Moreover, we demonstrated that stimulation of *RAET1* boosted NK cell activity, which could also reduce expansion of autoreactive lymphocytes.

This study illustrates how experimental models can contribute in identifying biologically relevant interactions. The importance of the *Klrk1-Raet1* interaction in MS and other diseases remains to be elucidated. Perhaps of greatest interest, the interaction highlights an interesting emerging mechanism involving NK cells. These mechanisms will be discussed in more detail later on in this thesis.

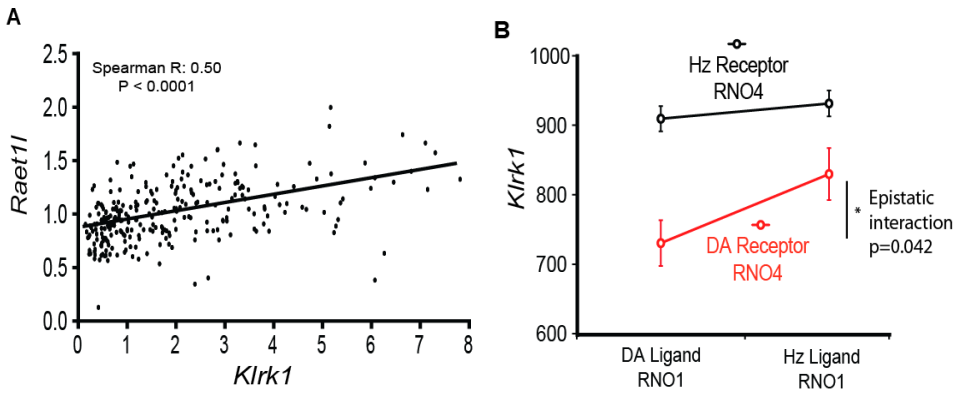

Figure 12. Epistasis between *Raet1* and *Klrk1*. Expression of the two transcripts correlated in the (DAxPVG)xDA backcross. Moreover, genotypes in *Raet1* modulated the expression of *Klrk1* and confirmed the epistasis. RNO1, RNO4 = rat chromosome1 and 4, respectively.

7.1.3. Shared genetic risk across diseases

In Paper IV we chose to study a model of another neuroinflammatory disease, GBS. We hypothesized that pathogenesis between EAN and EAE is in part shared. Given our knowledge of the genetic variance between DA and PVG rat strains, we established a (DAxPVG) G_{12} intercross, which was subjected to EAN. Overlap of QTLs between EAE and EAN would indicate shared pathogenic mechanisms across diseases. The study identified and refined a total of five QTLs, all being shared with other models of complex inflammatory diseases (Figure 13). The QTL exhibiting strongest linkage to clinical disease was *Ean6* on RNO12. We mapped down this effect to the neutrophil cytosolic factor 1 (*Ncf1*) as the disease-regulating gene, previously reported to regulate both EAE and experimental arthritis (220, 221). We also demonstrated how direct stimulation of the NADPH oxidase complex (NOXC), of which *Ncf1* belongs, ameliorated EAN. We therefore suggest a general role of *Ncf1* and the oxidative burst in the pathogenesis of experimental autoimmune animal models.

In addition to clinical parameters we used splenic production of IFNγ as a phenotype for the linkage study. Many of the shared QTLs linked to this phenotype indicating the existence of converging immune pathways that involve proinflammatory cytokine secretion. This also means that dissection of disease mechanisms in EAE or experimental arthritis will shed light on EAN pathogenesis, and *vice versa*.

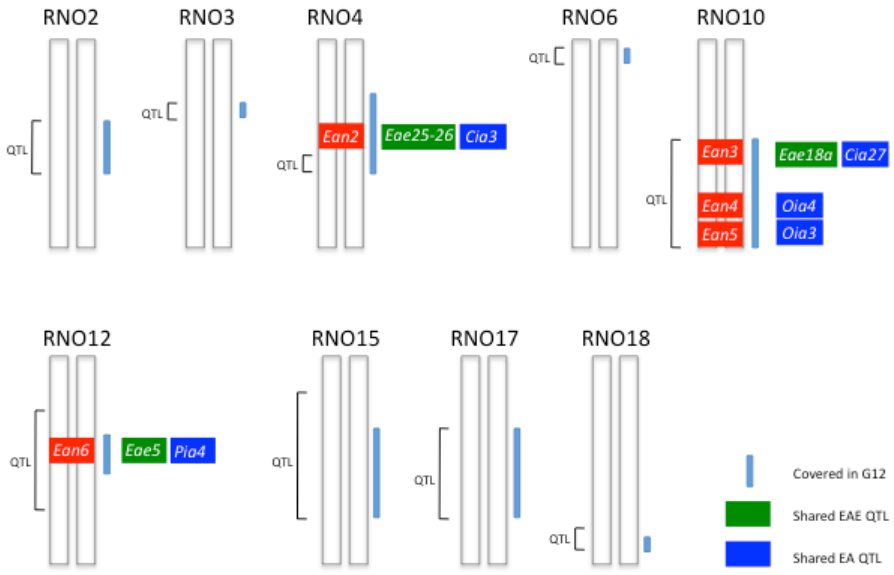

Figure 13. Shared QTLs between EAE, EA and the refined EAN loci *Ean2-6*. Blue bars denote the regions mapped in the EAN AIL.

Logically, we would expect to find both shared and non-shared loci. In this study, however, all EAN loci overlap those of other models. Although the same genes do not necessarily drive all shared QTLs, these findings strongly suggest that the QTLs regulate events occurring in the immune system, as these are most likely to be shared across inflammatory diseases. This would fit well with the human studies, which have thus far only identified immune risk genes for MS.

7.2. GENE DISCOVERY TIMELINE

As illustrated in Papers I-III and discussed in the *Methods* section, we rely heavily on congenic strains as a tool to fine-map disease QTLs. Depending on the genetic architecture of the QTL and the methodology employed, the timeline for identification of candidate genes has varied considerably (Figure 14). The strength of this approach is that multiple tools can be generated simultaneously for a number of QTLs, such as generation of AILs or shared congenic lines.

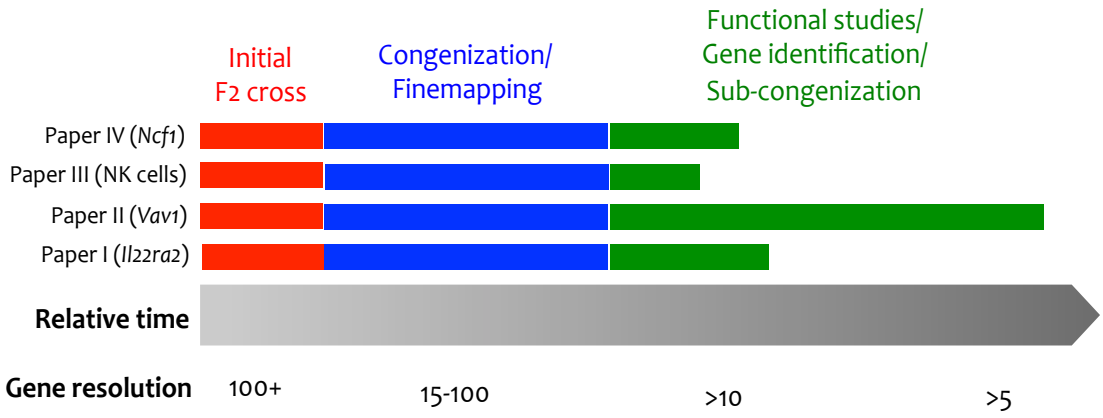

Figure 14. Timeline for gene discovery in Paper I - IV. Congenization and fine-mapping (blue lines) can often be performed simultaneously for multiple loci, e.g. congenization for Paper I and III. Moreover, the genes identified in Paper III might not need congenization and may be targeted using the emerging strategies in rats.

Smaller congenic strains require more generations of backcross breeding. The efforts to obtain the minimal congenic strains in Paper II, isolating a <1 Mb QTL including less than ten genes, largely explain the long timeline from study initiation to positional cloning of *Vav1*. This was further complicated by the lack of prior knowledge from an AIL or eQTLs on to where the most likely gene location was, as we had for the other studies. The *Vav1* study also constitutes the best

example of a systematic stepwise dissection of a QTL, wherein most other genes within the original QTL have been formally excluded. We were also hindered by the physical location of the QTL, close to the RNO9 centromere, resulting in low recombination frequencies. The importance of recombination frequencies for congenic breeding is illustrated in Figure 15, which compares *Eae4* with *Eae29*.

Another advantage both *Eae29* and *Ean6* offered over *Eae4* was the low gene density within the QTL confidence interval. This allowed more rapid positioning of candidate genes for *Eae29* and *Ean6*, whereas *Eae4* candidate genes had to be discerned through more detailed and tedious SNP haplotype-phenotype correlations.

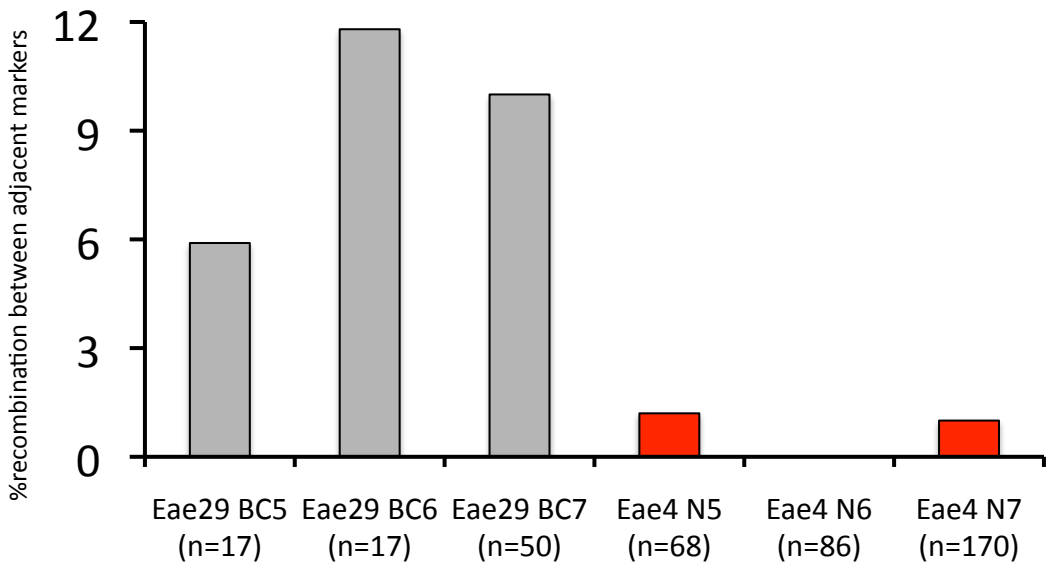

Figure 15. Recombination frequencies during subcongenization for the *Eae29* and *Eae4* alleles, respectively. Recombinations between the adjacent markers D1AO6 and D1Got11 (14.9 – 17.1 Mb) were assessed for *Eae29*, and between adjacent markers D9MJ280 and D9Rat45 (1 – 3.8 Mb) for *Eae4*. Grey bars represent *Eae29* recombinations for the last three generations of congenic breeding. Red bars represent *Eae4* recombinations in three representative generations of crossing between congenic lines and F_2 individuals.

7.2.1. Non-clinical phenotypes

In the *Vav1* study we rapidly discovered how TNF production of splenocytes correlated perfectly with disease susceptibility. We used this as an end-point phenotype in the sub-congenization of the initial DA.BN-*Eae4* congenic strain, thereby overriding the need to test each strain for difference in clinical disease and significantly reducing number of used animals and time necessary for testing new recombinants. Instead, the clinical difference could be confirmed in the

minimal congenic strain, R25. Without this approach the positional cloning of *Vav1* would have taken considerably longer time.

In Paper IV we also used lymphocyte activation as a phentype, but rather for detecting QTLs in the linkage study. IFNγ secretion by activated splenocytes reflects activity of mainly T cells and we therefore hypothesized that IFNγ secretion constitutes a less variable phenotype than clinical disease for the identification of QTLs important for T cell function. We identified four 'IFNγ' QTLs, which all overlap QTLs of other similar models, suggesting that they are truly disease-regulating.

7.2.2. Methodology

Considering the time and effort required producing congenic strains and testing candidate genes individually, it is important to improve the methodology in how we characterize phenotypes. Another issue is how we can formally exclude candidate genes within a QTL in an unbiased manner. In Paper III we could overcome some of these obstacles by using genome-wide transcript arrays combined with conventional linkage analysis to not only identify a genetic interaction that regulated expression of immune genes, but also to link this to a disease mechanism involving NK cells. We could therefore perform guided experiments to study the importance of the NK cell activity and lymphocyte expansion. By using genome-wide arrays in our congenic lines we will be able to see how whole networks are affected by our disease risk genes. Given the density of such generated data, the problem will rather be to identify the most disease-relevant time-point and tissue to study. The future will also lie in more efficient gene targeting that can be performed on any genetic background. ZFN technology and lentiviral transfection are examples of required methods, but also improved genome-wide pathway analysis will be crucial in efficient detection and characterization of risk genes. With genome sequencing becoming more feasible we will have access to multiple confirmed and putative targets to apply these technologies on.

7.3. PATHOGENIC MECHANISMS

7.3.1. IL22RA2 and innate immunity

The association of *IL22RA2* to MS illustrates the importance of cytokines and their receptors in MS. Moreover, the first non-HLA genes identified were also cytokine receptors, namely *IL2RA* and *IL7RA* (33, 36). Cytokine receptors have often been

linked with T cell fates; for example signaling through distinct receptors skews T helper cell proportions. Accordingly, IL22RA2 would be expected to involve T_H17 cell-mediated effects, given that these cells are major producers of IL22. A role for the IL22 system has also been suggested in other studies of EAE and MS (222, 223). However, in our D1cP congenic, T cell lymphocyte activation or expansion was unaffected compared to DA; instead macrophages were less activated. This implies a role for Il22RA2 involving innate responses.

As Il22RA2 is soluble, one cannot exclude the possibility of it acting as a ligand on an as yet identified receptor, possibly expressed on macrophages or microglia. IL22RA2 may alternatively function as a carrier protein for its ligand IL22 and thereby enhance long-range effects and stability. This has been observed for other cytokine – cytokine receptor systems (224-226). Assuming that IL22 is a pro-inflammatory cytokine, our data suggest an IL22-enhancing role of IL22RA2, as *Il22ra2* expression was higher in the susceptible DA strain. The mechanism could equally involve microglia, considering the differences in lesion activity between D1cP and DA. With this respect, it will be important to examine the relative impact of IL22RA2 on activation mechanisms versus that of phagocytic responses. Inflammatory responses may involve cytokine and receptor expression production that regulate antigen presentation, whereas differential phagocytic responses would rather imply a role in tissue destruction and disease chronicity. More experiments are needed to fully characterize the function of IL22RA2 in biology and disease.

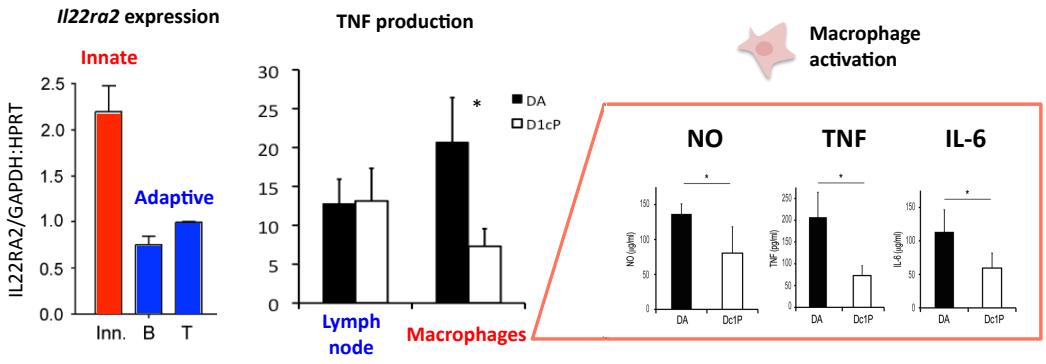

Figure 16. *Il22ra2* expression associates with macrophage activation. *Il22ra2* transcripts were elevated in the non-B non-T cell compartment. Moreover, activated macrophages from the susceptible DA strain secreted higher levels of TNF and other pro-inflammatory molecules compared to the more resistant D1cP strain, whereas stimulated LN cells did not.

7.3.2. VAV1 and adaptive immunity

VAV1 is an intracellular multifunctional adaptor molecule with a well-established role in T cell activation (227, 228). The same has also been reported for other surface receptors, including the B-cell antigen receptor, FcεRI, FcγRI/II/III, growth factor receptors, integrins, cytokine receptors and chemokine receptors (229). VAV1 transduces these receptor signals to various pathways leading to cell activation. We mainly studied the role of *Vav1* in T cell activation and expansion. We could demonstrate how genetic variations in *Vav1* regulated the inversely correlated production of pro-inflammatory cytokines such as IFNγ and TNF on the one hand, and the suppressive cytokine tumor growth factor beta (TGF-β) on the other (data not shown). The *Vav1* allele in our EAE-susceptible strains conferred higher VAV1 protein levels; it is also this allele that mediated higher pro-inflammatory cytokine expression. The same phenotypes were then confirmed in human blood and CSF; VAV1 expression correlates well with IFNγ and TNF expression and higher VAV1 expression is conferred by the risk polymorphism. Moreover, in rats the high expressing allele drove both an increased lymphocyte expansion upon re-stimulation with CNS autoantigens, and also lower proportions of natural T_{REGS} in several tissues. Collectively, our findings determine that natural variations in *Vav1* control the inherent ability of T cells to respond to stimuli which ultimately controls their ability to expand and mediate EAE (Figure 17). Apparently, the relative strength of this activation signal also regulates T_{REG} proportions. Our more recent experiments have also demonstrated how *Vav1* variants control the maturity of thymic T cells, a phenomenon that plausibly controls the early fate of T cells and promotes a skewing towards more T_{REGS}. We have also demonstrated that responses to B cell receptor stimulation are regulated by *Vav1* alleles. In the context of EAE, *Vav1* appears to control adaptive immune responses through several distinct mechanisms.

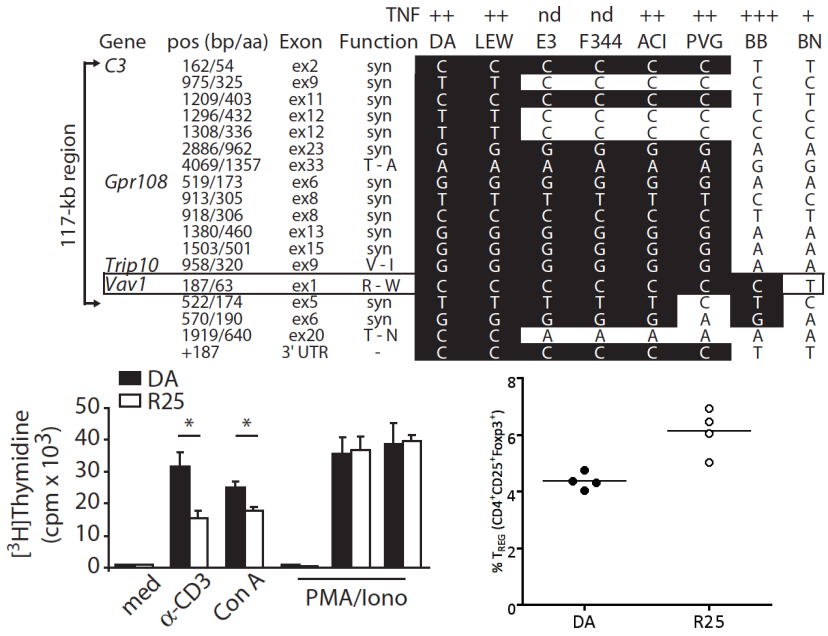

Figure 17. *Vav1* regulates T cell expansion and T_{REG} proportions. A SNP in exon1 of *Vav1* associates with deregulated TNF production and expansion of T cells, which inversely correlates with proportions of T_{REGS} in lymph nodes from immunized rats day 7 p.i. (p=0.02). The relative TNF expression of stimulated splenocytes across strains is denoted ranging from + to +++. Splenic T_{REG} proportions were inversely correlated with TNF expression for all analyzed strains.

7.3.3. NK cells reduce EAE

The epistatic interaction identified in Paper III highlights a novel mechanism of regulation of immune response by which NK cells can regulate autoimmunity. The current literature suggests that NK cells have a predominantly protective role in MS and EAE. NK cells have been described in experimental models to both mediate destruction of autoreactive cells and directly lyse dendritic cells (121, 230). Our data also demonstrate an inverse correlation between EAE susceptibility and splenic NK cell proportions. Similarly, decreased numbers of NK cells have been detected in MS patients (231). Therapies enriching for NK cell numbers also ameliorate MS through a mechanism that involves inhibition of autoreactive T cells (232).

Our findings suggest that one of the ways NK cells control disease is by targeting cells expressing the NK cell ligands retinoic acid early transcripts (*Raet1*). We determined this cluster of ligands and its cognate receptor Nkg2d to be more highly expressed in our resistant strains. We therefore propose a model in which overexpressed RAET1 ligands lead to increased NK cell numbers during development and become upregulated in activated immune cells that are subsequently cleared by poised NK cells expressing the activating NKG2D receptor (encoded by *KLRK1*). This in turn prevents disease exacerbation through

a mechanism involving reduced expansion of autoreactive lymphocytes (Figure 18). Concordantly, we demonstrate that *Raet1* ligands are produced in splenocytes and lymph node cells.

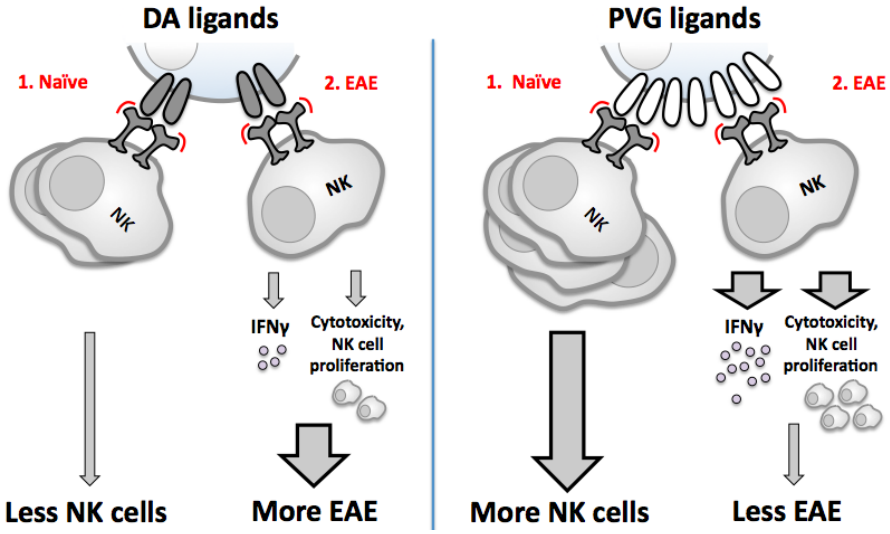

Figure 18. Proposed model of NK cell-mediated regulation of EAE through the RAET1-NKG2D system

7.3.4. Shared mechanisms across diseases

In Paper IV we identified *Ncf1* as a risk gene for EAN, already described as a risk gene for experimental arthritis (EA) and EAE. This finding strengthens the role of T cells in the pathogenesis of EAN. A high *Ncf1*-activity from APCs is thought to produce oxidative reagents which turn reduces T cell activation by altering their membrane potential. This has been demonstrated for EA and EAE, two diseases with a clear participation of activated T cells. EAN is primarily a model of AIDP, which is the most prevalent form of GBS in the Western hemisphere. It is therefore possible that the relative importance of T cells and their modulation by NOXC-stimulation is more pronounced in this subset of GBS patients. Interestingly, the protective role of NOXC stimulation in both GBS and MS has been suggested in a two Swedish studies in which higher NOXC activity in leukocytes correlated with milder disease (233, 234).

A similar disease-overlapping role is evident for *Vav1*. More recent data in our lab demonstrates that *Vav1* regulates EA, which has also been demonstrated in association studies in Swedish RA cohorts (data not included). The mechanisms involving pro-inflammatory cytokines, lymphocyte expansion and T_H cell subsets are also likely to control RA.

8. CONCLUSIONS AND FUTURE DIRECTIONS

The studies included in thesis have investigated distinct aspects of the genetic and pathogenic mechanisms regulating neuroinflammation. I have herein summarized the most essential conclusions and future experiments required to further advance our understanding.

- **EAE/EAN risk genes translate well to human disease**

Our translational studies (Paper I and II) collectively demonstrate that *IL22RA2* and *VAV1* regulate both EAE and MS. The relative strength of the rat QTLs *Eae29* and *Eae4* are also in concordance with the observed ORs of 1.18 and 1.26 for *IL22RA2* and *VAV1*, respectively. This supports EAE as a good model of MS and the usefulness of studying genetic regulation of experimental models in order to better understand human disease. Through use of whole-genome sequencing and transcript/protein profiling of the congenic lines and parental strains, we have a chance to not only identify the disease risk genes, but also to locate the exact variation controlling disease. This will be a considerable effort and will likely be achieved through large genetic consortia with access to modern techniques. It is also important that human and experimental genetic studies take advantage of each other's strengths. GWAS have superior resolution to detect risk SNPs and dozens of such SNPs are being discovered for various complex disorders (163). Upcoming large GWAS from MS is also predicted to unravel several novel risk genes. There is an inevitable need to study these functionally in patient tissue samples, but also in experimental models that are useful since conditions can be controlled and the relevant tissue is readily available. It will therefore be of great interest to functionally dissect MS risk genes in rat models but also to see how risk genes in the rat relate functionally to the ever-growing list of identified MS genes.

- **EAE/EAN pathogenic mechanisms translate well to human disease**

Papers II, III and IV identify genes important for several mechanisms including T_{REG} proportions, NK cell activity and the oxidative burst of the NOXC. We also demonstrate that an increased activity of these mechanisms associates with disease protection in our models. Importantly, these mechanisms have similar beneficiary roles in MS and GBS (232-235). This does not mean that they are regulated by the same risk alleles as in the rat, but that these phenotypes

constitute convergence points of central disease pathways. We must therefore continue to experimentally dissect the mechanisms regulated by risk alleles. Accordingly, risk genes identified in human cohorts should be studied functionally, both in humans and models, in order to pinpoint the specific pathways they regulate. The ultimate goal will be to discover the distinct genotype and disease mechanisms important for each patient, which should greatly improve therapeutic design and subsequent efficacy.

- **Rodent crosses and congenic lines are good tools for positioning of disease risk genes**

The list of formally proven risk genes in EAE, EAN or related models is steadily increasing (89-92, 221, 236, 237). The five candidate genes identified in my thesis support using intercrosses and congenic lines as tools in risk gene positioning. In particular, the DA and PVG strains with their opposing EAE susceptibilities and the high degree of natural genetic variation between them have been useful in this regard. Studying naturally existing genetic variations across inbred strains discern a more realistic and nuanced view of the mechanisms that risk genes control. This is relevant, as many of our EAE risk genes appear to have multiple functions that may not all be important for the disease. In such a scenario, looking at, for example, knockout strains would ablate all these mechanisms. This is not to reduce to importance of knockout strains, which together with transgenic and knock-in strains can offer powerful complements to congenic lines in follow-up functional studies of risk genes. Furthermore, the controlled genetic background in our system, which enables stratification of genotypes, is advantageous for studying epistatic interactions. We can thus study separate interactions that are truly important from those which are merely genetically regulated. Future studies must aim at identifying the level of redundancy and relative strength of observed epistatic interactions, which will likely explain part of the missing genetic variance in autoimmune disorders.

- **Shared genetic and pathogenic components across diseases**

As discussed earlier, we have observed many examples of shared genetic and pathogenic mechanisms across models. These can provide therapeutic targets for the specific diseases they regulate. TNF-blockage in RA and CD and α-VLA4 treatment in MS and CD are good examples of such targets (50, 138, 139, 238). We should therefore use our tools to study other chronic inflammatory diseases by testing our congenic strains in other inflammatory models. Conversely, disease

genes and mechanisms identified in other diseases should be evaluated in EAE and MS.

- **The identified pathogenic mechanisms involve both adaptive and innate immune cells**

Our findings suggest new perspectives for understanding the roles of several immune cells in the pathogenesis of EAE and MS. Among innate cells, NK cells regulate disease through targeting immune cells expressing RAET1 ligands, and macrophages can both drive disease through the activity of the soluble receptor IL22RA2, and ameliorate disease through oxidative burst in the interaction with T cells. Adaptive immune cells, B and T cells, drive disease through VAV1-mediated activation and expansion; the strength of this signal also appears to also impact on the proportions of T cell subsets, including T_{REGS} and T_H cells.

To summarize, in our studies we have used the EAE model to identify risk genes that modulate disease susceptibility and severity and also to determine which conserved immune mechanisms they regulate. These mechanisms are in turn likely to translate to humans with the goal of improving disease prognosis, better understanding of the pathogenesis and to tailor therapies.

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