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INTERLEUKIN-22 BINDING PROTEIN IN MULTIPLE SCLEROSIS AND EXPERIMENTAL INFLAMMATION MODELS

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Interleukin-22 binding protein in multiple sclerosis and experimental inflammation models

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By

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ABSTRACT

Multiple sclerosis (MS) is the leading cause of non-traumatic neurological disability in young adults. Although the cause of the disease is unknown, several genetic and environmental risk factors have been identified. By studying these risk factors in experimental systems we can learn more about the biological events that precede overt disease and then use this knowledge in the development of better and safer MS treatments.

The aim of the work presented in this thesis is to shed light on one of the established genetic risk factors, the single nucleotide polymorphism (SNP) rs17066096. Since this SNP is positioned close to the gene *IL22RA2,* we hypothesize that *IL22RA2* mediates the effect on MS susceptibility. The gene product of *IL22RA2* is interleukin-22 binding protein (IL-22BP), a soluble IL-22 antagonist. Consequently, in study I-IV below, we focus on the role of the IL-22-system in MS and experimental inflammation models.

In **study I** we reveal the role of IL-22BP in MS animal model experimental autoimmune encephalomyelitis (EAE). *Il22ra2*-deleted mice have less severe paralysis, immune cell infiltration, and demyelination compared to wild type mice.

In **study II** we further investigate the biology of IL-22BP in inflammation. We use the mouse strain from study I, but now in two skin inflammation models: contact hypersensitivity and an imiquimod-induced psoriasis model. In contrast to EAE, the *Il22ra2*-deleted mice have more severe disease in both skin inflammation models.

In **study III** we show that the risk genotype of rs17066096 is associated with higher expression of *IL22RA2* in monocyte-derived dendritic cells from healthy blood donors and that MS patients with more lesions on magnetic resonance imaging have higher cerebrospinal fluid levels of IL-22BP. Using an inducible *Il22ra2*-knockdown rat strain we show that a relatively modest decrease in *Il22ra2* expression is enough to make the rats resistant to EAE. Similarly, heterozygous deletion of *Il22ra2* in mice is sufficient to achieve a protective effect. Furthermore, we establish that the protective effect in *Il22ra2*-deleted mice is dependent on the presence of IL-22.

In **study IV** we investigate the effect of IL-22 signaling on the initiation of an adaptive immune response. Using the rat strain from study III, we show that knockdown of *Il22ra2* expression just prior to immunization causes a reduction in lymphocyte expansion, preferentially affecting B cells, as well as a reduction in antigen specific effector functions in B cells and Th1 cells.

In conclusion, we present data in support of a disease-promoting role for IL-22BP in neuroinflammation in three species. We show that the effect is dependent on IL-22, which consequently has a therapeutic potential.

LIST OF SCIENTIFIC PAPERS

I. **Hannes (Lindahl) Laaksonen**, André Ortlieb Guerreiro-Cacais, Milena Zeitelhofer Adzemovic, Roham Parsa, Manuel Zeitelhofer, Maja Jagodic, Tomas Olsson. *The multiple sclerosis risk gene IL22RA2 contributes to a more severe murine autoimmune neuroinflammation.* Genes and Immunity, 2014;15:457-465.

II. **Hannes Lindahl**, Elisa Martini, Susanna Brauner, Pernilla Nikamo, Irène Gallais-Serezal, André Ortlieb Guerreiro-Cacais, Maja Jagodic, Liv Eidsmo, Mona Ståhle, Tomas Olsson. *IL-22 binding protein regulates murine skin inflammation.* Experimental Dermatology, 2017;26:444-446.

III. **Hannes Lindahl**, André Ortlieb Guerreiro-Cacais, Mathias Linnerbauer, Nada Abdelmagid, Karolina Tandre, Sabrina Ruhrmann, Lars Alfredsson, Lars Rönnblom, Mohsen Khademi, Maja Jagodic, Tomas Olsson. *Multiple sclerosis risk variant results in higher expression of IL22RA2 that blocks the protective effects of IL-22 in experimental neuroinflammation.* Manuscript.

IV. **Hannes Lindahl**, André Ortlieb Guerreiro-Cacais, Maja Jagodic, Tomas Olsson. *IL-22 binding protein promotes lymph node chemokine expression and adaptive immune responses in rat.* Manuscript.

CONTENTS

LIST OF ABBREVIATIONS

APC	Antigen presenting cell
BBB	Blood brain barrier
CD	Cluster of differentiation
CIS	Clinically isolated syndrome
CNS	Central nervous system
CSF	Cerebrospinal fluid
DA	Dark Agouti
DC	Dendritic cell
EAE	Experimental autoimmune encephalomyelitis
EBV	Epstein-Barr virus
ELISA	Enzyme-linked immunosorbent assay
ENCODE	Encyclopedia of DNA elements
FACS	Fluorescence-activated cell sorting
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GWAS	Genome-wide association study
HHV-6	Human herpesvirus 6
HLA	Human leukocyte antigen
IFNγ	Interferon gamma
IL	Interleukin
IL-22BP	Interleukin-22 binding protein
IL22RA2	Interleukin-22 receptor alpha 2
ILC	Innate lymphoid cell
IMSGC	International multiple sclerosis genetics consortium
JC virus	John Cunningham virus
kb	Kilobase pairs
LD	Linkage disequilibrium
MACS	Magnetic-activated cell sorting
MBP	Myelin basic protein
MHC	Major histocompatibility complex

MΦ	Macrophage
MOG	Myelin oligodendrocyte glycoprotein
MRI	Magnetic resonance imaging
MS	Multiple sclerosis
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NK cell	Natural killer cell
PAMP	Pathogen-associated molecular pattern
PRR	Pattern recognition receptor
PVG	Piebald-Virol-Glaxo
RIS	Radiologically isolated syndrome
ROS	Reactive oxygen species
RRMS	Relapsing-remitting multiple sclerosis
shRNA	Short hairpin ribonucleic acid
SLE	Systemic lupus erythematosus
SNP	Single nucleotide polymorphism
SPMS	Secondary progressive multiple sclerosis
Tc	Cytotoxic T cell
TCR	T cell receptor
Th	Helper T cell
TLR	Toll-like receptor
TNF	Tumor necrosis factor
Treg	Regulatory T cell
VCAM-1	Vascular cell adhesion molecule-1
VLA4	Very late antigen-4

1 INTRODUCTION

The work presented in this thesis stems from previous studies by our group that gave rise to the following question: How does the interleukin-22 binding protein (IL-22BP) influence multiple sclerosis (MS) and its experimental models? The following section will provide background before proceeding with a discussion of the results. Topics covered will be: the biology and genetic variation of the immune system, interleukin-22 (IL-22) physiology and pathophysiology, neuroinflammation with focus on MS, and other inflammation models.

1.1 NEUROINFLAMMATION

1.1.1 Neuroimmunology

Clinical neuroimmunology encompasses the study of immune mediated neurological disorders, which are set apart from other inflammatory disorders by the following three characteristics: i) immune privilege, ii) limited tissue regeneration and iii) limited access by therapeutic agents.

1.1.1.1 Immune privilege

The concept of immune privilege was first described after experiments conducted by Peter Medawar in the 1940's[1]. It is based on the observation that tissue grafts placed in certain tissue environments fail to elicit an immune response and are not rejected. Organs that are considered immune privileged include the brain, eyes, testes and uterus. In the brain, the features that classically are proposed to constrain the activity of the immune system include:

- The blood-brain barrier (BBB) that keeps immune cells outside the CNS
- Limited lymphatic drainage
- Few antigen-presenting cells in the brain parenchyma
- Limited expression of major histocompatibility complex (MHC) molecules.

The BBB restricts the passage of cells, antibodies and other molecules from the circulating blood to the extracellular space in the CNS. It is formed by endothelial cells with tight junctions, even around the capillaries[2,3]. Apart from these endothelial cells, the BBB also consists of an endothelial basement membrane, scattered contractile cells called pericytes, a parenchymal basement membrane and finally a layer of astrocytic foot processes also known as the glia limitans. Many gases and lipid soluble molecules can diffuse freely across the BBB but other molecules essential for neural function – e.g. glucose and amino acids - are taken up by selective transport mechanisms[4,5].

The BBB is not an absolute immune cell barrier. Experiments in rodents show that encephalitogenic T cells transferred to the peripheral circulation of healthy animals do cross the BBB and enter the CNS where they cause neuroinflammation[6]. Furthermore, immune cell

surveillance has been shown to occur in the healthy CNS[7,8]. Parts of the intracerebral blood vessels, such as the circumventricular organs and the choroid plexus[9,10], more readily allow the passage of immune cells.

Lymphatic drainage from the cerebrospinal fluid (CSF) to the peripheral circulation has recently been well characterized in mice using modern microscopy technology[11,12]. Lymphatic vessels line the dural sinuses and drain to the deep cervical lymph nodes. These vessels give the immune cells in the CNS a route to travel to deep cervical lymph nodes, where an immune response can be elicited. It may be so that CNS antigens rather induce tolerance under these circumstances[13]. This conclusion recently became contested, however[14]. Interestingly, myelin-specific T cells are not only found in MS patients, but also in healthy individuals and naïve mice, in which case they do not cause disease[15,16].

The brain parenchyma is largely devoid of efficient antigen-presenting cells in the steady state. However, these cells do exist in the meninges and in association with blood vessels, where they are thought to activate T cells at initiation of disease[17–20].

1.1.1.2 CNS response to injury

Any form of significant injury to the CNS will result in a coordinated response from local cells, i.e. neurons and glia, and in most instances also from infiltrating immune cells[21,22]. The immune cells have a critical role in the defense against microbial pathogens, but also in clearing up debris that would otherwise interfere with the normal functions of the nervous tissue[23–25]. Fine-tuning this inflammatory immune response is very important in the CNS - perhaps more so than in other organs, because excessive activation will lead to damage that is likely to be irreversible, due to the very limited regenerative potential of neurons in the CNS. However, there are reports showing reversible axon damage in MS and EAE[26]. Primarily microglia, but to some extent also astrocytes, act as sensors of damage and recruiters of leukocytes to the CNS[27–30]. Astrocytes also respond in a characteristic way to limit the collateral damage to neighboring parenchyma by proliferating to enclose the lesion in a process called astrogliosis, similar to scar formation. This phenomenon was recognized early by histopathological studies and was for a long time viewed simply as a harmful response that limited synapse sprouting and axon growth. However, a large number of experimental *in vivo* studies have now shown that astrogliosis in many cases improves outcomes through what is interpreted as promotion of wound closure, neuroprotection, restoration of the BBB and resolution of inflammation[30,31].

1.1.2 MS - introduction

MS is a chronic inflammatory disease of the CNS and is the second most common cause of neurological disability in the young, after traumatic injuries[32]. It is typically characterized by recurring bouts of motor and/or sensory deficits, which are manifestations of immunological attacks on the myelin sheaths that surround axons in the CNS. If the axons remain intact after inflammation has resolved, the symptoms will remit after remyelination has taken place (Figure 1). Subclinical attacks on the CNS take place even before the initial presentation of

neurological symptoms. These can be demonstrated radiologically by magnetic resonance imaging (MRI). It is currently discussed whether radiologically isolated syndrome (RIS) is a useful term for this situation, which in extension would encourage a more active management of these patients[33]. The first clinical presentation consistent with MS is called clinically isolated syndrome (CIS), assuming that the diagnostic criteria for MS are not already fulfilled. Relapsing remitting MS (RRMS) is the classical disease phenotype, characterized by bouts of neurological symptoms followed by remissions that may be complete or incomplete. After approximately 10-15 years most RRMS have converted to secondary progressive MS (SPMS), characterized by a steady decline of neurological functions with or without superimposed relapses. However, this may no longer be the case with the more efficacious treatments that have become available in the last few years. Furthermore, approximately 5-10% of MS patients present with progressive disease directly from the outset, called primary progressive MS (not shown in Figure 1.). Brain atrophy can be detected early but accelerates as disease advances.
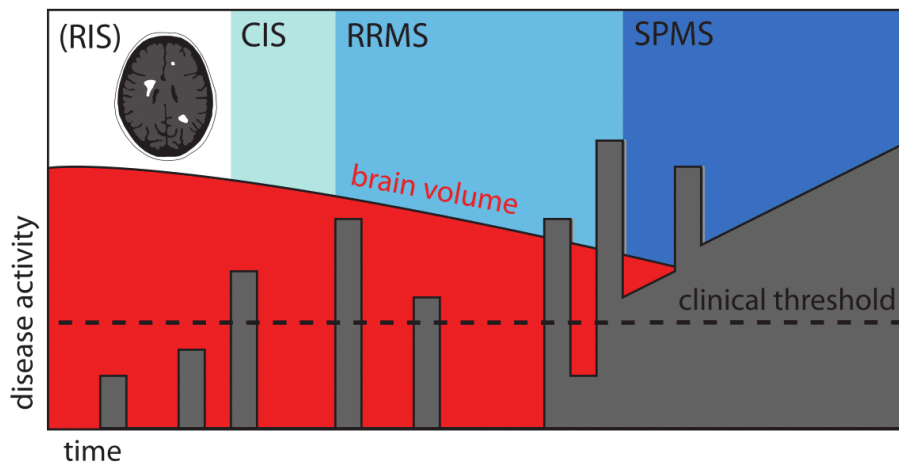

Figure 1. Clinical phenotypes of MS.

The prevalence of MS in Scandinavia is particularly high, at approximately 1-2 per 1000[34,35]. The worldwide prevalence of MS is much lower but it is difficult to arrive at an accurate number. Like most autoimmune diseases, there is a gender bias. In the case of MS, there are approximately 2-3 affected females per male. The explanation could be multi-factorial but a strong case has been built implicating sex hormones[36,37]. Females generally have higher antibody responses than males, a fact that is reflected by the strikingly higher incidence in females of the antibody-driven autoimmune disease systemic lupus erythematosus (SLE). During pregnancy there is a shift away from Th1 type immunity, further favoring Th2 and antibody responses, presumably for the purpose of promoting tolerance to the fetus[38]. This general shift in the immune system is believed to underlie the observed pregnancy-related

increase in relapse frequency for antibody-driven autoimmune disease (e.g. SLE and systemic sclerosis) and a decrease in relapse frequency for Th1/Th17-driven autoimmune diseases (e.g. MS and rheumatoid arthritis)[38], as well as the phenomenon of postpartum relapses[39]. Additionally, for MS the gender bias has increased over time, which may be an effect of changes in sunlight exposure and/or vitamin D levels[40,41].

1.1.3   MS - genes and environment

1.1.3.1   Genetic risk factors

The cause of MS is not known but it is believed that the disease is initiated in individuals for which the combination of genetic predisposition and environmental exposures reaches a threshold[42]. According to this model, there can be many different causes of MS. A simplified example: in one MS patient, genetic risk factors *1* and *2* in combination with environmental risk factor *A* was sufficient to reach the threshold of disease initiation and in another MS patient, genetic risk factor *3* in combination with environmental risk factors *B* and *C* was sufficient to cause the disease. A large amount of knowledge has accumulated about the genetic and environmental risk factors that influence susceptibility but those that influence severity and subphenotype are much less understood[43–48]. This, in turn is a consequence of a lack of good methods to assess severity, subphenotype and disease progression.

Familial clustering of MS was recognized early and the recurrence risk for first-degree relatives has previously been reported at approximately 3-5 %, 30-50 times the risk in the general population[49–52]. However, more recent data, without some of the bias inherent to the previous studies, estimate the recurrence risk at approximately 7 times the population risk for first-degree relatives[53]. From this observation alone, one cannot distinguish the relative importance of shared genetics or shared environment. The concordance rate for monozygotic twins has been reported at approximately 30 %, which is comparable to many other autoimmune diseases, e.g. rheumatoid arthritis and psoriasis[49–51,54,55]. Heritability, classically defined by comparing concordance rate in monozygotic and dizygotic twins, has been difficult to estimate with precision for MS, mainly due to relatively low prevalence[56]. Moreover, twin studies are generally problematic owing to small study groups and inability to distinguish genetic causes from intrauterine environmental causes. Stronger evidence for a genetic contribution comes from studies of adoptees and half-siblings. The susceptibility for first-degree non-biological relatives living with a person with MS is the same as for the general population[57]. The risk for siblings is approximately doubled compared to half-siblings[58].

Once a significant genetic contribution to disease susceptibility has been demonstrated the next step is to identify the genes, or more specifically the genomic sequence variants that mediate these effects. Disease associated variants can be classified as those that directly cause the disease, which result in Mendelian inheritance patterns, or those that just modulate a person's resistance to environmental disease triggers. The first type of variant is by necessity rare. Any *de novo* germline mutation having a large impact on MS susceptibility would

quickly have been lost because the carriers would not have been able to successfully produce and care for offspring. Consequently, it is perhaps not surprising that the identified genetic risk factors individually all have relatively small effects on MS susceptibility.

Early population based studies focused on candidate genes that were chosen based on the hypothesis of T cell mediated autoimmune demyelination. The first identified genetic risk factor mapped to MHC class I, which was later extended to class II as well[59,60]. Due to strong linkage disequilibrium (LD) in this region it has, to this day, not been possible to isolate the primary allele for this association. A haplotype including the DRB1*1501 allele is by far the most important genetic risk factor identified[61,62]. Interestingly, the MHC also includes the genes for the central driver of inflammation tumor necrosis factor (TNF) and MS autoantigen candidate myelin oligodendrocyte glycoprotein (MOG), none of which have however shown convincing associations to MS that are independent of MHC class II. The HLA molecules in the MHC region present antigens to the T cell receptor (TCR). However, studies of germline sequence variation in the TCR α and β chain genes have given little support for the involvement of the TCR in genetic predisposition to MS[63]. Similarly studies focusing on immunoglobulins have yielded inconsistent observations.

Association studies are hypothesis-driven population-based studies of candidate genes. An alternative approach is linkage studies, which are performed on MS families and identifies susceptibility loci in an unbiased manner. However, with the advent of high throuput SNP-genotyping, association studies are now performed that are essentially hypothesis-free. The genotyped SNPs are chosen based on strong LD with neighboring SNPs, thus serving as markers for haplotype blocks[64]. In these studies a few hundred thousand so called tagging-SNPs are genotyped from which one can, with reasonable certainty, assume the alleles of neighboring untyped SNPs. One distinct difference between these two approaches is that association studies will only be able to identify common variants. Linkage studies are required to identify rare variants. Now that several very large genome wide associations studies have been finalized it has become clear that the identified risk variants, now more than a hundred, all have small effects on disease susceptibility and the combined effects of these do not seem to correspond to the observed heritability[44,45,65]. Two hypotheses about the genetic determinants of MS susceptibility have been discussed i) common variants with small effects, which are the ones identified by performing association studies and ii) rare variants with large effects, which can only be identified by performing linkage studies. The later scenario, sometimes called the mutation-selection hypothesis, proposes that much susceptibility is due to variants of recent origin. These would exhibit great allelic heterogeneity, but perhaps not as pronounced locus heterogeneity. For comparison, the severe but fairly common Mendelian disease cystic fibrosis is due to single mutations in one gene, *CFTR*, but the number of known mutations add up to more than a thousand. These would not be identified by association studies. Resequencing of candidate regions, or perhaps of entire genomes, would be needed. Other hypotheses for the "missing heritability" are epistatic effects, i.e. the effect of a risk factor may be much larger in the presence of another risk factor, or epigenetic effects.

1.1.3.2 Molecular characterization of genetic risk factors

To date, more than a hundred SNPs have been associated with MS susceptibility and the number is expected to increase as further studies are published. Molecular characterization of these genetic risk factors has made relatively slow progress, however. Examples for which significant progress has been made include: the IL-7 receptor alpha chain (*IL7R*)[66], TNF superfamily 1A (*TNFRSF1A*)[67], the IL-2-receptor alpha chain (*IL2RA*)[68,69], and Tyrosine kinase 2 (*TYK2)*[70].

IL7R

The IL-7 receptor is a dimer of the IL-7 receptor alpha chain and the common gamma chain. IL-7 is important for differentiation and homeostasis of all lymphoid cells. Exaggerated IL-7 signaling has been shown to promote autoimmunity[71–74].An alternatively spliced, soluble form of the IL-7 receptor exists, which is produced by skipping exon 6. The risk-allele of the MS-associated SNP rs6897932, located in exon 6 of *IL7R*, results in a higher proportion of the soluble form of the IL-7 receptor being produced[66]. A proposed mechanism for the effect of an increase of the soluble IL-7 receptor on MS-risk is by its competition with the membrane bound receptor for circulating IL-7, thus leading to overall lower consumption of the cytokine. This in turn, increases concentrations of circulating IL-7, which has the potential to promote autoimmune processes.

TNFSF1A

TNFRSF1A encodes TNF receptor 1. The MS-risk allele of the intronic SNP rs1800693 leads to a skipped exon during splicing, which produces a soluble form of the receptor that blocks TNF-signaling[67]. The SNP is not associated with any of the other autoimmune diseases, like rheumatoid arthritis, psoriasis, Crohn's disease. This is mirrored by the treatment response to TNF-blockade, an established treatment for these diseases, but that exacerbates MS[75].

IL2RA

The IL-2-receptor alpha chain, also known as CD25, forms the high-affinity IL-2 receptor when incorporated with IL-2 receptor beta and the common gamma chain[76]. The MS-associated allele of the intronic rs2104286 increases cell surface expression of CD25, which results in more production of GM-CSF upon IL-2 stimulation[68,69].

TYK2

TYK2 is associated with the cytoplasmic tail of many cytokine receptors and is involved in signal transduction upon activation. MS-associated SNP rs3456443 causes a missense mutation in exon 21 of the gene *TYK2* that results in a change from a proline to an alanine. It does not affect TYK2 expression levels but does change activity of the gene[70]. The protective allele reduces TYK2 activity in T cells and results in a shift towards Th2 cytokine production.

1.1.3.3 Environmental risk factors

Environmental risk factors are more difficult to identify without bias. The most well established factors that increase the risk of MS on a population level are: smoking, late Epstein-Barr virus (EBV) infection, obesity, low vitamin D levels, and a striking geographic distribution that may be caused by variations in exposure to sunlight[42,77–82]. Although a large body of data exists on the matter, the underlying pathogenic mechanisms are largely unresolved.

Locus	Gene(s)	*p*-value	Odds ratio
1p36.32	*MMEL1*	1.3×10^{-26}	1.14
1p22.3	*DDAH1*	4.4×10^{-9}	1.09
1p22.1	*EVI5, RPL5*	1.4×10^{-26}	1.19
1p21.2	*VCAM1*	1.9×10^{-16}	1.10
1p13.1	*CD58*	5.0×10^{-42}	1.29
1q31.2	*RGS1*	4.8×10^{-20}	1.15
1q32.1	*C1orf106*	4.0×10^{-19}	1.13
2p13.3	*PLEK*	1.2×10^{-13}	1.10
2q37.1	*SP140*	4.2×10^{-23}	1.16
3p24.1	*EOMES*	1.5×10^{-15}	1.10
3q13.33	*CD86, TIMMDC1, IQCB1*	1.2×10^{-24}	1.13
3q25.33	*IL12A*	1.1×10^{-18}	1.11
5p13.2	*IL7RA*	4.3×10^{-17}	1.12
5p13.1	*PTGER4*	6.9×10^{-16}	1.21
5q31.3	*NDFIP1*	3.6×10^{-9}	1.07
5q35.3	*RGS14*	4.4×10^{-18}	1.12
6p21.32	*HLA-DRB1*	1.0×10^{-225}	2.75
6q15	*BACH2*	1.5×10^{-15}	1.14
6q23.3	*OLIG3, TNFAIP3, IL22RA2, AHI1*	1.6×10^{-23}	1.14

6q25.3	*TAGAP*	8.0×10^{-21}	1.12
8q24.21	*MYC, MIR1204, MIR1205, PVT1, MIR1208, MIR3686*	4.3×10^{-16}	1.12
10p15.1	*IL2RA*	2.3×10^{-47}	1.22
10q22.3	*ZMIZ1*	2.5×10^{-15}	1.10
10q23.33	*HHEX*	4.3×10^{-17}	1.11
11q12.2	*CD6*	3.7×10^{-23}	1.13
11q23.3	*CXCR5*	3.0×10^{-15}	1.10
12p13.31	*TNFRSF1A*	6.7×10^{-28}	1.14
12q14.1	*METTL1, CYP27B1*	5.0×10^{-11}	1.23
14q24.1	*ZFP36L1*	9.3×10^{-12}	1.11
14q31.3	*GALC*	2.4×10^{-20}	1.32
16p13.31	*CLEC16A*	6.4×10^{-46}	1.20
16q24.1	*IRF8*	5.9×10^{-12}	1.15
17q21.2	*STAT3*	3.7×10^{-20}	1.12
19p13.3	*TNFSF14*	1.7×10^{-24}	1.16
19p13.2	*TYK2*	1.8×10^{-14}	1.29
19q13.33	*DKKL1*	5.7×10^{-15}	1.11
20q13.2	*CYP24A1*	2.0×10^{-13}	1.09
20q13.12	*CD40*	7.7×10^{-16}	1.11
22q11.21	*MAPK1*	5.5×10^{-16}	1.10

Table 1. The most well-established MS-associated loci. The following loci have been identified in at least two independent genome-wide studies. One or several genes are listed for each locus, based on proximity to the associated SNP. The SNP with the highest *p*-value after combining available datasets is listed for each loci. All listed SNPs are intronic or intergenic, with the exception of rs41286801, which is located in the 3' untranslated region of *EVI5*. Adapted from Bashinskaya, *et al.*[83].

1.1.4 MS - pathogenesis

MS is widely regarded as a primary immune mediated disease[84], a hypothesis supported among other things by:

- MS lesions are characterized by early T cell infiltration[85,86],
- the vast majority of associated genetic variants are in close proximity to immune genes,
- and successful MS treatments all target the immune system.

Furthermore, MS is considered a primary autoimmune disease, supported among other things by:

- the most influential genetic risk factor is localized to the MHC,
- autoreactive T and B cells are frequently identified in MS patient blood samples,
- the many observed similarities to the autoimmune disease model EAE.

However, the primary autoantigen or antigens responsible for MS onset are not known. Unlike established autoimmune diseases like myasthenia gravis and neuromyelitis optica, which are known to be caused by autoreactive antibodies to acetylcholine receptors at the neuromuscular junction[87,88] and astrocytic water channel, aquaporin-4[89,90], respectively, MS can formally not be defined as a primary autoimmune disease. For the sake of discussion, an alternative hypothesis states that the disease trigger is neurodegeneration followed by secondary inflammation and autoimmunity. Any autoreactive T or B cells detected could be a result of bystander activation and may not even be driving the disease at all. Histopathological analysis of some of the earliest available MS lesions demonstrate cases of oligodendroglial pathology in the complete absence of immune infiltration[91].

Assuming a primary immunological trigger, two hypotheses exist regarding its nature: The immune system is triggered in the CNS or in the periphery. Postulated CNS triggers include a local viral infection. The hypothesis of a peripheral immune cell activation followed by trafficking to and reactivation in the CNS is analogous to the pathogenesis of the MS disease model EAE. Postulated peripheral triggers are viral or bacterial infection leading to cross-reactive immune cell specificities by molecular mimicry[92–94], bystander activation[95,96] or T cells bearing dual TCRs[97]. The case of Human herpes virus-6 (HHV-6) is interesting. Virions have been identified in MS lesions and sequence similarities with myelin basic protein (MBP) have experimentally been shown to result in T cell cross-reactivity[94]. This is example of molecular mimicry. Moreover, HHV-6 and other enveloped viruses have the capacity to

take up host proteins, which is another plausible mechanism how tolerance to self may be broken[98].

The primary feature of MS neuropathology is the MS plaque or lesion. These are confluent areas of demyelination, inflammation, oligodendrocyte and neuronal degeneration, and astrogliosis[99–102]. The lesions are found in both white and grey matter in the brain as well as in the optic nerves and the spinal cord. The inflammatory component, driven by adaptive immunity, is most pronounced in the early stages of disease. Early lesions are characterized by a permeable BBB, of which the radiologic correlate is contrast-enhancing lesions on MRI. These lesions tend to be dominated by macrophages and T cells, with CD8^{+} T cells outnumbering CD4^{+} T cells. It has, furthermore, been shown that CD8^{+} T cells isolated from MS lesions exhibit clonal expansion more frequently than CD4^{+} T cells[103]. B cells and plasma cells are also identified but to a lesser extent.

As the disease progresses, inflammation becomes less pronounced and fewer new lesions appear. At this stage, neuropathology is primarily characterized by brain atrophy and an cortical lesions, possibly driven by innate immunity and/or dysfunction in the neurons themselves[104–106]. Neuroaxonal loss is eventually noticeable also in so called normal appearing white matter and general brain atrophy accelerates seemingly independently of lesions and clinical relapses[107–110]. Measurement of brain atrophy has become an important parameter in clinical trials but can be to some degree confounded by the reduction in white matter volume due to resolution of inflammation, termed pseudoatrophy[111]. The proportion of B cells and plasma cells also increase during the later stages of disease[112,113]. Tertiary lymphoid structures in the meninges become more prevalent as well. In late disease inflammation appears to be contained within the CNS, as few immune cells are recruited from the periphery. This development mirrors the clinical transition from relapsing remitting disease to progressive disease. The CNS-intrinsic inflammatory component and the associated brain atrophy are essentially not influenced by established MS treatments. This lack of effective treatment options for progressive MS is one of the biggest and most pressing challenges facing research in this field as well as clinical practice.

Moreover, there are other neurological symptoms associated with MS that are not purely focal in nature and therefore somewhat neglected, perhaps due to difficulties in objectively assessing them. A form of primary fatigue is commonly seen which can be very debilitating[114]. Interestingly, also symptoms like fatigue and depression may be a manifestations of immunological processes in the CNS[115,116].

MS pathogenesis

1.

genetic predisposition

HLA variants have the largest genetic influence on MS susceptibility but there are also a large number non-HLA risk loci.

GC**G**TTA
CG**C**AAT

2.

environmental triggers

Established environmental risk factors include smoking, EBV infection, and vitamin D levels. They often interact with genetic risk factors leading to synergistic effects on MS susceptibility.
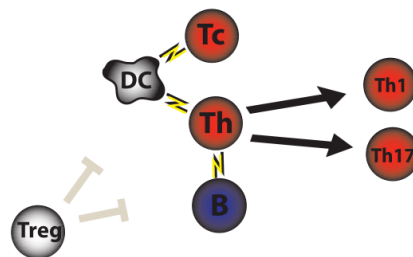
3.

failure of central tolerance

Autoreactive T and B cells escape central tolerance mechanisms in the thymus and bone marrow respectively. Antigen arrives in a lymph node via afferent lymphatic vessels. proposed mechanisms of activation include:
  - altered self-antigen
  - sequestered CNS antigen
  - molecular mimicry
  - bystander activation

antigen
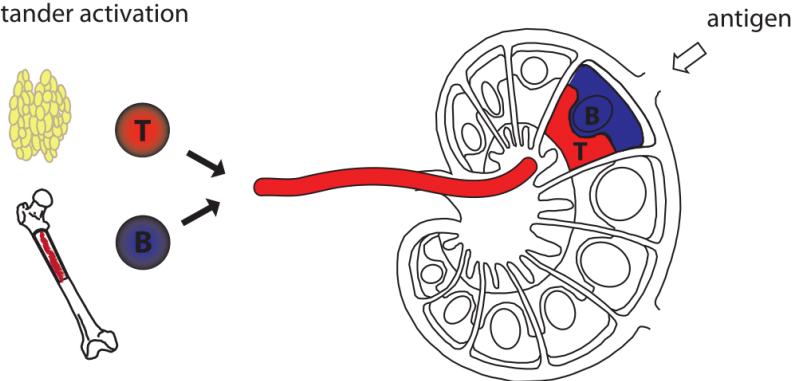
4.

failure of peripheral tolerance

Diminished T and B cell response to Treg suppression or hypofunctional Tregs lead to expansion of autoreactive lymphocyte clones.
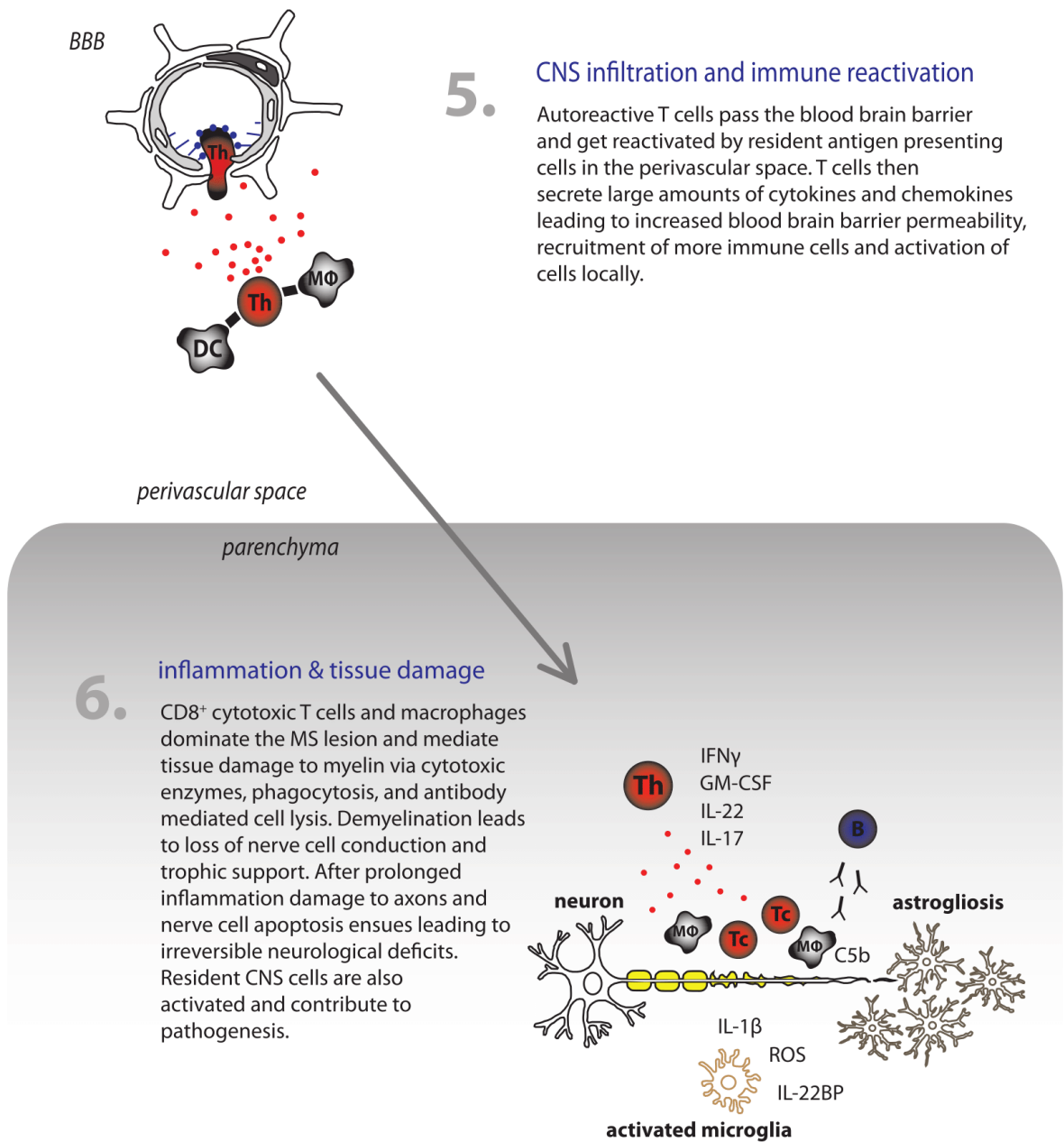
12

BBB

5. CNS infiltration and immune reactivation

Autoreactive T cells pass the blood brain barrier and get reactivated by resident antigen presenting cells in the perivascular space. T cells then secrete large amounts of cytokines and chemokines leading to increased blood brain barrier permeability, recruitment of more immune cells and activation of cells locally.

perivascular space

parenchyma

6. inflammation & tissue damage

CD8+ cytotoxic T cells and macrophages dominate the MS lesion and mediate tissue damage to myelin via cytotoxic enzymes, phagocytosis, and antibody mediated cell lysis. Demyelination leads to loss of nerve cell conduction and trophic support. After prolonged inflammation damage to axons and nerve cell apoptosis ensues leading to irreversible neurological deficits. Resident CNS cells are also activated and contribute to pathogenesis.

IFNγ
GM-CSF
IL-22
IL-17

neuron

astrogliosis

C5b

IL-1β
ROS
IL-22BP

activated microglia

Figure 2. The current view of MS pathogenesis

1.1.5 Experimental autoimmune encephalomyelitis (EAE)

Many pathophysiological research questions regarding MS have been addressed primarily in animal models. This has been necessitated by: the very limited access to human CNS tissue, large disease heterogeneity, and long preclinical disease stage. The most common disease model is EAE, which is induced in susceptible laboratory animals by breaking immunological tolerance to a CNS autoantigen.

EAE can be viewed as a group of models with differing characteristics depending on for example the species used, the autoantigen used, if active or passive immunization is performed. Several of the candidate autoantigens for MS have been used to induce EAE, for example MBP, myelin oligodendrocyte glycoprotein (MOG), and proteolipid protein (PLP). Interestingly, different peptides from these proteins can vary greatly in their encephalitogenic potential[117–119]. In work presented in this thesis, active immunization with MOG in C57BL/6 mice or Dark Agouti (DA) rats were used. Compared to most other proposed autoantigens in MS, MOG is a minor component of myelin. However, it is highly encephalitogenic in most laboratory animals and induction of EAE with whole myelin in MOG-deficient mice gives mild disease, showing that it is a major pathogenic component in autoimmunity towards myelin in mice[120]. Study results are inconclusive regarding the role of reactivity to MOG in MS patients[121–123].

To induce EAE, the autoantigen is emulsified in oil and injected subcutaneously to produce local slow release of antigen in a pro-inflammatory environment. C57BL/6 mice are relatively resistant to disease induction and MOG-EAE in this strain requires addition of heat-killed mycobacteria in the adjuvant as well as systemic administration of pertussis toxin, the latter repeated after 2 days. Mycobacteria are a classic inducer of Th1 type immunity. Interestingly, EAE can also be induced with the mycobacteria exchanged for *Citrobacter rodentium*, an inducer of Th17 type immunity, yielding a less severe disease course[124]. Proposed mechanisms of action for the pertussis injections in the context of EAE include: i) transiently opening the BBB, ii) inhibition of regulatory T cell activity, and iii) promotion of Th17 differentiation[125–127]. Once the emulsion is injected, dendritic cells (DC) take up the antigen, are activated by ligation of pattern recognition receptors (PRR), upregulate MHC class II, and migrate to the draining lymph nodes where they present the antigen to circulating CD4$^+$ T cells. The T cells are activated, expand in numbers, reenter the circulation, and infiltrate the CNS where they are reactivated by local APCs. The resulting inflammation leads to recruitment of monocytes, neutrophils, CD4$^+$ T cells, and CD8$^+$ T cells. Naïve T cells with other specificities are also recruited, which can become activated through epitope spreading[128,129]. EAE can also be passively induced by adoptive transfer of activated encephalitogenic T cells.

1.1.6 T and B lymphocyte effector mechanisms in MS and EAE

Although many immune cells have been shown to influence EAE and similarly been implicated in MS, CD4^{+} T cells have received the most attention. The relative importance of T helper cell subsets and their effector molecules in EAE and MS has been debated for some time[130].

EAE was initially believed to be driven by Th1 cells and the signature cytokine IFNγ. This was partly based on the ability of Th1 cells to induce passive EAE[131], identification of IFNγ secretion from T cells infiltrating the CNS, and the observed resistance to EAE in mice with targeted deletion of *tbet*, master transcription factor for Th1 cells, as well as mice with deletion or antibody-mediate neutralization of subunit p40 of Th1 promoting cytokine IL-12[132–134]. Consequently, MS has also been viewed upon as a Th1 mediated disease, which is supported by the detection of IL-12 and IFNγ in lesions and CSF from MS patients[135,136]. Furthermore, administration of IFNγ to MS patients in the setting of a small trial caused disease exacerbation[137]. It was therefore surprising when subsequent studies showed that the other IL-12 subunit, p35, as well as IFNγ was redundant or even protective in EAE[138–141]. The explanation for these contradictory findings came when it was shown that IL-12p40 was a common subunit for both IL-12 and IL-23, the latter a factor that promotes Th17 differentiation[142]. Mice deficient in IL-23 subunit p19 were shown to be resistant to EAE induction, which ignited great interest in Th17 cells in EAE and MS. Just like IFNγ, none of the signature cytokines of the Th17 subset is necessary for EAE induction[143–145].

There is currently renewed interest in the cytokine granulocyte-macrophage colony-stimulating factor (GM-CSF), which appears to be critical for EAE induction[146,147]. More recent reports have shown that GM-CSF production by both Th1 and Th17 is necessary for disease induction[148,149]. However, in C3HeB/Fej mice, which differently from other mouse strains develop inflammation in both spinal cord and brain, full EAE susceptibility has been reported despite absence of GM-CSF[150]. It may be so, that no single mediator is completely necessary for EAE or MS to develop.

In contrast, an increasing number of reports suggest that the balance between encephalitogenic Th1 and Th17 responses influences the pathological manifestations of EAE and MS[151]. In EAE, an increased Th17:Th1 ratio is associated with inflammation predominantly in the brain and a decreased Th17:Th1 ratio favors inflammation in the spinal cord[152]. Several studies in mouse EAE have later shown that IFNγ acts to suppress inflammation in the brain but not in the spinal cord[153,154]. In contrast, a recent study showed that myelin specific peripheral blood T cells from MS patients with predominant spinal cord inflammation had higher Th17:Th1 compared to patients with predominant brain inflammation[155]. It is still unclear whether the T cell phenotype in the periphery accurately reflects that in the CNS or is inverted as Th1 cells are mobilized to brain, in which case the human data would be consistent with previous mouse data. It is also not known whether the spinal cord and brain have different susceptibility to Th1 and Th17 responses or if the T cells locally are differentially polarized by the respective microenvironment. There is mouse data

that support the first scenario, using the C3HeB/Fej mice. After immunization the CD4$^+$ T cells in these mice recognize two distinct MOG epitopes, MOG$_{79-90}$ and MOG$_{97-114}$. Adoptive transfer of only MOG$_{79-90}$ specific T cells predominantly yields inflammation in the spinal cord whereas transfer of MOG$_{97-114}$ T cells predominantly results in inflammation in the brain[156]. Moreover, the MOG$_{97-114}$ specific T cells displayed a higher Th17:Th1 ratio after *in vitro* stimulation, compared to the MOG$_{79-90}$ specific T cells. When drawing conclusions regarding the effects of IFNγ and IL-17 in EAE one must bear in mind the plasticity of T helper cells in general and in particular that there seems to be a switch from Th17 to Th1 polarization after entry in to the CNS[157].

The involvement of B cell in MS has not been studied to the same extent as that of T cells. Observations that support a role for B cells in MS pathogenesis include:

- identification of myelin specific antibodies in CSF and blood from MS patients[158,159],
- presence of oligoclonal intrathecal antibodies in most MS patients, which serves as a useful diagnostic biomarker,
- and evidence for clonal expansion of B cells in the CNS of MS patients[160,161],
- depletion of B cells using anti-CD20 monoclonal antibodies is one of the most effective treatments for both RRMS and progressive MS[162–166].

Interestingly, anti-CD20 treatment does not deplete plasma cells, making it plausible that the B cells influence MS by other mechanisms than production of antibodies, for example cytokine production or antigen presentation. Both of these explanations have received experimental support. The effect of anti-CD20 treatment in mice is dependent on B cell IL-6 production, which promotes Th7 responses[167]. B cells are the most abundant MHC class II-expressing cell in the naïve mouse CNS[168]. In the same report, it is further shown that in the absence of B cells, adoptively transferred T cells infiltrate the CNS but have a diminished capacity to recruit more cells from the periphery.

1.1.7 MS - treatments

After interferon beta-1a was approved in the mid-nineties, as the first drug to treat MS[169], the treatment options have expanded greatly. More than 10 disease-modifying drugs have been approved to date.

Interferon beta-1a is a naturally occurring cytokine with antiviral and anti-proliferative effects. It is available as subcutaneous or intramuscular injections for the treatment of MS. A pegylated form is now available that only needs to be administered every two weeks[170]. Many immunological effects of Interferon beta-1a treatment have been observed but their relative contribution to the treatment effect is not known[171–174]. It has a modest effect on relapse rate and MRI activity but no effect on disease progression. Side effects are mild and mainly involve influenza-like symptoms and skin-reactions at the injection site.

Glatiramer acetate is a mixture of polypeptides that includes four amino acids that recur in the MS candidate autoantigen MBP. Several mechanisms of action have been proposed e.g. partial induction of tolerance in MBP-specific T cells, thus acting as an *altered peptide*

ligand[175,176], and induction of glatiramer acetate-reactive Th2 T cells[177]. Glatiramer acetate is administered daily as a subcutaneous injection and the efficacy and safety profile is similar to interferon beta-1a[178].

Teriflunomide is taken orally and inhibits dihydro-orotate dehydrogenase, a key enzyme in the *de novo* pyrimidine synthesis pathway. It is believed to inhibit proliferation of activated T and B cells without affecting the homeostatic proliferation of resting lymphocytes[179]. As a result, it does not increase occurrence of serious infections[180]. It also induces an anti-inflammatory shift in T cell polarization and immunoglobulin isotypes. The efficacy and safety profile is similar to interferon beta-1a[181].

Dimethyl fumarate is a small molecule derived from fumaric acid, an intermediate in the citric acid cycle. The mechanism of action is not clear but may involve an antioxidant effect by activation of the transcription factor nuclear factor E2 related factor 2 (Nrf2) and also an anti-inflammatory effect through inhibition of the transcriptional regulator NF-κB. It is taken orally and reduces relapse rate and MRI activity[182,183]. A reduction in disease progression, i.e. increased time to disability, was shown in one trial[182]. A small number of cases of progressive multifocal leukoencephalopathy (PML), an adverse effect with high mortality caused by reactivation of latent John Cunningham (JC) virus infection in the CNS, has been reported in the treatment of MS patients with dimethyl fumarate[184].

Fingolimod is a sphingosine-1-phosphate analogue that is taken orally. It acts as an antagonist of sphingosine-1-phosphate receptors. Fingolimod ligation leads to the receptor being internalized and degraded. As lymphocytes depend on cell surface expression of sphingosine-1-phosphate receptors to egress from lymphoid tissue, this treatment result in them being trapped and consequently less lymphocytes will be present in the circulation. Like dimethyl fumarate, fingolimod has been shown to reduce disease progression in one trial[185,186]. In a head to head comparison with interferon beta-1a, fingolimod treatment resulted in approximately half the number of relapses per year[187]. But fingolimod treatment is on the other hand associated with more disconcerting side effects, like bradycardia, heart block, macular edema, and infections as well as some very serious rare adverse effects, most notably, generalized varicella zoster infection, herpes encephalitis and PML.

Natalizumab is a humanized monoclonal antibody that blocks α-4 integrin, which is a component of very late antigen 4 (VLA-4) present on lymphocytes. It is given intravenously every 4 weeks. Natalizumab inhibits the interaction between VLA-4 and its ligand, vascular cell adhesion molecule 1 (VCAM-1), expressed on endothelial cells, thus preventing lymphocytes from crossing the blood-brain barrier. It is one of the most effective disease modifying treatments for MS with a clear effect on disease progression shown in one trial[188]. However, more than 600 MS patients have developed PML during natalizumab treatment with a mortality rate of more than 20 %[189]. By excluding patients with high JC virus-antibody blood titers or increasing monitoring by MRI, the risk of PML has significantly been reduced[190,191].

Alemtuzumab is a humanized monoclonal antibody directed against CD52, which is expressed on lymphocytes and monocytes. It is given intravenously for five consecutive days and then another three consecutive days a year later. This results in a very long-lasting reduction in B and T cells, with T cells recovering slower than B cells. One trial has shown a significant effect on disease progression[192]. The safety profile is an issue, however, which makes frequent clinical monitoring necessary. Apart from an expected increase in susceptibility to infections, approximately 30 % develop secondary autoimmune disorders, primarily affecting the thyroid gland[192,193].

Daclizumab is a humanized monoclonal antibody directed against CD25, a component of the IL-2 receptor, thus acting as an IL-2 antagonist. It not only reduces the circulating levels of $CD4^+$ and $CD8^+$ T cells but also expands a population of $CD56^{bright}$ natural killer (NK) cells, which may be more important for the clinical effect in MS[194]. Monthly subcutaneous injections of daclizumab has been shown to be superior to interferon beta-1a but does not reduce disease progression. It is a relatively new drug that has recently been approved in the USA and Europe for the treatment of MS[173].

Ocrelizumab is a monoclonal antibody directed against CD20, which is expressed on B cells. It was developed in light of the efficacy of rituximab in MS[162,164,195] and the CD20 epitopes of the two antibodies overlap. It acts by depleting CD20-expressing cells, i.e. B cells but not plasma cells. Ocrelizumab is a humanized antibody, thus expected to be less immunogenic compared to rituximab, which is a mouse chimeric antibody. Ocrelizumab is recently approved in the USA for the treatment of MS and is also the first treatment approved for the treatment of primary progressive MS[165,196].

1.2   IMMUNOLOGY

The research presented in this thesis is mainly within the field of immunology, in regards to questions addressed and methods used. Now follows an overview of the immunological scope of the work.

The immune system consists of a network of specialized cells and molecules that have evolved to protect multicellular organisms from pathogens. These cells and molecules typically interact in complex and dynamic ways, of which our understanding is far from complete, despite having accumulated a tremendous amount of detailed knowledge. In addition to fighting off infections, the immune system suppresses the development of cancer and has also been shown to interact with other body systems, such as the metabolic, endocrine, and nervous system. However, the work discussed in this thesis will be centered on the situation when disease is caused by the immune system itself i.e. inflammatory and autoimmune diseases.

It is useful to think of the immune system as consisting of two lines of defense against invading pathogens - innate and adaptive immunity. Adaptive immunity is more effective at

clearing infections but takes several days to develop. In the meantime, innate immune mechanisms will attempt to stop or at least delay the infection and simultaneously start the process of mounting an adaptive immune response directed specifically to the invading microorganism. The two systems do not work in isolation, however. Signals from the innate immune system activate and shape the adaptive immune response and a major part of adaptive effector mechanisms is to focus and amplify the activities of innate immune cells.

1.2.1 Innate immunity

Key elements included in the concept of innate immunity are:

- *Physical barriers* – most prominently the epithelial layers lining surfaces facing the outside world,
- *Chemical and mechanical barriers* - such as acidic pH, antimicrobial peptides, and secretions with directional flow that wash away invaders.
- *Inflammatory response* – characterized by recruitment of fluid, molecules, and cells to the site, thus signaling to the rest of the body that a threat is present,
- *Phagocytosis* – rapid ingestion of bacteria by cells such as neutrophils and macrophages.

1.2.1.1 Epithelial barrier of the skin

The skin consists of the epidermis and underneath it, the dermis. The epidermis is composed of several layers of tightly packed cells of which the outermost layers are dead cells filled with keratin that makes the skin a waterproof barrier. The dermis is mostly connective tissue and contains blood and lymphatic vessels, hair follicles, sweat and sebaceous glands, as well as immune cells such as DCs, macrophages, and mast cells.

1.2.1.2 Antimicrobial proteins and peptides

Epithelial cells at barrier surfaces constitutively produce a broad range of molecules that kill or inhibit growth of bacteria or fungi. These show some degree of specificity, seen for example in the case of psoriasin, which is expressed in the skin. This antimicrobial protein does not affect skin commensal *Staphylococcus aureus* but rapidly kills intestinal commensal *Escherichia coli* if it happens to end up on the skin. The related antimicrobial, also from the family of S-100 proteins, does however kill both bacterial species[197]. Antimicrobial peptides are generally smaller than 100 amino acids and are a primitive form of innate immunity even found in plants and some fungi. The main types found in humans are the defensins and cathelicidin. They can disrupt membranes of bacteria and fungi and then enter the cell where they exert other effects such as inhibiting the synthesis of DNA, RNA, and proteins[198].

1.2.1.3 Toll-like receptors

Inflammation and phagocytosis depend on the recognition of conserved structural elements present on pathogens but absent in humans, so called pathogen associated molecular patterns (PAMP). PAMPs are recognized by a wide range of extracellular and intracellular pattern recognition receptors (PRR), that help the immune system to distinguish between what is self (part of the body) and non-self (an invader that should be eradicated). There are a number of

PRR-families but the best characterized are the toll-like receptors (TLR)[199,200]. Ten TLRs have been identified in humans, each with specificity for certain conserved microbial elements. The TLRs are either cell surface receptors or localized to intracellular vesicles and they are most highly expressed by sentinel cells like macrophages and dendritic cells. TLRs are expressed in the CNS by microglia, the primary resident sentinel cell, but also by astrocytes, oligodendrocytes and neurons[201].

1.2.1.4 Chemokines

The chemokines are a family of small proteins that are secreted into the extracellular space for the purpose of attracting other cells. A concentration gradient is established from the site of production that attracts cells bearing the corresponding chemokine receptor, a process called chemotaxis. Specificity of leukocyte chemotaxis is achieved by the existence of many different chemokines and chemokine receptors that are selectively expressed in a dynamic way. The chemokines are divided into two groups: the homeostatic chemokines, which are continuously secreted in certain tissues and facilitate immune cell surveillance in the steady state, and the inflammatory chemokines, which are induced as a part of an inflammatory response. The B cell-attracting chemokine CXCL13 has attracted much attention as a potential biomarker for MS and may promote the formation or tertiary lymphoid follicles in the CNS characteristic of progressive disease[202–206].

1.2.1.5 Innate immune cells

All immune cells as well as erythrocytes and thrombocytes develop from the same origin, the hematopoietic stem cell, which resides in the bone marrow. When a hematopoietic stem cell starts differentiating, the first fate-determining step is to become either a common myeloid progenitor that later gives rise to erythrocytes, granulocytes, monocytes, and macrophages or to become a common lymphoid progenitor that later gives rise to T cells, B cells, and NK cells. Myeloid cells and NK cells are part of the innate immune system.

Neutrophilic granulocytes (neutrophils) constitute the majority of circulating immune cells. They are the first cells to be recruited during an immune response and the rapid increase in circulating granulocytes is used clinically as an indication of infection. Neutrophils have in their cytoplasm granules that contain various preformed proteins ready to be expelled, some resulting in direct damage to pathogens and others resulting in recruitment of other cells, for example macrophages and more neutrophils.

Monocytes constitute 5-10 % of circulating cells. They are a heterogeneous population that can be divided into inflammatory monocytes, which enter tissues rapidly and can differentiate into macrophages with high phagocytic capacity, and patrolling (non-classical) monocytes, which may in contrast act to dampen inflammation[207]. DCs are also a heterogeneous population of cells that can develop from either the myeloid progenitor, lymphoid progenitor or directly from monocytes. They phagocytose pathogens in the tissues, migrate to secondary lymphoid organs and present antigens to CD4$^+$ T cells to initiate an adaptive immune response. Other cells that can act as APCs are macrophages and B cells.

20

1.2.2 Adaptive immunity

The T and B lymphocytes are the orchestrators of adaptive immune responses. They have several important features in common. Randomly assembled antigen-receptors, stringent selection to exclude autoreactive clones, high threshold of activation, and immunological memory that enables a more rapid and effective response the next time the same antigen is encountered.

1.2.2.1 T cells

T cells are divided into T helper (Th) cells, distinguished by cell surface expression of CD4 and cytotoxic T cells (Tc) distinguished by cell surface expression of CD8. Th cells recognize antigens bound to MHC class II on APCs and primarily act by releasing cytokines that activate other cells of the immune system. Tc cells recognize antigens bound to MHC class I on most nucleated cells and primarily act by killing the cells displaying the antigen. Th cells have been the subject of intense research aiming to characterize distinct subsets based on the cytokines they produce and the signaling events that lead to their differentiation. In relation to autoimmune disease, the Th1 and Th17 subsets are most frequently discussed and are generally regarded as pathogenic. In relation to protective immunity to infection, Th1 cells mount immune responses to intracellular pathogens and Th17 appears to be important for extracellular pathogens and fungi.

Another group of T cells frequently discussed in relation to autoimmune disease is the regulatory T cells, which by different means act to suppress lymphocyte activation and consequently inflammation.

All the T cells discussed so far have a TCR that is composed of an alpha chain and a beta chain, sometimes called αβT cells to distinguish them from T cells with a TCR composed of a gamma chain and a delta chain. The γδT cells have features of both adaptive and innate immune cells and are currently the subject of much research but their role in autoimmune disease is only beginning to be elucidated.

1.2.2.2 B cells

B cells include B-1 B cells, B-2 B cells, marginal zone B cells, and regulatory B cells. B-2 B cells are by far the most common B cell, and is usually implied when discussing simply B cells. Although B cells also secrete cytokines and act as antigen presenting cells, the major activity is to produce antibodies. They display on their surface a B cell receptor, which has the same specificity as the antibodies that they will produce if activated. Once B cells exit the bone marrow they are directed towards secondary lymphoid organs as a consequence of their expression of the chemokine receptor CXCR5 whose ligand, CXCL13, is constitutively secreted there by follicular dendritic cells. The B cell receptors bind antigens independently of any other cell type but in general they need help from T cells that recognize epitopes from the same microbe/source, to become activated. Once activated, some B cells will go through

a processes in the lymphoid organ to optimize antibody specificity (somatic hypermutation) and to change the type of antibody produced (isotype switching).

1.3   INTERLEUKIN-22

1.3.1   The IL-22-system

Cytokines are soluble mediators that all cells of the immune system use to some extent to shape the immune response. The term interleukin was proposed in 1979 as a classification of cytokines that are secreted by leukocytes and mediate signals to other leukocytes[208]. As new cytokines were discovered that fit this criterion they were named interleukin and a number, based on the order they were discovered. The definition of interleukins is however somewhat outdated, because as more knowledge has been gained about these cytokines it has become clear that also other cells produce them and respond to them.

1.3.1.1   IL-22

IL-22 was described in 2000 as a cytokine expressed by T cells[209]. It is now known that many immune cells of both the innate and adaptive immune system produce IL-22[210]. Most well studied is the contribution from Th17[211–214] cells and innate lymphoid cells[215–218]. Moreover, a distinct T helper subset that expresses IL-22 but not IFNγ, IL-4, or IL-17 has been described, termed Th22[219–221]. Fate-mapping shows that *in vitro* generated Th22 cells have not previously expressed IL-17[222]. They have a marked plasticity towards IFNγ production under Th1 polarizing conditions. IL-22 expression in non-hematopoietic cells has been reported as well[223]. Unlike most other cytokines, IL-22 acts primarily on non-hematopoietic cells[224,225].

1.3.1.2   IL-22R

The IL-22 receptor (IL-22R) is a heterodimer consisting of the subunit IL-22R1 (transcribed from the gene *IL22RA1*) and the ubiquitously expressed IL-10R2 (transcribed from the gene *IL10RB*)[226–230]. The receptor is expressed on epithelial and parenchymal cells in a range of organs, most notably the barrier surfaces skin, lungs and gastrointestinal tract, but also other organs like kidney, pancreas, thymus, liver and synovial tissue[217,231–234]. There are also reports of IL-22R expression on immune cells but these findings need further confirmation and characterization before the significance of this can be appropriately assessed[235–242].

1.3.1.3   IL-22BP

The soluble binding protein IL-22BP binds to IL-22 with very high affinity[243,244]. The binding site of IL-22BP on IL-22 overlaps with that of IL-22R1, thus effectively stopping IL-22 from activating the receptor[230]. IL-22 is one of few cytokines that have a dedicated inhibitor, implying that it has been particularly important for the organism to be able to turn off unwarranted IL-22 signaling at certain time points of an immune reaction or at certain

locations[245]. The cellular source of IL-22BP has mostly been reported as being APCs, but recently also T cells and eosinophils have been shown to express IL-22BP specifically in the gut[246–250].
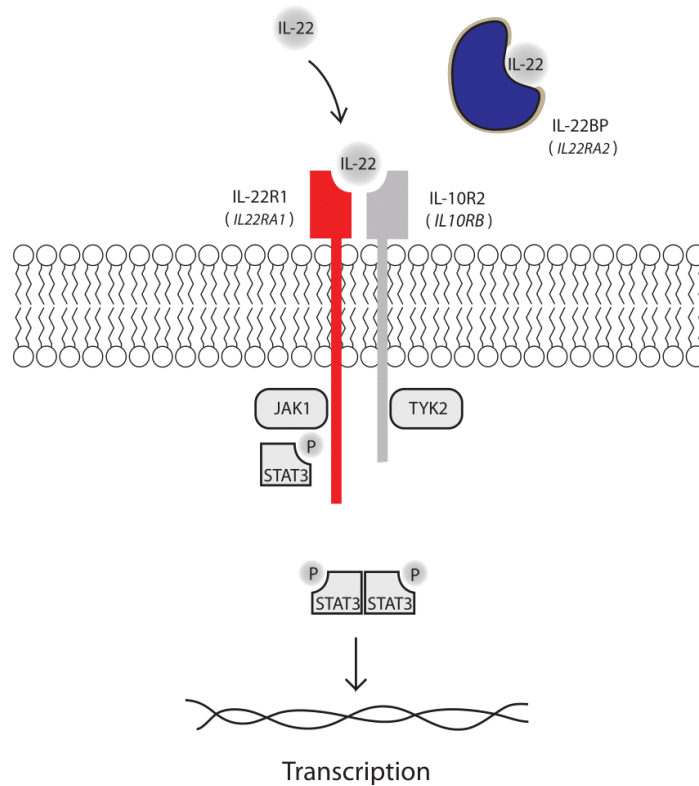
Figure 3. IL-22 signaling

1.3.2 IL-22 physiology and pathophysiology

Typically, the effects of IL-22 on tissues can be summarized as proliferative, regenerative, and tissue protective but it also induces innate immune mechanisms such as anti-microbial peptides and chemokines[210,251]. IL-22 levels have been correlated to many inflammatory states and expression is essentially always upregulated in these studies.

1.3.2.1 skin

There is great interest in the role of IL-22 in the skin and all the effects mentioned above have been demonstrated in IL-22-stimulated keratinocytes. Moreover, IL-22 results in delayed keratinocyte differentiation, which is one of the hallmarks of the pathology of psoriasis[212,252–255].

1.3.2.2 liver

In the liver, IL-22 has been shown to induce production of acute phase reactants by the hepatocytes but also confers upon them protective and regenerative effects in several experimental models of injury such as infection, alcohol mediated damage, autoimmunity, and ischemia[256–262].

1.3.2.3 thymus

After radiation injury to the thymus IL-22 acts to regenerate the tissue and restore function relating to T cell development[263].

1.3.2.4 gastrointestinal tract

In the gastrointestinal tract, IL-22 has an important role in maintaining epithelial barrier integrity. One study show that innate lymphoid cell (ILC) derived IL-22 contains *Alcaligenes* species in the gut and thus prevents systemic inflammation[217]. However, the consequences of IL-22 deficiency is much greater in the context of intestinal inflammatory pathology, exemplified by markedly exacerbated colitis in *Il22^{-/-}* mice infected with *Citrobacter rodentium* compared to wild type mice[264–268]. IL-22 supports the epithelial cells lining the gastrointestinal tract by inducing expression of genes that regulate proliferation, wound healing, and apoptosis. If the proliferative effect of inflammation-induced IL-22 is not limited by IL-22BP neoplasia can result, as shown in a mouse model of inflammation induced tumorigenesis[248]. IL-22 may also regulate tight junctions between the epithelial cells[269].

1.3.2.5 CNS

The potential effect on tight junction integrity is interesting in relation to neuroinflammation. One report has shown that both IL-17 and IL-22 are able to disrupt BBB tight junctions[270]. This implies that IL-22 may act to increase susceptibility to and/or severity of neuroinflammation. However, IL-22 deficient mice have the same disease course of EAE as wild type mice[145]. Another report has shown that astrocytes express IL-22R and receive trophic support from IL-22 and yet another reports IL-22R expression on oligodendrocytes and increased apoptosis upon IL-22 stimulation[271,272]. So, the role of IL-22 in neuroinflammation is still very much unclear.

1.4 SKIN INFLAMMATION

1.4.1 Structure of the skin

The skin consists of three layers: epidermis, dermis, and subcutis. The epidermis is mainly composed of keratinocytes with the important addition of Langerhans cells, a population of skin-resident DCs. Keratinocytes differentiate as they move from the most basal layer of the epidermis towards the surfaces of the skin at which point they lose their nuclei and organelles and become interlocked with each other in a highly hydrophobic matrix. The dermis consists mostly of collagenous connective tissue interspersed with blood vessels, immune cells, hair follicles, sweat glands and sebaceous glands. Subcutis, also called hypodermis, is a layer of fat that makes up the innermost layer of the integumentary system.

1.4.2 Immune system of the skin

Skin associated lymphoid tissue, just like its counterpart in the intestines, is in a state of relative tolerance necessitated by symbiosis with abundant microbiota[273]. A prominent feature of the immune compartment of the skin is a population of resident effector memory T cells that are believed to be non-recirculating. They bear the skin migratory receptor cutaneous lymphocyte antigen (CLA), which binds to E-selectin on cutaneous blood vessels. Furthermore, expression of chemokine receptor CCR10 enables homing towards CCL27 produced by keratinocytes during non-inflammatory states. Keratinocytes are major producers of chemokines upon inflammation. In the case of a Th1 response, IFNγ induces keratinocyte production of CXCL9, CXCL10, and CXCL11, which recruit more Th1 cells. Similarly, IL-17 yields production of CCL20, CXCL1, CXCL2, and CXCL8, which recruit Th17 cells and neutrophils. The major APCs of the skin are Langerhans cells and myeloid DCs.

1.4.3 Psoriasis

The prevalence of psoriasis shows great regional variability but is approximately 2-3 % in Europe and North America[273]. Psoriasis vulgaris is the most common type and presents with large plaques of erythematous desquamating lesions characterized by infiltration of immune cells. The severity of psoriasis is commonly assessed by approximating the percentage of the body surface that is affected. The disease etiology is complex and not fully understood.

2 AIMS OF THE THESIS

When the work included in this thesis started in 2010, rat *Il22ra2* had attracted our interest as the possible mediator of an effect on EAE mapped to a locus on chromosome 1[246]. Moreover, *IL22RA2* had also been defined as the candidate gene for the MS-associated SNP rs17066096[44]. The literature on *IL22RA2* and its gene product IL-22BP was scarce and no other links to neuroinflammation had been reported. The literature on IL-22 was much more extensive, but still only a few indications of what role this cytokine may have in MS or EAE were available. Most notably, there was the negative report in which *Il22*-deletion was shown to have no effect on EAE in mouse[145], which suggested IL-22 independent effects of IL-22BP in the context of neuroinflammation.

The overall aim of this thesis was to understand the role of IL-22BP in MS pathogenesis.

The specific aims were:

1. to determine if deletion of *Il22ra2* or specific reduction of its expression influence EAE,

2. to determine if any such influence is dependent on the presence of IL-22,

3. to characterize IL-22BP/IL-22-mediated effects on the immune system,

4. and to detemine if the genotype of MS-risk variant rs17066096 influences *IL22RA2* expression.
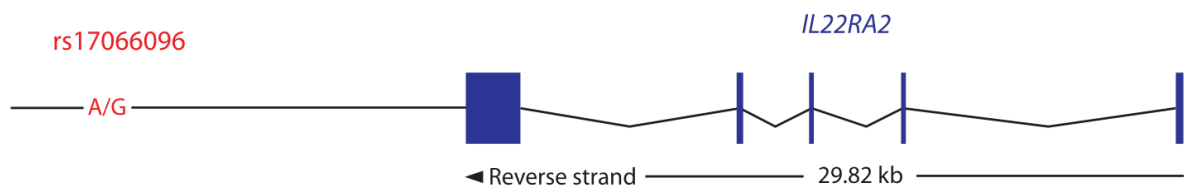
Figure 4. MS-associated SNP rs17066096 is located approximately 14 kb downstream from *IL22RA2*. Blue bars represent exons.

3 METHODOLOGICAL CONSIDERATIONS

A discussion of the methods that were used in this thesis follows below. Detailed descriptions are found in the respective materials and methods sections of paper I-IV.

Ethics statement

All procedures performed using animals, human samples, or clinical data were approved by the appropriate ethical review boards. All human subjects that have contributed samples to these studies have given their written or oral consent prior to inclusion.

Mouse and rat strains

Specific disruption of a gene or gene product of interest, while keeping all else equal is a well-tried principal of experimental research aiming to understand biological processes. With such tools at your disposal, you can address the role of a biomolecule or cell type in specific contexts. There are a number of principally different genetic models available with distinct advantages and disadvantages, meaning that careful attention must be paid to the requirements of the project.

In our laboratory, we have a long tradition of working with rats, mainly for the purpose of locating and studying natural genetic variants that influence EAE. This can be done by intercrossing inbred rat strains with different susceptibility to EAE, thus producing heterogeneous offspring as a result of random recombinations during meiosis. Inducing EAE in a large number of offspring enables linkage analysis in a way similar to how genes responsible for Mendelian genetic diseases are localized to a specific region of a chromosome. The effect of the linked region can then be confirmed by producing a congenic strain. This is done by intercrossing the two parental strains and repeatedly selecting offspring with the chromosomal fragment of interest and backcrossing these with wild type rats of the other parental strain. Using this approach, we reported an effect on EAE that came from a region in the beginning of chromosome 1 including the gene *Il22ra2*. This was published before the work on this thesis started[246].

For the follow-up studies included in this thesis, we commissioned a company to produce an inducible *Il22ra2*-knockdown rat strain that makes use of *tetracycline-controlled transcriptional activation*. Administration of the tetracycline-analogue doxycycline in the drinking water induces expression of a short hairpin RNA (shRNA) that specifically hybridizes with *Il22ra2* mRNA that subsequently becomes degraded by the cellular microRNA machinery. This results in a significant drop in *Il22ra2*-mRNA levels within 1-2

days after the start of doxycycline administration yielding knockdown efficiencies in the range of 50-90 %. From our perspective, the advantages of this genetic model were:

- that we continued working with a rat strain of the same genetic background as the one in which we had observed an effect on EAE coming from chromosome 1,
- we avoided potential compensatory effects by the complete lack of *Il22ra2* from conception to adult rat,
- we could study the role of *Il22ra2* at different time points during the disease course,
- reducing expression of a gene rather than deleting it completely more closely resembles the situation of human natural genetic variation that we ultimately want to understand,
- and that gene targeting in rat was not available at the time.

The major disadvantage of working with rats in general when investigating disease mechanisms is the scarcity of reagents and commercially available transgenic strains. Moreover, the literature is dominated by information from studies of mice, which can not always be extrapolated to rat.

To expand the number of tools available to us, we decided to also study *Il22ra2* using mice in parallel and acquired a conventional *Il22a2* knockout mouse strain. This enabled us to later also acquire an *Il22* conditional knockout mouse strain and produce the *Il22ra2/Il22* double knockout used in paper III.

EAE

EAE is a disease model with several similarities to MS. Various autoantigens can be used to induce EAE but in this thesis we have used only MOG. For EAE in rat, MOG is emulsified in incomplete Freund's adjuvant and injected subcutaneously at the tail base. For EAE in mouse, MOG is emulsified in complete Freund's adjuvant, i.e. it also includes heat killed mycobacteria, and is injected similarly, followed by an intraperitoneal injection of pertussis toxin, which is repeated on day 2.

Skin inflammation models

Oxazolone model: The small molecule oxazolone is an irritant that is often used to induce delayed type hypersensitivity (hypersensitivity type IV) in experimental animals, which is regarded as a model of atopic dermatitis. Mice are sensitized by applying oxazolone on shaved skin of the back. After 6 days, an immune reaction is elicited by applying oxazolone on one of the ears. The degree of inflammation is quantified by measuring the ear thickness 24 hours later with a high precision caliper.

Imiquimod model: One of the more frequently used murine models of psoriasis is induced by daily applications of imiquimod cream on shaved skin[274]. The main readout is thickening of the epidermis, which is assessed on histological sections. Imiquimod is a small molecule that is used as a topical treatment of various skin conditions including cancerous and precancerous lesions. It is an immune modifier that acts by activating TLR7 on innate immune cells.

In vivo cytokine treatment

As a confirmatory experiment, recombinant rat IL-22 or PBS was injected subcutaneously and the effects were compared to those seen when knocking down *Il22ra2*.

Real-time quantitative PCR

Gene expression has been used extensively throughout the thesis to assess cellular responses in experimental settings. It is assumed that a change in mRNA quantities for a particular gene also will be reflected on the protein level. Comprehensive studies have shown a moderate correlation between mRNA and protein copy numbers when broadly assessing several tissues and cell lines with a median Pearson correlation coefficient of 0.6[275]. However if a gene-specific factor is introduced the same value increases to 0.93. This means that mRNA levels reflect changes in protein levels well in qualitative terms, i.e. let us know if there is an increase or decrease in translation, but less well in quantitative terms[275]. Naturally, having data on protein levels is generally preferred. But protein quantification methods are often associated with significant obstacles and therefore need more extensive protocol optimization, for example regarding sample preparation or antibody staining, making them impractical in certain circumstances. In this thesis, real-time quantitative PCR has been used as a screening tool or when protein quantification has not been attainable or practical.

Flow cytometry

Although any cell can potentially be analyzed by flow cytometry, it requires that you can prepare a single cell suspension, which imposes a challenge when investigating most tissues. Immune cells are more or less migratory and therefore only have loose attachments to their surroundings. For this reason, lymphoid tissue can easily be disintegrated yielding free single cells. However, light treatment with enzymes may be needed to increase the yield of certain cells, such as APCs. Getting immune cells from other tissues generally requires more extensive mechanical and enzymatic manipulation, both of which compromise cell viability and cell surface marker integrity. In this thesis digestion of CNS, lymphoid and skin tissue has been used for analysis by flow cytometry.

Cell sorting

Antibody based sorting of cells has been performed using the technologies fluorescence-activated cell sorting (FACS) or the simplified magnetic-activated cell sorting (MACS). FACS makes use of a flow cytometer and allows for a large degree of control over the sorting. It is indispensable when complex gating is required to define the population of interest or to get accurate information regarding sorting purity. MACS uses magnetically labeled antibodies that can be separated using simple equipment. It allows for less control but is simple and can more easily be performed in sterile conditions, important if the cells are to be cultured afterwards.

Isolation of peripheral blood mononuclear cells and in vitro stimulation

Peripheral blood mononuclear cells were isolated from heparinized blood samples or buffy coats using Ficoll gradient centrifugation. CD14$^+$ monocytes were isolated using MACS beads followed by differentiation *in vitro* using recombinant cytokines and other stimuli.

ELISA

Enzyme-linked immunosorbent assay (ELISA) has been used to determine the concentration of circulating antibodies or other proteins in serum, plasma, or CSF.

Western blot

In paper II, changes in mRNA expression of *Il1b* and *Cxcl2* in skin biopsies were confirmed on the protein level using western blot.

Histopathology and immunofluorescence

To corroborate the difference in EAE severity in paper I, mouse spinal cord was stained with hematoxylin and eosin to assess inflammation and Luxol fast blue to assess demyelination. Immunofluorescence staining of mouse IL-22BP was performed on naïve lymph nodes. In paper II, hematoxylin and eosin staining was performed on skin biopsies from the two disease models. In paper III, immunocytochemistry was performed on cultured mouse oligodendrocytes to visualize cell surface expression of IL-22R. In paper IV, immunofluorescence staining was performed on draining lymph nodes from MOG-immunized rats to visualize T cells, B cells, and germinal centers.

SNP genotyping

In paper II, SNP-genotyping was performed using TaqMan SNP-genotyping assays.

4 RESULTS AND DISCUSSION

The results of papers I-IV will be discussed in the following section.

4.1 IL-22BP INCREASES SEVERITY OF MURINE NEUROINFLAMMATION

Background: We had previously reported a quantitative trait locus (QTL) on rat chromosome 1 with influence on EAE severity[246]. In that study, a congenic rat strain was created, to confirm the effect of the QTL, by breeding so that the chromosomal fragment of interest from the relatively EAE-resistant PVG strain was isolated on DA genetic background. This congenic strain has milder EAE compared to wild type DA rats, consistent with the linkage data. The congenic fragment includes several genes, of which *Il22ra2* appeared as the most plausible gene responsible for the observed effect on EAE. This was based on lower *Il22ra2*-expression in the protected congenic strain compared to wild type DA rats as well as reports of its involvement in innate immunity. Furthermore, we showed, in a Nordic cohort, that SNPs near *IL22RA2* associate with MS risk. Shortly after, data from a large scale GWAS performed by the International MS Genetics Consortium (IMSGC) were made public showing that rs17066096, a SNP 14 kb downstream of *IL22RA2* strongly associate with MS susceptibility[44]. The role of *IL22RA2* in MS warranted further investigations and the next step was to definitely confirm the involvement of *Il22ra2* in EAE.

Hypothesis: Mice with a complete deletion of *Il22ra2* have less severe EAE.

Main results: Il22ra2$^{-/-}$ mice subjected to MOG-induced EAE had a slightly earlier onset of disease and a similar severity at peak of disease compared to littermate wild type controls. In contrast, after the peak of disease the knockout mice had a better recovery. We did not see any changes in the cellular composition of secondary lymphoid organs in untreated mice or significant effects on T cell phenotype in draining lymph nodes during priming, day 7. However, *Il22ra2*$^{-/-}$ mice had less demyelination and inflammation on spinal cord histopathology and assessment of CNS infiltrating cells by flow cytometry showed a significant decrease in Ly6C$^+$ inflammatory monocytes.
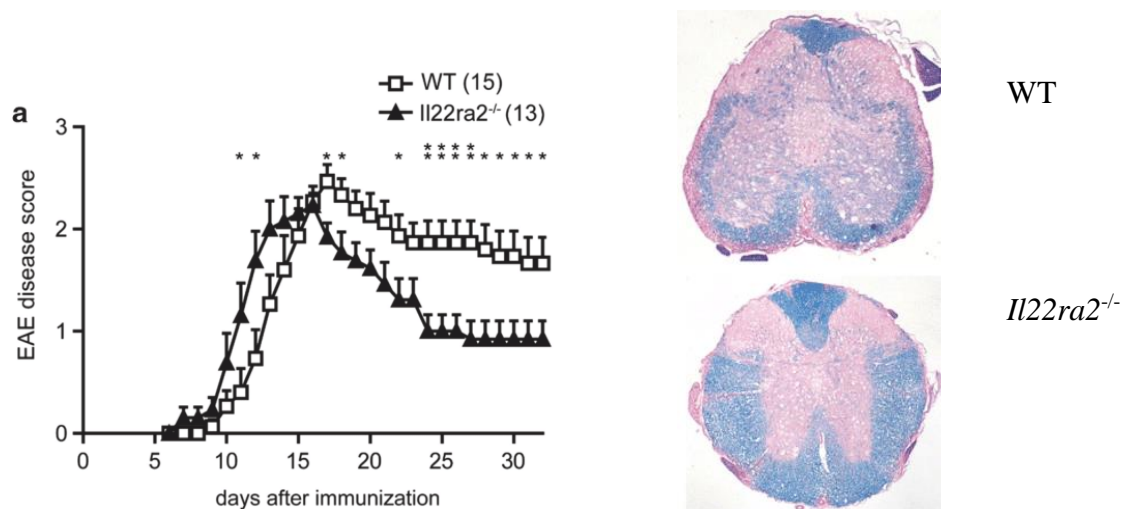
Figure 5. *Il22ra2*^{-/-} mice had less severe clinical signs of EAE and less demyelination in the spinal cord compared to wild type mice. * denotes a *p*-value < 0.05 and ** is a *p*-value < 0.01 using Mann-Whitney U test.

IL22RA2 codes for the soluble protein IL-22BP, which acts as an IL-22 antagonist by binding tightly to it so that it cannot dock with the signaling membrane-bound IL-22R. When the work started for this paper Th17 cells had relatively recently been identified as an important pathogenic factor for EAE[142,276,277]. IL-17 and IL-22 are signature cytokines of Th17 cells, which had spurred a few investigations into the role of IL-22 in EAE and MS[145,270]. Most notably, one paper had shown that mice with a constitutive deletion of *Il22* have no apparent EAE phenotype. The authors for that paper correctly concluded that IL-22 is not necessary for IL-22 induction but in the literature the message tended to be skewed towards IL-22 not having any role in EAE and initially that was also how I regarded those results. To try to reconcile these findings with the protective effect of IL-22BP-deletion we postulated the existence of an alternate ligand for IL-22BP and proceeded by trying to find evidence for this using immunoprecipitation. Recombinant mouse IL-22BP or antibodies for mouse IL-22BP were used as bait and lymph node tissue lysate or serum were added, using various immunoprecipitation reagents. The goal was to catch proteins that interacted specifically with IL-22BP or already formed IL-22BP-ligand complexes, which then could be eluted, visualized on a protein gel, and identified by mass spectrometry. These were exciting experiments to perform considering the potential implications of any positive results. However, after a few months without progress the research question was abandoned. Nevertheless, the experience gave me valuable insight into protein chemistry and some of the specific challenges inherent to methodologies in that field.

Similarly, the question of what cell type(s) express IL-22BP was not easily answered. In a first screen of *Il22ra2* mRNA expression in a panel of tissues in wild type mice we observed highest expression in lymphoid organs and we proceeded to define the cellular source. For soluble proteins, such as IL-22BP, antibody staining tends to be more challenging and if positive staining is achieved it may not clearly indicate which cell is the producer. Furthermore, no IL-22BP-antibody staining had been reported in the literature at this time. We therefore initially attempted to localize *Il22ra2* expression in rat lymph node using *in situ* hybridization. Despite some promising initial results, a robust assay could not be established. Instead, we chose a different strategy and FACS-sorted cell populations from mouse lymph nodes based on expression of various combinations of cell surface markers. This way, *Il22ra2* expression was localized to both the CD11b$^+$ CD11c$^+$ as well as the CD11b$^+$ CD11c$^-$ populations, which can be broadly interpreted as myeloid cells. Although positive antibody staining of mouse IL-22BP in lymph node section was achieved later and included in the paper, co-staining for CD11b was not possible due to protocol incompatibilities.

4.2   IL-22BP DECREASES SEVERITY OF MURINE SKIN INFLAMMATION

Background: We had now shown that *Il22ra2*$^{-/-}$ mice have less severe disease in EAE, suggesting a protective role for IL-22[278]. In contrast, there is strong evidence for IL-22 having a disease-driving influence on psoriasis, through its direct effects on keratinocytes[251,252]. The role of IL-22BP in skin inflammation had not previously been investigated. Paradoxical effects of cytokines on different inflammatory diseases have been observed previously, most notably for TNF. TNF-neutralization is a very successful treatment for rheumatoid arthritis and inflammatory bowel disease but increases disease activity in MS[75].

Hypotheses: 1) Mice with a complete deletion of *Il22ra2* will have increased severity of disease in experimental models of skin inflammation and 2) The MS-risk associated allele of rs17066096, which is located near *IL22RA2*, will have a protective influence on psoriasis.

Main results: Il22ra2$^{-/-}$ mice had more severe disease in oxazolone-induced contact hypersensitivity. To investigate the generalizability of this observation we included also the psoriasis model imiquimod-induced psoriasiform inflammation. Similarly, the *Il22ra2*$^{-/-}$ mice had more severe disease, evidenced by increased epidermal thickness but also more infiltrating neutrophils, and higher expression of neutrophil attracting factors in the skin, such as IL-1β and CXCL2. In the oxazolone-model we did not see any difference in immune cell infiltration, suggesting that different pathways are involved in the two models. We performed a genetic association study but could not demonstrate an opposite influence of rs17066096 on

MS and psoriasis. However, a trend was observed suggestive of reduced severity of psoriasis in carriers of the MS risk allele.
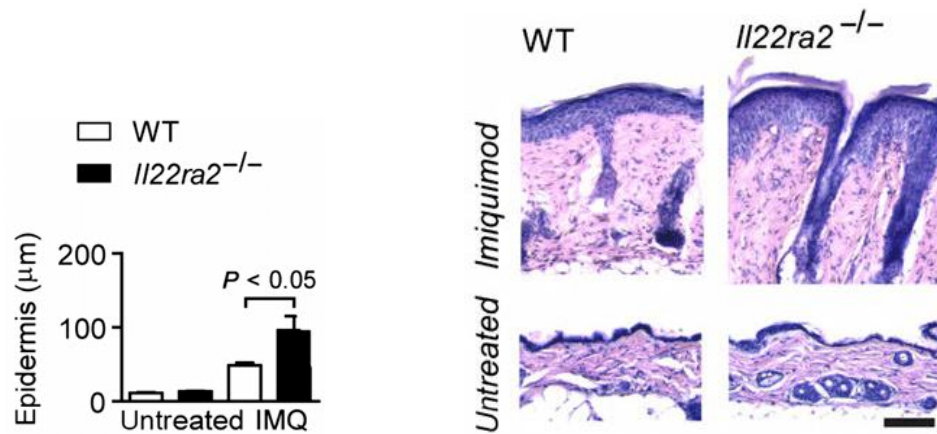

Figure 6. *Il22ra2*^{-/-} mice had more severe histopathological manifestations of imiquimod-induced psoriasiform inflammation, assessed by measuring epidermal thickness. *P*-values were calculated using unpaired Student's t test. Representative images of hematoxylin and eosin stained skin is shown. Scale bar is 100 μm.
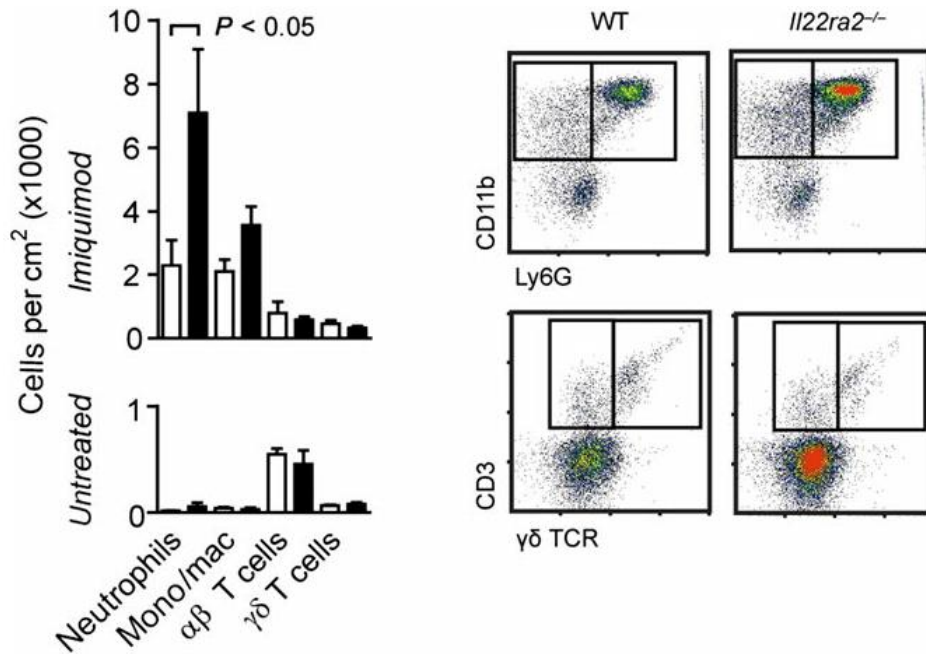
Figure 7. *Il22ra2*^{-/-} mice had more infiltration of neutrophils in the inflamed skin in the context of imiquimod-induced inflammation. Live CD45^{+} cells were separated into CD11b^{+} Ly6G^{-} monocytes/macrophages, CD11b^{+} Ly6G^{+} neutrophils, CD3^{+} γδTCR^{-} αβT cells, and CD3^{+} γδTCR^{-} γδT cells. *P*-values were calculated using unpaired Student's t test.

Our study of the role of IL-22BP in skin inflammation started with a number of small experiments using the oxazolone-model. These experiments were primarily motivated as a form of control of our genetic model - if IL-22BP is successfully deleted in the *Il22ra2*^{-/-} mice we expect to see increased severity of IL-22-driven pathology. Although there was little information on IL-22 in relation to contact hypersensitivity, we chose this model as it was easy to perform. The results clearly showed increased severity of disease in the *Il22ra2*^{-/-} mice, as expected. Considering that the role of IL-22BP in regulating the pathological effects of IL-22 in any model of skin inflammation had not been addressed, we started collaboration with a skin inflammation research group to investigate this further.

We could show that IL-22BP is an important regulator of inflammation in mouse models of psoriasis and atopic dermatitis. It was satisfying to see that the effect of IL-22BP on imiquimod-induced psoriasiform inflammation was later confirmed in a report published shortly after ours[279,280]. This is important information in relation to psoriasis and other inflammatory disorders of the skin, in which inhibition of IL-22 is discussed as therapy, suggesting another way of modulating the effects of this cytokine.

There are a limited number of cytokines that are known to have a dedicated antagonist molecule. One can speculate that evolutionary pressure has resulted in the use of IL-22BP as an important break in the IL-22-system, as too much will lead to pathology exemplified by psoriasis and to little will increase to risk of other pathologies exemplified by MS. It would not be surprising then if the expression of IL-22BP was genetically regulated, resulting in genotypes with opposite risk of psoriasis and MS. However, we could not provide strong evidence for this hypothesis but this does not exclude that such relationships exist. A larger cohort of psoriasis samples and more sub-phenotypes of both diseases may be necessary and possibly genotyping of other SNPs.

Marker	mild cases a/b	severe cases a/b	P	OR (CI)
rs17066096	288/1084	249/845	0,3	0,9 (0,74-1,09)
rs202573	400/972	432/964	0,3	1,09 (0,93-1,28)

Table 2. Carriers of the MS-risk associated (minor) allele of rs17066096 were less frequent in psoriasis patients with severe disease in the studied sample but not to the degree that statistical inference could be made. Abbreviations: *a/b*, number of minor/major alleles; OR, odds ratio; CI, confidence interval. *P*-values were calculated using the Chi-square test..

4.3   IL-22BP LEVELS ARE ASSOCIATED WITH MS RISK

Background: IL22RA2 is the candidate gene for an MS-risk locus defined by genetic marker rs17066096[44,45]. The gene product IL-22BP promotes disease severity in mouse EAE[278].

Hypotheses: 1) The disease predisposing G allele of rs17066096 will give higher expression of IL-22BP and 2) the protective effect of *Il22ra2*-deletion in mouse is dependent on IL-22.

Main results: Although human circulating immune cells did not express *IL22RA2 ex vivo*, monocytes differentiated *in vitro* with IL-4 and GM-CSF did. This combination of cytokines is known to result in a DC-like phenotype. Interestingly, the expression of *IL22RA2* was amplified even further by the addition of the retinoic acid receptor agonist AM580 throughout the differentiation process. Using this *in vitro* expression system, we saw that

cells from healthy blood donors that carry the disease predisposing G allele of rs17066096 had higher expression of *IL22RA2*. No significant differences were observed when performing the experiment on samples from MS patients, however. To link subtle changes in *IL22RA2* expression to neuroinflammation we performed EAE in a rat strain that enables inducible knockdown of *Il22ra2* through doxycycline controlled expression of a gene-specific shRNA *in vivo*. Knockdown of *Il22ra2* had a dose-dependent protective effect on EAE. Using mouse strains with deletion of *Il22ra2*, *Il22*, or both genes, we saw that the protective effect of *Il22ra2*-deletion was indeed lost in the absence of *Il22*. IL-22BP protein was present in rat and human CSF and levels correlated with disease course and number of MRI lesions, respectively. By sorting CNS cells from naïve mouse brains we saw that *Il22ra2* was expressed by microglia.

In this paper we addressed all stated aims of this thesis. First off, rs17066096 is located 14 kb downstream of *IL22RA2* and is associated with MS risk - but does it influence *IL22RA2* expression? This is a key question in the thesis but two aspects in particular made it difficult to address: 1) *IL22RA2* is not expressed in cells that are easily accessible for study and 2) the minor allele frequency of this SNP is relatively low ($\approx$ 0.23) with only about 5 % in a randomly selected group of individuals being homozygous carriers. To circumvent this, we wanted to establish an *in vitro* assay to study *IL22RA2* expression in peripheral blood cells. By searching the gene expression omnibus (GEO) DataSets, a searchable database that contains curated datasets from gene expression studies, we found that human monocytes differentiated with GM-CSF and IL-4 express *IL22RA2* and that expression is amplified by including retinoic acid receptor agonist AM580. After some optimizations we chose this as our assay. Next step was to collect blood samples and freeze the cells to be able to run all samples in parallel. However, we needed to preselect individuals based on rs17066096 genotype to have sufficient numbers of homozygous carriers of the risk allele. This could fortunately be arranged because many MS patients that regularly come to the neurology clinic have contributed samples for genomic studies and therefore already have been genotyped for rs17066096. Recruitment was done by research nurses and the collection of blood samples from 10 homozygous individuals took more than 6 months. Similar logistics were needed to collect samples from healthy individuals but this time collaboration was set up with the neighboring city Uppsala. An infrastructure was in place there in which individuals who were regular blood donors also had been densely genotyped, including rs17066096. Whenever an individual with the right genotype came to donate blood, a buffy coat was sent to our lab.
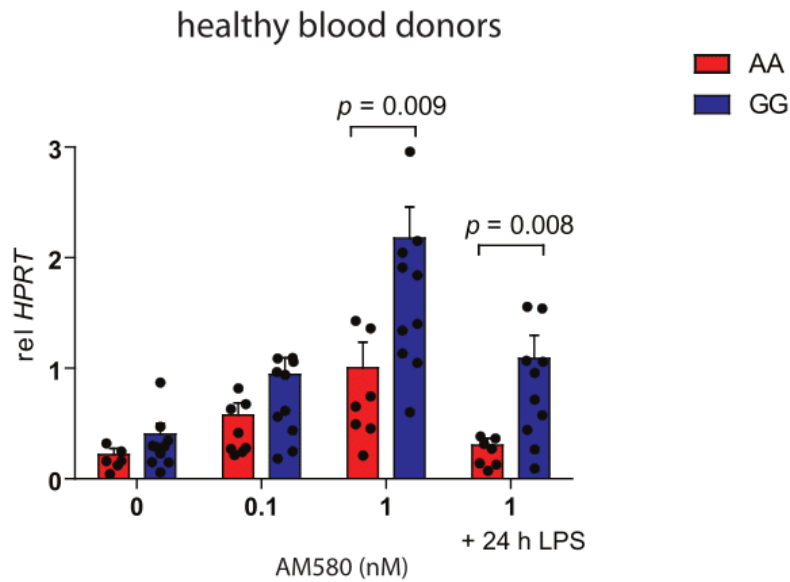
Figure 8. Monocytes from healthy blood donors carrying MS-risk allele rs17066096[G] expressed more *IL22RA2* after *in vitro* differentiation with GM-CSF, IL-4, and AM580. *P*-values were calculated using unpaired Student's t test.

We next resolved the question that arose in the beginning of the project - is the protective effect of knocking out *Il22ra2* in EAE mediated by IL-22? The available literature on IL-22 offered very little that could explain the disease course seen in the *Il22ra2*^{-/-} mice in paper I, which had spurred our attempts to find other ligands for IL-22BP. But by also deleting *Il22* we could see that the protective effect of *Il22ra2* deletion was lost.
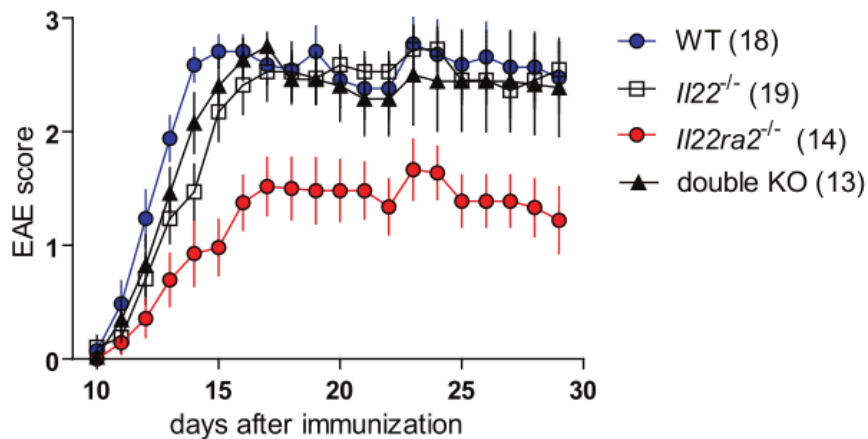
Figure 9. The protective effect of *Il22ra2*-deletion was lost in the absence of *Il22*.

Some inconsistencies are apparent when comparing the EAE disease course in the rat and mouse experiments in this paper as well as in previous papers. In paper I, the *Il22ra2*^{-/-} mice had a slightly earlier onset that was statistically significant. At peak of disease the two groups are equal but right after that, the knockout mice clearly have a better recovery. This disease course was consistently seen, and although only data from male mice are reported in the paper, the same pattern was seen in experiments using females. Two plausible explanations for the earlier onset are 1) IL-22 is often regarded as a pro-inflammatory cytokine and could therefore boost or accelerate immune cell priming and 2) IL-22 has been suggested to open the blood brain barrier in the setting of a human *in vitro* model, which could explain the earlier onset of paralysis. The question of the blood brain barrier was intriguing but the observed effect was deemed to be too small to be feasible to investigate further. We wanted to address the issue of immune cell priming by adoptive transfer EAE but had at that time difficulties setting up the model in the lab. However, in paper III the EAE disease course in the *Il22ra2*^{-/-} mice looks different. The onset is the same as wild type mice and the protective effect is apparent earlier, so that also the peak of disease is distinctly lower. The explanation for this difference is elusive but may involve subtle differences in several parameters such as: interbreeding with two other mouse strains with slightly different C57BL/6 background, an effect of the cre-enzyme, breeding has moved to a different room in the animal facility, or different MOG batch. Interestingly, the EAE disease course in these experiments is more similar to that seen in rats. Using the knockdown rat we see a dose-dependent effect on EAE severity and susceptibility, where 20 mg/l of doxycycline administered before immunization gives a disease course similar to the knockout mice and higher doses make the transgenic rats completely resistant. The reason for this dramatic effect in rats that likely still has some IL-22BP production intact when complete knockout in mouse gives a milder phenotype is intriguing. Several plausible explanations can be suggested for this but perhaps most importantly that constitutive knockout animals develop without the targeted gene from the gamete stage to adulthood and have during that time the possibility to compensate for the lost function in ways that are difficult to predict. Other plausible explanations include the use of a different species and a different immunization protocol. It is interesting to compare these experiments with those in the previous publication using the congenic strain, Dc1P. This is the rat strain that included a large number of genes from the protected PVG on DA background, which had lower *Il22ra2* expression and relatively mild EAE[246]. Compared to DA controls, disease course in Dc1P is characterized by a similar onset and then a trend for a lower severity during the peak of disease but significant difference only at the chronic stage of EAE, i.e. somewhat in between what we see in all the later experiments in mouse and rat.

4.4 IL-22BP FACILITATES IMMUNE CELL PRIMING

Background: IL-22 is implicated in the pathogenesis of several inflammatory diseases but its effect on B and T cell activation and phenotype is little studied.

Hypothesis: IL-22 influences the priming phase of an adaptive immune response.

Main results: Il22ra2 was highly expressed in naïve lymph node tissue. Upon immunization of rats with MOG in incomplete Freund's adjuvant, expression of *Il22ra2* decreased reaching nadir at day 7, which coincided with a peak in *Il22* expression. In the late stage of EAE, *Il22ra2* expression in spleen correlated with transcripts related to B cell function, suggesting that common pathways are involved.
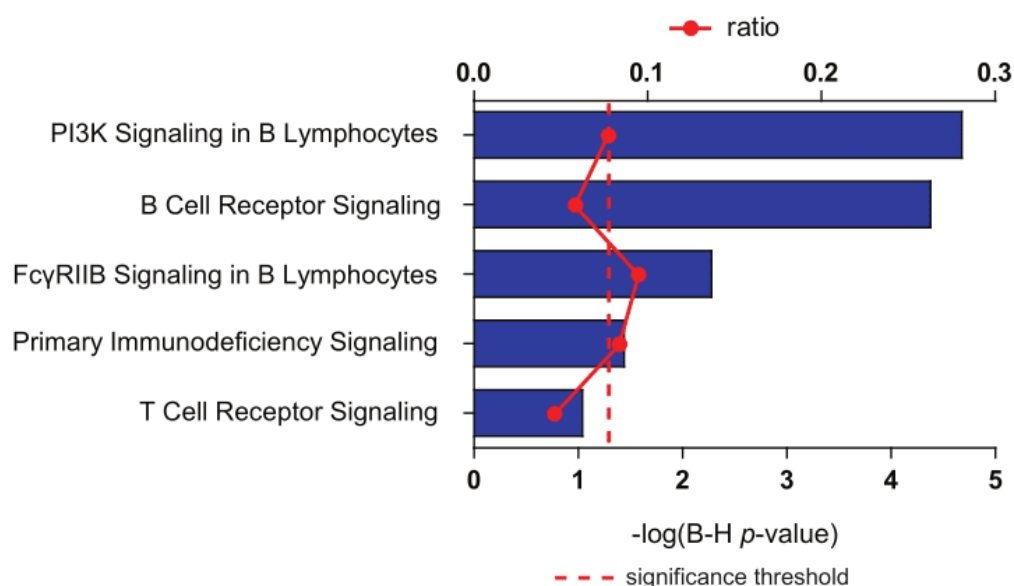

Figure 10. Using the Ingenuity Pathway Analysis (IPA) software, *Il22ra2* expression in rat splenic tissue from day 35 of EAE correlated with B cell related transcripts. *P*-values were calculated using Fisher's exact test with Benjamini-Hochberg correction for multiple testing.

Using the inducible *Il22ra2*-knockdown rat strain from paper III, we showed that reduced *Il22ra2* expression during immune cell priming resulted in smaller lymph nodes with a proportional reduction in B cell numbers. Expression of B cell-attracting *Cxcl13* and several other chemokines were reduced in naïve lymph nodes in rats after knockdown of *Il22ra2* expression. Injection of recombinant rat IL-22 subcutaneously during priming also reduced expression of lymph node *Cxcl13* in the draining lymph nodes. Both B and T cell effector functions were affected by reduced expression of *Il22ra2* during immune cell priming. There was a trend suggestive of less expression of activation markers CD80 and CD86 in lymph node B cells, and less MOG specific IgG2b in serum, both from day 7 after immunization. At

44

the same time point, we observed less IFNγ$^+$ T cells and more Foxp$^+$ regulatory T cells after *in vitro* restimulation of lymph node cells with MOG.
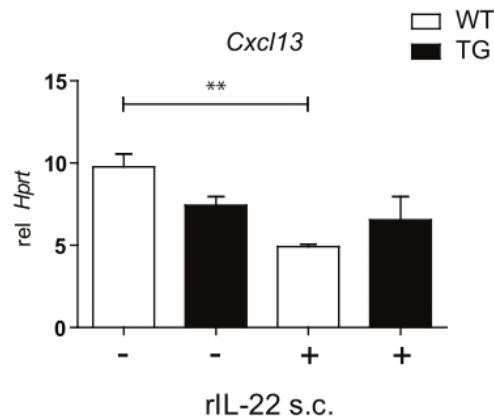

Figure 11. Knocking down *Il22ra2* expression before immunization with MOG lead to a reduction in *Cxcl13* expression day 7 after immunization. A similar effect was seen when recombinant rat IL-22 was injected subcutaneously at immunization and again day 4. *P*-values were calculated using one-way ANOVA and Tukey's post hoc test.
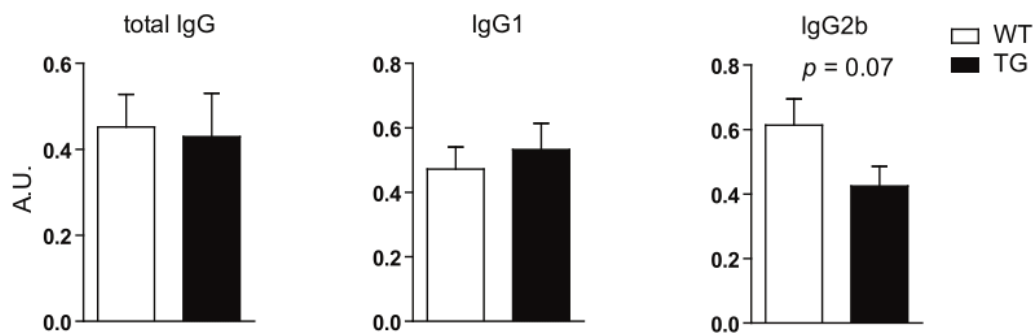

Figure 12. Serum anti-MOG antibodies day 7 after immunization showed a reduction specifically in IgG2b when *Il22ra2* expression was knocked down prior to immunization. *P*-values were calculated using unpaired Student's t test.
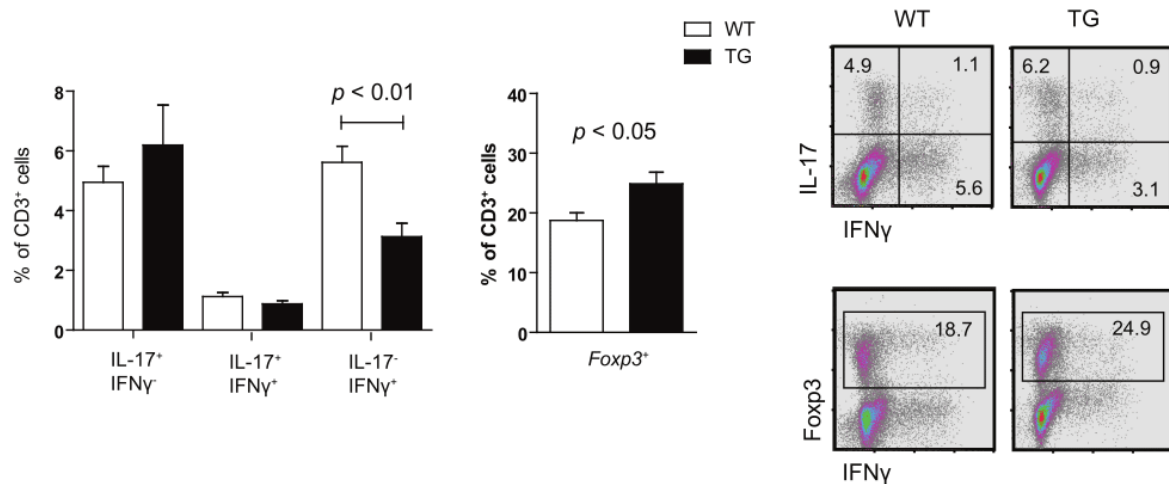
Figure 13. Lymph node T cells taken day 7 after immunization and restimulated with MOG *in vitro* expressed less IFNγ when *Il22ra2* expression has been knocked down prior to immunization. *P*-values were calculated using unpaired Student's t test.

One of the first things that struck us when starting our investigations of IL-22BP is that when comparing the tissue distribution in naïve rats and mice, *Il22ra2* expression is highest in lymph nodes, where we detect very little transcripts for *Il22* or IL-22R subunit *Il22ra1*. Upon immunization with MOG expression of *Il22ra2* decreases, which is mirrored by an increase in *Il22* expression. It seems that an intricate system is in play allowing for IL-22 signaling to occur during immune cell priming, which is controlled by IL-22BP. There was no literature available on IL-22 mediated effects in secondary lymphoid organs so to explore this we used a dataset produced earlier in the lab consisting of gene array results from spleen samples harvested from late stage EAE from 150 rats. Surprisingly, we found that the expression of *Il22ra2* in these samples correlated very distinctly with expression of B cell related genes. This seemed unrelated to the data we were getting using the knockout mouse as no striking differences had been seen in B cell numbers or serum antibody concentrations. Consequently, we decided to elaborate on this finding in a separate publication addressing the role of IL-22BP in the priming phase of an adaptive immune response.

5 CONCLUDING REMARKS

In this thesis, the roles of IL-22 in several inflammatory disease models have been addressed, for the most part indirectly by manipulating the endogenous antagonist molecule IL-22BP. The work was primarily motivated by MS-associated genomic variation close to its gene, *IL22RA2*. We have accumulated evidence that consistently suggest a disease-driving role for IL-22BP in autoimmune neuroinflammation, in three species:

- a congenic rat strain with a natural variant of *Il22ra2*, which results in lower expression of the gene, has less severe EAE (not included in this thesis),
- a transgenic mouse strain with a specific deletion of *Il22ra2* has less severe EAE,
- a transgenic rat strain with induced knockdown of *Il22ra2* has less severe EAE
- the protective allele of MS-associated SNP rs17066096 is associated with lower expression of *IL22RA2*, under specific circumstances *in vitro*.

We also show in mice that the effect is dependent on the presence of IL-22, suggesting a protective role for this cytokine in EAE and possibly also MS.

This is contrasted with psoriasis, where IL-22 directly promotes the cardinal manifestations of the disease. We confirm this in a mouse model of psoriasis and show that IL-22BP is an important regulator of skin inflammation. We could not link rs17066096 genotype to psoriasis susceptibility or severity, however.

We show that IL-22BP is present in the CSF of rats and humans and that the transcript is expressed in microglia in mice. Suggesting a possible role in the CNS in the case of MS. An alternative explanation, although not mutually exclusive, is the broad dampening effect on adaptive immune activation in peripheral lymph nodes that we have characterized in rat. We have recently detected *IL22RA1* expression on a subset of human T cells *ex vivo* and are currently investigating this further as a possible mechanism for the results reported in relation to EAE and MS. We find it plausible that IL-22 may act directly on T cells to diminish their encephalitogenic potential.

Relatively simple experiments that could have been done for this thesis but were not due to time constraints include:

- conditional knockout of *Il22ra2* using cre-lines specific for immune cells, myeloid cells, and microglia to locate the cellular source responsible for the effect seen in EAE,
- and conditional knockout of *Il22ra1* using the *Vav1*-cre strain, to test the wide-spread notion that IL-22 generally does no act directly on immune cells, as well as other cre-strains to narrow down the responding cell responsible for the effect of IL-22 seen in EAE.

We have established IL-22BP as a pathogenic factor in autoimmune neuroinflammation in rat and mouse, making it a potential therapeutic target for MS that warrants further investigation. Important outstanding questions include:

- What pathway or mechanism is involved in the protective effect of IL-22 on EAE.
- What would be the most appropriate strategy to achieve this effect pharmacologically, neutralizing IL-22BP antibody? IL-22 receptor agonist? Other targets downstream of the IL-22 receptor?

Another major question is: what are the downstream signaling effects of the different alleles of rs17066096? Also, rs17066096 may not be the causative SNP, but in strong LD with it. One can argue that, as the effects of IL-22BP are unraveled and more evidence is gathered regarding its role in MS and EAE, the importance of getting to the bottom of this question may lessen. However, as a basic science question it is tremendously important. Based on the data available today from the very ambitious project encyclopedia of DNA elements (ENCODE), rs17066096 does not appear to lie in close proximity to any known regulatory elements[281]. Understanding how rs17066096, or a linked SNP, influences the risk of getting MS could elucidate general mechanisms of value for investigations into the genetic risk factors of any disease.

6 ACKNOWLEDGEMENTS

Sent: den 7 januari 2009 21:49
Subject: forskningsprojekt

Hej!

Jag heter Hannes och är läkarstudent på
termin 9. Jag läste neurologikursen i höstas
och blev såld på ämnet. Skulle det vara
möjligt att ordna ett projekt åt mig i din
forskargrupp?

Vänliga hälsningar
Hannes

Subject: RE: forskningsprojekt
Date: Thu, 8 Jan 2009 08:35:03 +0100

Hej,

Går nog att ordna.

Kom gärna hit och besök oss.

Bästa hälsningar

Tomas

…and the rest is history.

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