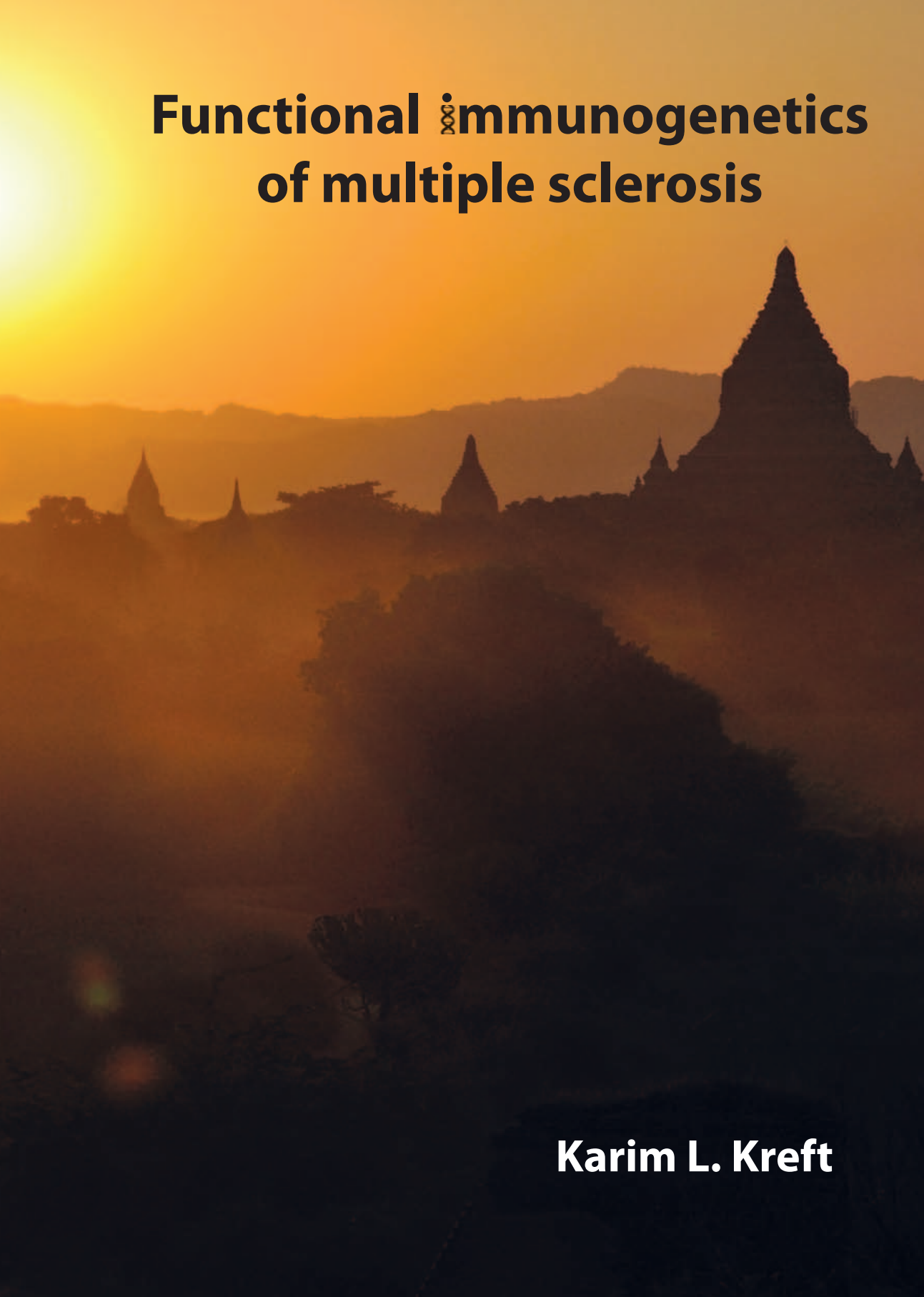
Functional immunogenetics of multiple sclerosis



Karim L. Kreft

Stellingen behorende bij het proefschrift
Functional immunogenetics of multiple sclerosis

1. De IL-7Rα signaleringsroute is in MS patiënten verschoven naar versterkte cellulaire responsen, die kunnen bijdragen aan het ontstaan van de ziekte (*dit proefschrift*)

2. CLEC16A verzorgt het endosomale transport en de regulatie van HLA klasse II expressie en heeft hiermee een niet-klassieke functie van C-type lectines (*dit proefschrift)*

3. De bijdrage van de MS risicopolymorfismen aan het ontstaan van een versterkte humorale immuniteit tegen het Epstein Barr virus eiwit EBNA-1 is beperkt (*dit proefschrift*)

4. De toegenomen expressie van het kinesine kif21b in de cortex van Alzheimer patiënten vergeleken met MS patiënten wordt grotendeels verklaard door de onverwacht hoge expressie van kif21b in astrocyten in Alzheimer (*dit proefschrift*)

5. Single nucleotide polymorphisms (SNP) met een minimaal effect op het risico om een ziekte te krijgen, kunnen grote biologische effecten hebben (*dit proefschrift*)

6. Het falen van verschillende clinical trials in MS met middelen gericht tegen de effecten van specifieke functionele T-helper cellen, geeft aan dat de kennis omtrent de immunopathologische mechanismen in MS beperkt en mogelijk bevooroordeeld is

7. Directe ex vivo karakterisering van T cellen uit inflammatoire milieus is klinisch relevanter dan het kwantificeren van weinig frequent aanwezige functionele T-cel subsets na in vitro polarisatie

8. Het merendeel van het klinisch wetenschappelijk bewijs is toepasbaar voor de minderheid van de patiënten

9. De aanzienlijke overlap van genetische associaties tussen verschillende autoimmuunziektes betekent niet zonder meer dat polymorfismen in een locus ten grondslag liggen aan basale mechanismen die betrokken zijn bij de ontwikkeling van autoimmuniteit

10. Het gebrek aan inzicht in de rol van antigeenpresentatie in MS staat in schril contrast met de ruim dertig jaar bekende en consistent robuust gerepliceerde HLA klasse II associaties met het risico om MS te ontwikkelen

11. Wetenschap is net als fotografie: het overzien van de grote lijnen zonder daarbij de details uit het oog te verliezen

Karim L. Kreft, Rotterdam, 8 januari 2014

Functional immunogenetics of multiple sclerosis

Functionele immunogenetica van multiple sclerose

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Functionele immunogenetica van multiple sclerose

Proefschrift

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Voor Peetje en Omi, die te vroeg heen gingen.

Chapter 1

General introduction

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Preface

Complex autoimmune diseases like multiple sclerosis (MS) result from many genetic factors with a relatively minor contribution interacting with several environmental factors.

It is estimated that the genetic component of MS contributes approximately 25-30% of the a priori risk to develop MS. For over three decades, the only genetic factor robustly associated with MS was carriership of certain HLA class II alleles, with odds ratios (OR) around 6 for homozygous risk carriers of HLA-DRB1*1501. From 2007 onwards, genome wide association studies (GWAS) have identified 57 single nucleotide polymorphisms (SNP) individually associated with a modestly increased risk (OR between 1.08-1.22) to develop MS.

GWAS currently have a limited role in clinical application, for example to predict whether or not a person will develop MS, for the prognosis of a MS patient regarding the development of sustained neurological deficits, or for individualized treatment. Nevertheless, GWAS are important to identify genes involved in the underlying immunopathogenesis of MS. Additionally, GWAS will increase our knowledge of general mechanisms contributing to the development of autoimmunity in general. Therefore, understanding the functional alterations caused by these SNP is pivotal to gain insight into how the genetic component contributes to MS risk. Therefore, functional (immuno)genetic studies are necessary and currently only a very limited number of immunogenetic studies in MS have been performed.

In this thesis, we set out to investigate functional consequences of the SNP in the interleukin 7 receptor alpha chain (IL-7Rα), C-type lectin 16A (CLEC16A) and kinesin family member 21b (kif21b). We selected these SNP of the earliest MS-associated SNP, because the IL-7Rα is important in T-cell biology and T-cells are major players in the immunopathogenesis of MS. CLEC16A was selected because it is associated with several autoimmune diseases (type I diabetes mellitus, celiac disease, primary biliary cirrhosis among others) and therefore likely to contribute to autoimmunity by a generalizable mechanism. Additionally, the function of CLEC16A is currently unknown. Kif21b was selected, because it is an important cellular transporter and therefore likely to be involved in the poorly understood neurodegenerative component of MS. In order to investigate whether these SNP alter the expression, localization, transport or function of the protein, we used functional (cellular) immunological and cell biological assays, both at the mRNA and protein level. The results obtained were subsequently stratified according to carriership of the SNP risk allele in both MS patients and healthy controls (HC). Moreover, we assessed whether MS patients have aberrancies in the underlying pathways compared with HC. Lastly, using all currently available MS risk SNP, we performed an unbiased screen to gain insight into which genes are involved in the aberrant humoral immune response against the Epstein Barr virus (EBV), which is a well-validated and important environmental risk factor for MS with an OR around 4.5.

The general introduction provides an overview of clinical, epidemiological and immunopathological findings in MS. The genetic contribution to MS and the function of all 57 MS risk SNP are discussed. Lastly, considerations for functional immunogenetic studies are reviewed.

Multiple sclerosis

Clinical aspects and diagnosis of MS

MS is a devastating neurological disease and the most common cause of disability among young adults. Most MS patients present with a clinically isolated syndrome (CIS), which is characterized by a subacute episode of neurological complaints lasting at least 24 hours and presumably caused by an inflammatory event in the central nervous system (CNS). The main locations of a CIS are the optic nerve, spinal cord and brainstem [1]. The clinical evaluation of a patient with a CIS consists of the assessment of a magnetic resonance imaging (MRI) scan, sometimes in parallel with a lumbar punction for cerebrospinal fluid (CSF) analysis, according to the McDonald criteria, currently with the 2010 revision [2]. Typical MRI lesions are found in the white matter of brain, mainly around the ventricles, juxta-cortical and infratentorial [3]. The lesions are mainly located around (small) blood vessels in the brain. Some lesions are enhancing upon gadolinium administration, which indicates that the functionality of the blood-brain barrier (BBB) is compromised. A patient fulfilling the MRI Barkhof criteria for MS has an approximately four-fold increased risk to develop MS [4]. Analysis of the CSF includes assessment of intrathecal immunoglobulin (Ig) production, visualized using iso-electric focusing and/or the calculation of the IgG-index. CIS patients who have oligoclonal bands have an approximately ten fold increased risk to develop MS [5]. The diagnosis of MS can be made conclusively when a patient presents with a second attack of neurological disability or with new lesions on MRI. Most MS patients have the relapsing remitting form of MS. This form is characterized by episodes of neurological complaints (relapses), which (partly) resolve, followed by periods with relatively normal neurological functioning (remission). The majority of patients with relapsing remitting MS (RRMS) will eventually progress to secondary progressive MS (SPMS), with accumulation of neurological disability over time and a decline in neurological function. The relapse frequency is much lower compared with the relapsing-remitting phase and relapses are often completely absent in the secondary progressive phase. On the other hand, a small proportion of patients is diagnosed with the primary progressive form of MS (PPMS) in which the disease is characterised by a progressive decline in neurological functioning from the onset of the disease without relapses. MS is a very heterogeneous disease, with some patients relatively mildly affected for many years while other patients have a very rapid progression. In addition, the number of relapses and the time to develop sustained neurological disabilities varies considerably between patients [6]. MRI abnormalities in MS do not correlate with the severity of the disease, a phenomenon called the clinico-radiological paradox. Currently, no good model to predict the severity of the disease course after a CIS is available.

Epidemiology of MS

Worldwide, MS affects approximately 2.5 million individuals. The incidence of MS approximately correlates with the distance from the equator, with the highest incidences in the Scandinavian countries, Canada and in Australia [7]. Even within Australia and New Zealand, a latitude gradient is

observed [8]. The prevalence of MS is increasing during the last decades from approximately 50 per 100.000 persons around 1950 to slightly above 100 per 100.000 persons in 2010. The increasing prevalence is especially observed in females [9]. Before puberty, the increase occurs mostly in both genders, but after puberty it is especially observed in women. The cause of this phenomenon is largely unknown, but hormonal factors might contribute [10]. Children born around May have a significantly increased risk to develop MS compared with children born around November [11]. Lastly, based on migration studies, the risk to develop MS depends on the place where a child grows up. Children born in low-risk areas migrating to high-risk areas have a higher chance to develop MS and vice versa [12, 13]. These facts point to the importance of environmental factors (including infections) in the development of MS or the interaction between the environment and MS-associated genes.

Pathology of MS

The three hallmarks of the pathology of MS are inflammation, demyelination and axonal damage. Additionally, astrogliosis is observed leading to scar formation (sclerosis) and also the development of atrophy in both the white and grey matter became more prominent with new MRI techniques [14].

Demyelination occurs mostly in areas around small blood vessels in the white matter of the CNS. Myelin is important for the protection of the axon and for the velocity of nerve conduction, eg. to accelerate action potentials arising from the neuron to the target cell. Demyelinating white matter (WM) lesions in MS are heterogeneous, but generally speaking are all inflammatory [15]. In demyelinating lesions, many types of immune cells and mediators are found. Their relative contribution depends on the type of lesion. There are claims that CD8+ cytotoxic T-cells outnumber CD4+ T-helper cells [16, 17]. Some lesions contain immunoglobulin and complement depositions. In addition, many activated microglia cells (the macrophages of the brain) and invading macrophages are found, some containing myelin debris with a foamy morphology [18]. The classification of lesions in the white matter is therefore based on characteristics regarding demyelination and the number of the infiltrating inflammatory cells and the level of HLA-II expression. This classification represents roughly the chronological development of demyelination, leading to scar formation by reactive astrocytes in areas with extensive astrogliosis.

In demyelinated areas, the axon is poorly or not protected and the signal transduction may be impaired. Axonal pathology is also observed, especially in active and chronic active MS lesions, with immune cells in close proximity to damaged axons [19-21]. In a histopathological study, the number of amyloid precursor protein (APP, a marker for axonal damage) positive axons in remyelinated lesions correlated with the number of macrophages and $CD8^+$ T-cells [22]. Additionally, axonal loss and neurodegeneration are observed already early in the disease course and are likely involved in the development of neurological impairment. Axonal pathology might result from demyelination by the lack of trophic and structural support, ion and energy imbalance and/or immune-mediated damage [23], but it should be noted that axonal damage might at least partially be independent of demyelination.

Some regeneration in the CNS of MS patients takes place, for example remyelination of axons and redistribution of sodium channels along the axons [24]. However, these repair mechanisms are only partial and cannot completely compensate for the existing damage. It is currently thought that the accumulation of axonal loss with only partial CNS recovery eventually leads to the development of SPMS.

In addition to the hallmarks of MS pathology, inflammation, demyelination and axonal degeneration, atrophy is another important factor, contributing to the development of neurological decline. Atrophy is already occurring very early in the disease course. In CIS patients, atrophy is already observed [25]. The amount of atrophy also correlates with the MS functional composite (MSFC), a multidimensional scoring system to measure disability and cognitive function [26, 27].

MS is classically thought to be a disease of the white matter (WM) of the brain, the part of the CNS with the most myelin expression. However, recent insights into grey matter (GM) pathology have emerged. The amount of GM demyelination and atrophy may even be more extensive than WM demyelination [28, 29]. From post-mortem studies, it appeared that GM demyelination was mostly non-inflammatory, because of the relative absence of immune cells in these lesions. However, recently it was shown in a biopsy study that in the early phase of MS, also GM pathology is inflammatory. In early GM demyelination, abundant numbers of T-cells and activated microglia cells or macrophages were found [30]. Hypothetically, GM demyelination is initiated by an inflammatory insult, which disappears over time, while the inflammatory component of white matter demyelination is more continuous. Furthermore, post-mortem assessment of the meninges of MS patients revealed follicle-like structures containing numerous B-cells. The presence of these B-cell follicles, which can be regarded as tertiary lymphoid structures, correlates with accelerated disease progression and the development of sustained disability. These follicle-like structures resemble germinal centers, including dividing B-cells [28]. In the pathology of the GM, also neuronal loss is observed, even in non-demyelinated areas in the cortex. This neuronal loss might be a consequence of axonal pathology, local meningeal inflammation or the production of soluble immune mediator in these B-cell follicles, chronic microglia activation or mitochondrial dysfunctioning [31, 32]. As GM demyelination in port-mortem studies is generally non-inflammatory, the current classification of GM lesions is based on the localisation of the demyelinating lesions rather than on pathological characteristics of the lesions as used in the classification of WM lesions (Table 1).

Table 1. Characteristics of white and grey demyelinating lesions used in this thesis

White matter			Characteristic [33, 34]		Grey matter	Characteristic [34, 35]
		HLA-DR	Acid phosphatase	Oil Red O		
NAWM		-	-	-	Type I	Mixed grey and white matter lesion
Pre-active lesion		++	+	-	Type II	Lesion solely in grey matter, mostly small lesions
Active lesion		+++	++	+++	Type III	Subpial demyelinating lesion
Chronic active lesion	Hypercellular rim	+++	++	+++	Type IV	Grey matter demyelinating lesion stopping exactly at the border of the cortex and the white matter
	Hypo-cellular rim	+	+	+/-		
Chronic inactive		+	+/-	-		

Immunology

The current paradigm for the role of immunity in MS is that (auto-reactive) immune cells are triggered in the periphery, subsequently migrating across different tissue barriers to access the CNS. In the brain parenchyma, they re-encounter antigen presenting cells (APC), presenting currently unknown self peptides to immune cells. These APC express costimulatory molecules, reactivating the T-cells, which are subsequently causing damage to the myelin sheaths around the axons [36]. A variety of leukocyte subsets has been implied in the disease course of MS. For many decades, CD4+ T-helper cells were implicated to mediate the CNS damage. Until the last decade of the previous century, mainly the IFN-γ producing Th1-cells were suspected to be involved in the pathogenesis of MS. With the identification of IL-17 producing Th17-cells, this new subset was implied to contribute to the pathogenesis of MS [37]. Recent insights showed that Th17 cells are important for the initiation of the disease through the entry into the CNS via the choroid plexus, whereas Th1 cells might be more important for the continuation of experimental autoimmune encephalomyelitis (EAE) [38]. However, this concept has been challenged with the demonstration that Th1 cells are found earlier in the CNS in EAE [39]. In MS, several reports suggest that the Th17 pathway is important in the pathogenesis [40]. However a phase II clinical trial using Ustekinumab, a monoclonal antibody against the p40 subunit of IL-12 and IL-23 inhibiting both Th1 and Th17 cells, failed to show efficacy in MS patients [41]. In contrast with the abundance of CD4 T-cell research in MS, relatively little attention is given to CD8+ cytotoxic T-cells. Clonally expanded CD8+ T-cells are found in CSF [42] and MS lesions [43], containing granules with cytotoxic substances like granzymes polarised towards the axons. These cells are found in areas with damaged myelin sheaths [44]. Several studies have investigated whether autoreactive T-cells are present in MS patients. In a subgroup of patients, T-cells reactive against myelin components, like

myelin oligodendrocyte glycoprotein (MOG) [45], myelin proteolipid protein (PLP), myelin basic protein (MBP) [46] and myelin-associated glycoprotein (MAG) [47] have been found. However, in healthy controls, these T-cells have also been described and therefore are likely part of the normal immune repertoire [48]. It might be that these potentially auto-reactive T-cells are kept under control by regulatory T-cells in healthy persons, while this process fails in MS patients.

Alternative to aberrant effector T-cell activation, failure of the inhibiting function of regulatory CD4+ T-cells might contribute to the pathogenesis of MS [49]. Recently, also CD8+ regulatory T-cells have been described. In addition to the role of T-cells in MS immunopathogenesis, also B-cells and macrophages are implicated. Macrophages phagocytose myelin debris, becoming foamy macrophages with anti-inflammatory properties [18]. These foamy macrophages might also be important for tissue repair. Also microglia have tissue repairing capacities. Alternatively, activated microglia may be involved in tissue destruction in the CNS and thereby contributing to neurodegeneration [50]. B-cells have for long been neglected in MS. One of the first diagnostic tools for MS was the assessment of CSF for the presence of oligoclonal IgG bands. These oligoclonal bands (OCB) is an indication that immunoglobulins are produced intrathecally. Moreover, the IgG-index can be calculated. This is the ratio between (concentration IgG CSF/ concentration IgG serum)/ (concentration albumin CSF/ concentration albumin serum) and a raised index is also indicative intrathecal IgG synthesis [51]. More recently, using molecular and sequencing techniques, it was shown that B-cells and plasma cells are clonally expanded in MS patients and that these clonally expanded B-cells are producing the OCB [52, 53] . Interestingly, a recent phase II clinical trial using Rituximab, a monoclonal depleting antibody against CD20, a B-cell specific marker, showed a spectacular and rapid reduction of disease activity, both clinically and on MRI [53]. The levels of immunoglobulins remained stable, indicating that the production of immunoglobulins by B-cells and plasma cells are not the most important contribution of these cells to the immunopathogenesis in MS. Subsequent functional studies showed that the main effect of B-cell depletion was due to decreased expression of lymphotoxin and tumour necrosis factor (TNF) alpha. Both CD4 and CD8 T-cells from patients who received Rituximab were almost completely anergic, also for antigenic-independent stimuli [54]. Additionally, also the presence of autoantibodies has been investigated in MS patients. Some studies have found antibodies directed against myelin components, mainly anti-MOG antibodies, and that the presence of these antibodies predicts conversion from CIS to MS [55]. However subsequent studies failed to validate this study [56]. Additionally, several studies have shown that the recognition and binding of these antibodies differed between experimental procedures (Western Blotting, ELISA or cell lines expressing MOG) [57, 58]. Additionally, also in paediatric MS anti-MOG antibodies were observed in a subset of children and the titers of these antibodies fluctuated during the disease course [59]. In a very recent and elegant study, KIR4.1 was found to be a target of serum IgG of MS patients. Anti-KIR4.1 antibodies were found in approximately half of the MS patients and in none of the healthy controls. Injection of these antibodies into wild-type mice induced neuropathology on glia cells and complement activation [60]. Currently, these findings await independent validation.

Current and developing therapies

Currently, in addition to symptomatic treatment, four disease-modifying treatments are available. The oldest chronic treatment is the use of IFN-β1a. A dozen mechanisms of action on its effects in MS have been proposed [61]. Glatiramer acetate is a homologue to MBP and the mechanism of action is thought to arise from immunomodulatory properties [62]. Both treatments have been shown to be safe and relatively effective, reducing the relapse rate by approximately 30%. More recently, Natalizumab was approved. This monoclonal blocking antibody against VLA-4 prevents mononuclear cells migrating from the blood into the CNS. This inhibition reduces CNS tissue damage. Natalizumab reduces the clinical relapse rate by 68% [63]. The major drawback of Natalizumab is the increased risk to develop progressive multifocal leucoencephalopathy (PML) [64], which is around 4 per 1.000 patients after two years of treatment [65]. PML is caused by the JC polyoma virus and this opportunistic infection is most probably caused by decreased immune surveillance by T-cells. Lastly, the first oral drug in MS, Fingolimod was approved. This is an antagonist of the S1P receptor family. Blocking of S1PR prevents lymphocytes from leaving the secondary lymphoid organs, thereby limiting their migration to the CNS. Two fatal infections during this treatment have been observed thus far, both caused by herpes simplex infections [66].

New drugs under investigation include several B-cell depleting agents (monoclonal depleting antibodies against CD20, a pan B-cell marker), monoclonal antibodies against CD25, the IL-2Rα (Daclizumab), against CD52 (Alemtuzumab) and Fumarates. Currently, no treatment strategies to limit neurodegeneration exist. All drugs currently being investigated are targeting the inflammatory component of the disease. Developing strategies to slow down the progression rate or halt the development of irreversible neurological damage are necessary to limit neurodegeneration.

Etiology

Environmental factors, infections and genetic predisposition contribute to the risk to develop MS. Environmental factors which are implicated MS etiology include low levels of vitamin D in peripheral blood, and smoking. The observed differences in the risk to develop MS stratified according to month of birth might reflect differences in levels of ultraviolet B exposure and thereby levels of vitamin D during pregnancy [67]. Numerous infections and pathogens have been implicated in the etiology of MS. Currently, the association between MS and Epstein Barr virus (EBV) is well validated. From epidemiological studies, a history of infectious mononucleosis (IM) increases the risk to develop MS two-fold and this appears to be one of the strongest risk factors for the development of MS [68]. However, the exact role of EBV in the immunopathogenesis of MS is currently unknown. Additionally, the risk to develop an MS relapse is approximately two-fold increased during or until five weeks after an upper respiratory tract infection [69]. Multiple mechanisms might contribute to this phenomenon, one is bystander immune activation of autoreactive T-cells. In a Lewis rat EAE model, autoreactive T-cells residing transiently in the lung changed their gene-expression, and were subsequently reactivated. Additionally, these T-cells upregulated important chemokine and adhesion receptors

and subsequently migrated to the CNS [70]. The current tendency that the incidence of MS is increasing during the last decades, underscores the importance of environmental factors, as this rapid increase cannot be explained by changes in genetics. The complex interplay between environmental factors in a genetically susceptible individual will probably result in the development of MS.

Genetics

Since half a century, the importance of genetic predisposition to the risk to develop MS is known, but the mechanisms are poorly known. The first line of evidence for a role of genes in the risk to develop MS came from twin and family studies, although it should be emphasised that MS is not a classical heritable disease. The life-time risk to develop MS in the Western World is around 1 per 1.000. If a person has a first degree relative with MS, the chance to develop MS increases to 2 per 1.000, whereas in a monozygotic twin the unaffected twin has a chance of ~30% to develop MS [7]. Based on these studies, it is expected that maximally 30% of the disease variance can be explained by genetic associations, whereas the remaining risk might be explained environmental factors, infectious agents and interactions between factors.

For several decades, the identification of genes involved in MS was restricted to HLA class II alleles, especially the HLA-DRB1*1501. This haplotype is carried by approximately 30% of the Caucasian MS patients and around 12% in the general non-affected population. Although multiple HLA risk associations for MS have been established during several decades, the mechanisms by which these HLA alleles attribute to the development of MS remains largely unknown. It should be noted that having the HLA risk alleles is neither necessary nor sufficient to develop MS [71]. Importantly, only a part of individuals with certain HLA-alleles will develop MS. Additionally, also some HLA class I alleles are associated with MS risk. The combination of different HLA alleles can enhance or decrease the risk to develop MS. This phenomenon is called epistasis [72].

Recent major advances in the understanding of the genetic complexity of MS came from GWAS, which identified SNP associated with a modestly increased risk to develop MS.

Genome wide association studies

A SNP is change of one nucleotide at a certain position on a chromosome. It occurs approximately every 1.250 base pairs and some regions in the genome contain more SNP than others. In the current version (build 138) of the dbSNP database (www.ncbi.nlm.nih.gov/projects/SNP/), over 62 million SNP in the human genome have been described, of which approximately 44 million are validated. A single diploid human genome contains between three to four million SNP [73, 74]. The substitute nucleotide has to be prevalent in at least 5% of a homogeneous population. The frequency of a given SNP is tested in a disease population and compared with a healthy control group, known as the common disease-common variant hypothesis. This hypothesis states that complex polygenic diseases are largely due to common genetic variants with small to modest odds ratios [75].

Table 2. Overview genome wide association studies in MS

Number cases/ controls and population	Newly found genes	Newly suspected genes *	Validated genes	Non-validated genes	Year	Study design	Reference
6942/5418	IL-2Rα IL-7Rα	CLEC16A RPL5 DBC1 CD58	N/A	N/A	2007	GWAS	[77]
3908/3204	IL-7Rα	N/A	IL-7Rα	N/A	2007	Genomic convergence	[78]
2450/2634	IL-7Rα	N/A	IL-7Rα	N/A	2007	Candidate gene	[79]
975/1500		SLC4A5 FLJ10902 FLJ10204 INPP5A LRP5 ZNF45 GIPR SAP102	IL-7Rα	Not mentioned	2007	GWAS***	[80]
2679/3125	kif1b	None	Not mentioned	Not mentioned	2008	GWAS	[81]
617/617	None	None	Not mentioned	Not mentioned	2008	GWAS	[82]****
3507/8204 (931 trios)	kif21B TMEM39A C16orf75 PRM1	N/A	CLEC16A CD58 IRF8 MMEL1 (unpublished data)	N/A	2009	Validation and candidate study	[83]
3874/5723		METTL1 CYP27B1 CD40	CD58 EVI5-RPL5 IL2RA CLEC16A IL-7Rα TYK2	Kif1b CD226		GWAS	[84]
978/883	PTPRU PARK2 RDH10 JRKL RPS29	C20orf46 GPC5 PDZRN4 CSMD1 SLC25A36 SH3GL2 EN1 MGC45800 ZIC1 DDEF2 TRIB2	CD58 IL-7Rα IL2RA CLEC16A	None	2009	GWAS	[85]
5213/4453 (2053 trio families)	Tyk2	IFI30 FCRL3 SLC4A5	IL-7Rα	N/A	2009	Candidate	[86]
4839/9336	TNFRSF1A CD6 ICSBP1/IRF8	IL12A OLIG3/TNFAIP3 PTGER4 RGS1 IL12B BCL2 NEDDL4 PPA2 STAT3	IL-7Rα IL-2Rα CLEC16A CD58	kif1b (not assessed in replication study)	2009	Meta-analysis**	[87]
3454/3416	IL7 SOCS1	PRKCE BCL2	Tyk2	N/A	2010	Candidate	[88]
2657/2877	CBLB	N/A	Not mentioned	Not mentioned	2010	GWAS	[89]
592/825	None	VAV2 ZNF433	None	None	2010	GWAS	[90]
4638/10275	STAT3	N/A	Not mentioned	Not mentioned	2010	GWAS	[91]
8439/9280 (608 trios)	N/A	N/A	TNFRSF1A IRF8 CD6 (borderline)	Not mentioned	2012	Validation study	[92]
9772/17376	29 genes	N/A	23 genes	Not mentioned	2012	GWAS	[93]
8499/8765 (958 trios)	N/A	N/A	5 genes from previous GWAS with sub-genome wide significance	N/A	2013	Candidate study; meta-analysis	[94]

The HLA class II associations consistently replicated are not indicated. Only candidate gene studies that have subsequently been replicated in a GWAS were taken into account.

* The classification of a gene as suspected based on the observed p-value differs between studies since the definition used by authors varies.

** Suspected genes were initially assessed given their association with other inflammatory and autoimmune diseases.

*** Non-synonymous SNP (nsSNP) tested with a Cochran-Armitage Trend test with significant p-value $<10^{-3}$

**** Underpowered study

N/A, not applicable

Genome wide association studies (GWAS) are large efforts, including tens of thousands of patients and controls. It should be noted that currently, only approximately 20% of the variance in the human genome is assessed in GWAS. On a small chip, up to one to two million single nucleotide polymorphisms (SNP) can be assessed in one experimental run. Given the fact that an enormous number of SNP is tested simultaneously, the chance of a false positive association is high. Therefore, a p-value of approximately $5*10^{-7}$ is generally used as a cut-off for statistical significance [76]. The use of a validation cohort is now generally required to ascertain that a SNP has a true association with a disease.

The first GWAS on MS was published in 2007. Two genes were found to be significantly associated with a modest risk to develop MS and several others showed a trend towards significance. The majority of genes were immune related. Subsequently, nine GWAS were performed and these studies increased the number of SNP associated with MS. Consecutive GWAS validated most SNP from previous GWAS (Table 2). In 2011, the largest GWAS for MS was published. It validated 88% of the previous associations and found 29 new SNP associated with an increased risk to develop MS. It should be noted that an individual SNP contributes very modestly to the increased risk to develop MS (OR around 1.1-1.2), compared with the HLA-II associations (OR around 6). Functional insights into the mechanisms how these polymorphisms affect the risk to develop MS and how they would be involved in the (immuno)pathogenesis of MS is currently largely lacking.

Lessons learned from GWAS

Most of the SNP associated with an increased risk to develop MS in the largest GWAS to date, are genes involved in immune development, functioning and signalling. Some genes have several different functions within the immune system (Table 3, Figure 1).

Lymphoid and myeloid cell development

SNP associated with an increased risk to develop MS are found are found in genes important in leukocyte precursor cells. In the bone marrow, multiple polymorphisms are involved in lymphoid and myeloid cell development. For example, MYB is an important regulator of haematopoiesis, especially for myeloid cells, and indispensable for yolk sack myeloid cells like microglia cells [97]. Moreover, HHEX is important for the development of B-cells [98]. Interestingly, polymorphisms are also found in both IL-7, expressed by stromal cells in the bone marrow, and the IL-7Rα expressed on developing lymphoid cells. Signalling via the IL-7/ IL-7Rα axis in the large pre-B-cell stage is important for the rearrangement of the immunoglobulin κ light chain. Defects in this pathway lead to immunodeficiency, especially to severe combined immunodeficiency (SCID). Moreover, also for the development of mature T-lymphocytes signalling via this pathway is important. In the thymus, IL-7 is expressed by stromal cells. The early T-cell lineage progenitors (ETP) derived from the bone marrow migrate to the thymus, where they mature into the double negative stage of thymocytes. The IL-7Rα is expressed on these cells and is important for the rearrangement of the T-cell receptor (TCR). Thymocytes downregulate the IL-7Rα in the double positive

state and when they mature into naive T-cells, they re-express the IL-7Rα [99]. The thymus is an important organ for the selection of non-autoreactive T-cells, as well as the induction of anergy in auto-reactive T-cells. THEMIS, another MS susceptibility gene, is important for late thymocyte development and is also involved in the positive selection of T-cells [100]. It is thus tempting to speculate that aberrancies in the development of lymphoid cells caused by these polymorphisms are important for early development of autoreactive T- and B-cells contributing to the development of CNS autoimmunity.

Table 3. Multiple sclerosis susceptibility genes

Function	Susceptibility genes
Neurons	MMEL1, kif21b*, TAGAP, ZNF746, GALC, SOX8
Vitamin D metabolism	CYP27B1, CYP21A1
Ligands	CD58
Receptors	MERTK, PTGER4
Adhesion molecules	VCAM1, CD6
Signalling	RGS1, PTGER4, CBLB, TYK2, MALT1, MAPK1, RPS6KB1, DKKL1****, PLEK, MERTK, BACH2
Cytokines and and their receptors	IL12A******, IL12B******, IL7, IL-2Rα, CXCR5, TNFRSF1a, IL-7Rα, IL22Ra**, TNFRSF6b
Transport molecules	EVI5, ARL61P4, kif21b
Transcription factors	EOMES, NFKB1, MYC, HHEX, ZFP36L1, BATF, STAT3, MYB, ZMIZ1, IRF8, SOX8, MAPK1
Transcriptional activity	BACH2, ZMIZ1, ZFP36L1, BATF, MYB, MYC, ZBTB46*****
Costimulation	CD40, CD86, CLECL1, TNFSF14
Cell cycle	MERTK, RPS6KB1
Apoptosis	BACH2, MYC, SOX8, ZFP31C1, ZNF36, TNFRSF6B***
Proteolysis/ lysosomes	MALT, ZNF46, MANBA*****
Phagocytosis	MERTK
Endosomal maturation	CLEC16A
Autophagy	CLEC16A
Mitochondrion	SCO2
Lymphoid development	THEMIS
Cytoskeleton remodelling	PLEK
Unknown	PVT1, MPHOSPH9, MPV17L2, DKKL1, SP140, TMEM39A

* The susceptibility SNP (rs12122721) that has been assigned to this gene, actually lies nearby the open reading frame (C1orf106).
** IL-22Ra is a receptor antagonist
*** Counteracts TNFSF14 and FASL (CD154) mediated cell death
**** Inhibitor of WNT signalling
***** rs228614 attributed to NF-kB1 and MANBA; rs6062314 attributed to TNFRF6B and ZBTB46 [95, 96]
****** IL12A also known as IL12p35 and IL12B as IL12p40

Immune cells in the periphery

Mature naive T-cells that have left the thymus are subsequently circulating in peripheral blood and are entering the secondary lymphoid tissues. Here, T-cells might encounter antigen presenting cells (APC) like monocytes, macrophages and dendritic cells (DC). In the immune system, ZBTB46 also known as BTBD4, is a transcription factor specific for conventional DC, not expressed in other myeloid or lymphoid cells [101]. In the interaction between APC and T-cells, multiple genes are associated with an increased risk to develop MS. Firstly, there is the classical genetic association between HLA-II molecules and MS. These HLA-II molecules are important for the presentation of antigens to the T-cell receptor. Secondly, multiple co-stimulatory molecules like CD40 and CD86 are able to give a second signal. Interaction between CD86 on an APC and CD28 on the T-cells is important for T-cell activation. The interaction between CD40L (also known as CD154) on T-cells and CD40 on B-cells is an important factor for B-cell growth, differentiation, isotype switching of immunoglobulins and cytokine production, which is in turn able to influence T-cell polarisation and function. Moreover, APC are able to produce IL-12, which is a T helper-1 (Th1) polarizing cytokine. Recently, it was shown in experimental autoimmune encephalomyelitis (EAE, the animal model of MS) that IL-17 producing T helper cells, named Th17 cells, are thought to initiate break down and damage to the blood brain barrier, after which Th1 cells are thought to sustain the damage to the CNS [38]. Interestingly, the MS associated risk gene STAT3 is an important transcription factor for Th17 cells [102]. CLECL1, expressed on B-cells and APC, is C-type lectin like molecule involved in both co-stimulation to T-cells and in the polarization of T-cells into IL-4 producing T helper-2 (Th2) cells [103]. Moreover, it phosphorylates MAP kinase (MAPK1) and is involved in enhancing HLA-DR expression without affecting the expression of co-stimulatory molecules indicating that it might have a regulatory role in immunity [104]. Anti-inflammatory regulatory T-cells, named Treg, are implicated in the immunopathogenesis of MS. One of the main features of Treg is the high expression of the IL-2Rα (CD25) and low expression of the IL-7Rα (CD127), both genetically implicated in MS. The balance between different types of T helper subsets is probably very important for the balance of tolerance and autoimmunity [105]. One of the other interesting pathways important in this balance is the IL-7/IL-7Rα axis. IL-7 is expressed in secondary lymphoid tissues by fibroblastic reticular cells [99]. For T-cells in the periphery, signalling via the surface bound IL-7Rα is important for the formation of immunological memory and homeostatic proliferation of potential auto reactive T-cells. After activation of T-cells by an APC, the T-cells start to proliferate and differentiate into effector or memory cells. In this process CD58, another risk gene associated with MS, is upregulated. CD58 is also known as lymphocyte function-associated antigen 3 (LFA-3), which is an activation and memory marker for T-cells [106].

Lymphoid cells in the CNS

After activation of the T-cells in the periphery, activated T-cells are expressing very late antigen 4, VLA-4, enabling themselves to bind to vascular cell adhesion molecule 1 (VCAM-1), another gene associated with MS. After binding of VLA-4 to VCAM-1, which is expressed on endothelial cells in the brain, T-cells are able to migrate over the blood-brain barrier into the CNS. Noteworthy, Natalizumab (Tysabri) is an

effective and approved treatment for MS and this monoclonal blocking antibody is directed against VLA-4 [107]. In the CNS, the activated T-cells encounter APC and might be re-activated, after which they start to clonally expand and cause damage to the myelin sheets. Recently, it was shown that this mechanism is also applicable to CD8 T-cells [108]. Although much of the research on the immunology of MS has focused on CD4+ T-helper cells, it has been noticed for already a long time that CD8+ cytotoxic T-cells outnumber CD4+ T-helper cells in MS brains. In addition, it was shown that CD8 T-cells are oligoclonally expanded in the brain of MS patients. Interestingly, EOMES was found in the recent GWAS. EOMES is the essential transcription factor for the effector function of CD8 T-cells [109]. Moreover, in CD4+ T-helper cells, EOMES induces IFN-γ responses and suppresses IL-17 production by binding to the RORγC and IL-17 promoters.

Recently, in post-mortem tissue the existence of B-cell follicles in the meninges of MS patients was shown. These follicles are specialized tertiary lymphoid tissue, consisting of follicular helper T-cells (Tfh), B- and plasma cells [28]. Tfh have high expression of the chemokine receptor CXCR5 [110], another candidate gene for MS. Interestingly, B-cells are able to produce CXCL13, the ligand for CXCR5. In addition, it was shown that Tfh are expressing the tumour necrosis factor (TNF) family member 14 (TNFSF14, also known as LIGHT), another MS susceptibility gene. LIGHT and lymphotoxin (LT) binds to the lymphotoxin beta receptor (LTβR) which is an important factor for the formation of lymphoid follicles [111]. B- and plasma cells are the source of oligoclonal bands and the raised IgG-index, often observed in MS and commonly used as diagnostic tools for MS. Moreover, B-cells are capable to present antigens to T-cells and might thus serve as antigen-presenting cells in the brain to re-activated T-cells, entering the brain after peripheral activation. Noteworthy, a recent phase II trial with Rituximab, a monoclonal depleting antibody against CD20, a pan B-cell marker was an effective treatment for MS compared with placebo [53]. Validation of these results in phase III trials is awaiting. Moreover, it was shown that the working mechanism of action of Rituximab is probably due to depletion of B-cells and thereby inhibiting T-cell responses [54, 112].

Immune signalling

In multiple pathways and signalling cascades, MS risk genes are found. For example in cytokine signalling, risk genes influence both receptors and ligands, and also adaptor proteins like STAT3 and TYK2. In APC, multiple risk genes are involved in signalling, for example MERTK. This is a tyrosine kinase receptor, which mediates phagocytosis of apoptotic materials, and down regulates the activation of macrophages. Moreover, it is involved in homeostatic regulation of APC and is able to induce a tolerogenic phenotype in DC via blockade of NF-κB, one of the MS risk genes [113]. Moreover, after the bindings of its ligand protein S and GAS6, MERTK auto phosphorylates and induces the phosphorylation of MAPK1 and MAPK2 among others. MAPK1 is another MS risk gene. MAPK1 is involved in the regulation of the cell cycle [114]. Moreover, it is a suppressor of gene transcription, including IFN-γ inducible genes. Lastly, MAPK1 phosphorylates and regulates CIITA, the master transcription factor for HLA class II expression and functioning [115]. CBL-B belongs to the family of Cbl, which are molecular

adaptors playing a key role in the regulation of tyrosine kinase-dependent signalling. CBL-B is described to inactivate NF-κB. Moreover, it inactivates T-cells until the second signal for activation (the first signal is HLA-TCR interaction, the second signal co-stimulation) is received [116]. The combination of these two mechanisms, provides reduction of T-cell activation. Moreover, it also reduces B-cell functioning by negatively regulating B-cell proliferation [117].

Linking MS risk genes with environmental risk factors

From epidemiological studies, it is well known that the aetiology of MS probably consists of a combination of genetic susceptibility and environmental triggers. Infections are a well-known risk factor to develop MS. One of the strongest risk factors for the development of MS is a history of infectious mononucleosis (IM). Several studies have linked Epstein Barr virus (EBV) to MS [118]. Recently, it was demonstrated that gut microbiota in EAE are important for the development of dysfunction in mice [119]. The SNP in IL-22RA is interesting, since IL-22 is important in the promotion of antimicrobial immunity, but also in the regulation of inflammation and tissue repair in the mucosal environment [120].

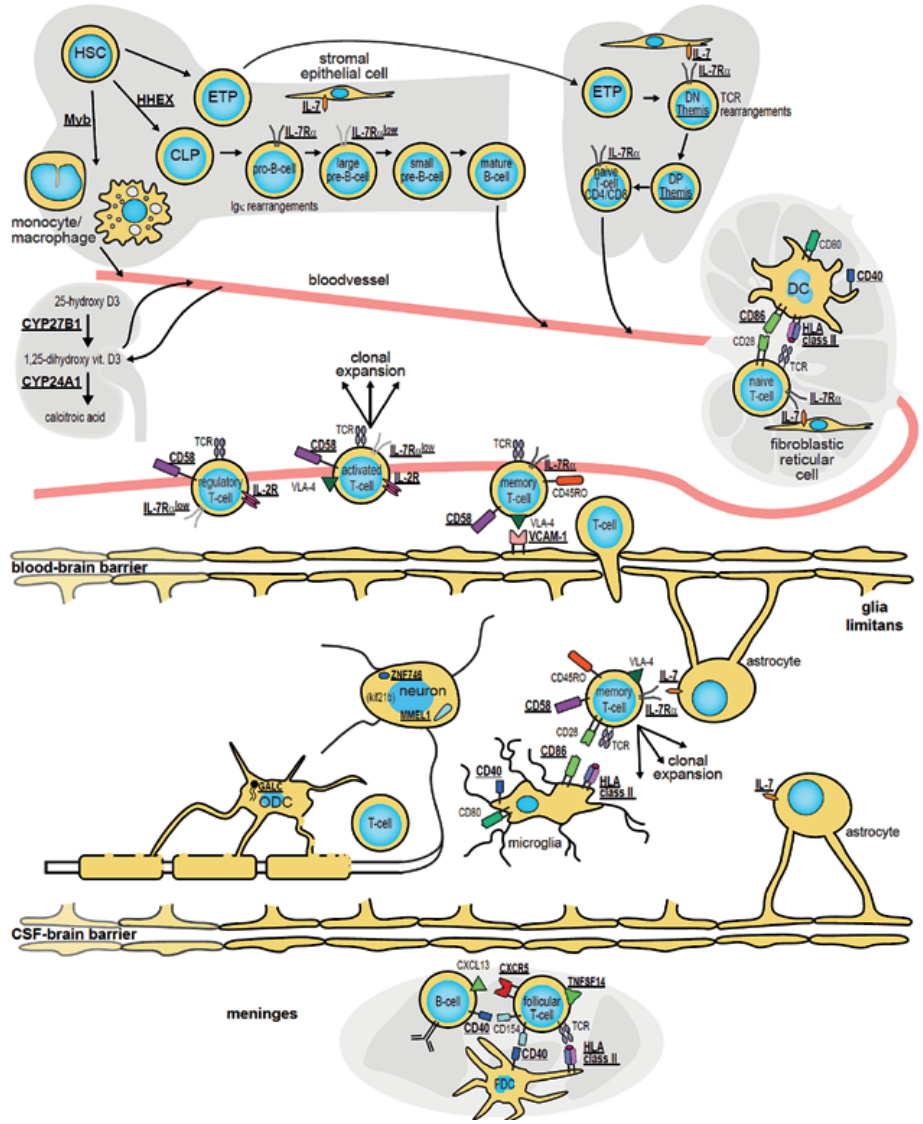
Other environmental risk factor have epidemiologically been linked to MS, such as vitamin D as an modulator of the immune response, both on antigen presenting cells as well as on T-cells. Interestingly, two SNP in the vitamin D metabolism were found associated with MS. One SNP is located in CYP27B1 gene, which is involved in metabolizing the inactive form of vitamin D (25-hydroxy D3) into the bioactive vitamin D (1,25-dihydroxy D3). Another SNP is located in CYP24A1 gene, which is involved in the inactivation of bioactive form into calcitroic acid. Aberrancies in this pathway, in combination with differences in sunlight exposure, might be an explanation for the observed latitude gradient of MS over the world, with the highest incidence of MS far away from the equator.

Genes with functions within the CNS

From the recent large-scale GWAS in MS, one observation is the relatively lack of genes with presumed functions in the CNS. However, some genes have been described to have a function in the CNS. GALC encodes the lysosomal enzyme galactocerebrosidase, which digest galactocerebroside. Galactocerebroside is the major substrate in Schwann cells and oligodendrocytes (ODC), the myelinating cells of the nervous system [121]. Mutations leading to deficiencies in activity of galactocerebroside are found in globoid cell leukodystrophy (GLD, also known as Krabbe's disease), a devastating disease in young children leading to severe neurological complaints through progressive demyelination. Several studies have investigated whether MS patients have antibodies against GALC, but none of these studies have found anti-GALC auto-antibodies [122, 123]. Moreover, incubation of primary human ODC with the serum of MS patients did not result in ODC cytotoxicity nor morphological changes [124].

Mutations in MANBA, the gene encoding beta-mannosidase, are involved in the development of beta-mannosidase, a rare autosomal recessive lysosomal storage disease [125]. This disease is associated with several neurological diseases, for example neonatal onset epilepsy and hydrocephalus [126], spinocerebellar ataxia [127] and severe leucoencephalopathies [128]

Figure 1. Presumed functional role of MS risk SNP



Development of precursor cells in the bone marrow and thymus, followed by activation of lymphoid cells in the secondary lymphoid tissue and the migration into the CNS and the formation of B-cell follicles in the meninges. Vitamin D metabolism is shown in the kidney. Abbreviations: CLP, common lymphoid progenitor; ETP, early thymic progenitor; HSC, hematopoietic stem cell.

MMEL1, membrane metallo-endopeptidase like 1, also known as neprilysin 2 (NEP2) is expressed in the pituitary glands, testis and in the brain in several neuronal populations [129]. Relatively little is known about its function, but it is involved in the degradation of beta-amyloid [130]. Decreased expression of MMEL1 is observed in the brain of Alzheimer's patients (AD) and inhibitors of MMEL1 peptidase activity leads to increased deposition of beta-amyloid depositions in mice and humans [131].

ZNF746 (also known as PARIS) is another MS risk gene, which is also implicated in neurodegeneration. Recently, it was shown that PARIS is involved in the pathogenesis of Parkinson's disease. ZNF746 accumulates rather specifically in the striatum and substantia nigra, but not in the cerebellum or cortex. It suppresses the transcriptional activity of peroxisome proliferator-γ coactivator-1α (PGC-1α), by binding to its promoter and thereby inhibiting the transcription of NRF-1 [132], which is involved in cellular metabolism and mitochondrial biosynthesis [133]. Overexpression of PARIS leads to dopaminergic neuronal cell death in the substantia nigra and thus to neurodegeneration [132]. Mitochondrial dysfunction has also been implicated in the pathogenesis of MS. In active MS lesions, there is a loss of several important mitochondrial proteins. In chronic inactive lesions the number and the activity of mitochondria increases [134, 135]. Recently, it was shown that there are extensive deletions in mitochondrial DNA (mtDNA) in neurons of MS patients. The cause of these deletions is currently unknown, although it is speculated that this occurs secondary to inflammation [136]. It should be mentioned that the inflammatory process in MS may cause damage to the mitochondria, for example via reactive oxygen species and nitric oxide. Currently, from GWAS only one SNP in a mitochondrial gene (SCO2 gene) is associated with MS. Moreover, some candidate gene studies regarding mtDNA SNP have been performed in MS. A SNP in the ***mt-ND5*** gene (nt13708G/A SNP) is associated with both adult [137] and paediatric MS [138]. This SNP causes an amino acid change, but if this is affecting mitochondrial functioning is currently unclear. Another mtDNA SNP (rs659366 in the promoter of the UCP2 gene) is associated with MS. The G-allele of this SNP has an increased prevalence in MS patients. This is associated with a decreased expression of UCP2 gene, which increased ATP levels [139]. Increased ATP levels are associated with pro-inflammatory response via activation of the inflammasome. As the frequency of MS in females is increasing, it is worthwhile to further study mtDNA, as this is per definition maternally transmitted. It should be noted that substantial evidence exist that MS is also maternally transmitted [140, 141].

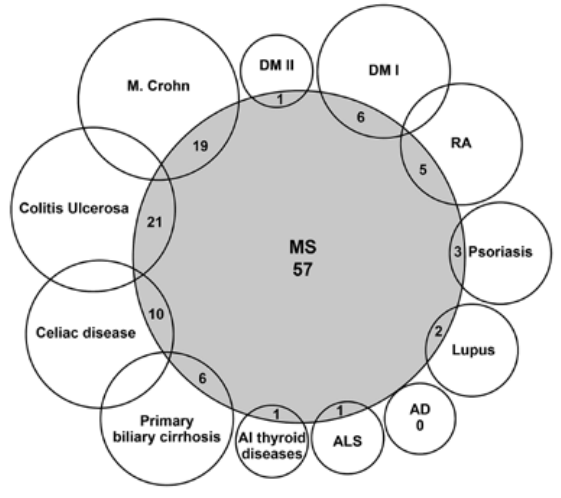
Lastly, several studies associated kif21b with MS. However, in the latest GWAS, not kif21b, but the nearby located open reading frame (C1ORF106) was found to give rise to this signal. It remains to be validated whether the association in this region comes from kif21b or the ORF. Nevertheless, kif21b is an interesting candidate. Kif21b belongs to the family of kinesins, which are responsible for intracellular transport of protein and organelles [142]. The exact cargo of the transporter kif21b is currently unknown, but it is known that this kinesin is expressed in dendrites of neurons [143]. Alterations in these important transport molecules might be related to neurodegeneration in MS. Moreover, kif1b was also found in a GWAS [81], although subsequent studies failed to replicate this finding [144]. Also candidate studies have implicated another kinesin, namely kif5a, to be associated with MS [145].

Genetic overlap with other autoimmune diseases.

Clinical and epidemiological observations imply that several immune-mediated inflammatory and autoimmune diseases can occur simultaneously in a single person or in closely related family members. Most frequently observed other autoimmune disease in MS patients are thyroid disease (both M. Graves and Hashimoto's disease pooled), inflammatory bowel disease (both ulcerative colitis and M. Crohn) and psoriasis and type I DM [146]. However, it should be noted that caution regarding the overlap between autoimmune diseases is necessary. Several examples of biases include publication, ascertainment and misclassification bias. Additionally, only the use of autoantibodies does not prove an autoimmune disease and patient questionnaires are unreliable to determine if a MS patient has another autoimmune disease. Many of the autoimmune diseases have a strong genetic component on the disease risk [93, 147-149].

Risk genes and pathways shared between these diseases may explain the observed co-morbidities between MS patients and other autoimmune diseases. Through GWAS as many as 140 susceptibility loci have thus far been identified for immune-mediated and autoimmune disorders [150]. There is evidence that genetic variation predisposing to one disease enhance or decrease the risk of another disease [151]. For example, the type I DM susceptibility genes CLEC16A and CD226 also influence the susceptibility to develop MS [152].

Figure 2. Genetic overlap between different neurological and autoimmune disease.



Increasing size of the circle represents more overlap in genomic loci between autoimmune and neurodegenerative diseases (note that the circles are not to scale). The number of shared genes is indicated in the overlap between MS and another disease. Please note that the tripartite overlap is not showing complete overlap between the diseases for the sake of clarity. Abbreviations: AD, Alzheimer's disease; AI, autoimmune thyroid disease; ALS, amyotrophic lateral sclerosis; DM, diabetes mellitus; MS, multiple sclerosis and RA, rheumatoid arthritis. References of the GWAS used in this figure are listed in Table 4. A slightly modified version of this figure is published in Multiple sclerosis immunology – a foundation for current and future treatments, chapter 10, genetics of multiple sclerosis

SNP increase the susceptibility of an individual to develop a disease, but protect against another [153], for example the HLA-class II alleles. Most auto-immune diseases show association with HLA-II. In MS, the disease risk is substantially increased in carriers of HLA-DRB1*1501. In type I DM, this gene has a protective effect. In Sardinia, HLA-DRB1*1501 is uncommon in general population and other HLA alleles contributes to the risk to develop MS. Because the type I DM protective HLA alleles are uncommon in the Sardinian population, co-morbidities between MS and type I DM occurs [154]. This is also observed for SNP in other autoimmune diseases, for example the IL18RAP locus being associated with celiac disease and protective against type I DM [151].

When all currently known associated genes (with a p-value $<10^{-7}$) for seven common autoimmune diseases are visualized together, the extent of sharing can clearly be appreciated. Between MS, inflammatory bowel diseases (both ulcerative colitis and M. Crohn) and celiac disease, a strong overlap in risk genes is observed (Figure 2). Interestingly, between MS and other neurodegenerative diseases (Alzheimer's disease, AD and amyotrophic lateral sclerosis, ALS), only one gene overlaps with ALS (Table 4), suggesting that the immune component of MS is the most important one in the pathogenesis. Genes most frequently associated with several autoimmune diseases are BACH2, CLEC16A, IL2RA, IRF8, TYK2 and ZMIZ1 (functions shown in Table 3).

Table 4. Overlapping loci between MS and other (autoimmune) diseases

Disease (number of overlapping genes)	Genes	References
Ulcerative colitis (21)	C1ORF106 (kif21b), CD6, CD40, CXCR5, GALC, IL2RA, IL7RA, IL12B, IRF8, MANBA, MAPK1, MMEL1, NFKB1, PTGER4, RPS6KB1, STAT3, TNFRSF6B, TYK2, ZBTB46, ZFP36L1, ZMIZ1	155-160
M. Crohn (19)	BACH2, C1ORF106 (kif21b), CD6, CD40, CXCR5, GALC, IL2RA, IL12B, IRF8, MAPK1, PTGER4, RPS6KB1, SP140, STAT3, TAGAP, TNFRSF6B, TYK2, ZFP36L1, ZMIZ1	156, 159-168
Celiac disease (10)	BACH2, CLEC16A, IL12A, MMEL1, PLEK, RGS1, TAGAP, THEMIS, ZFP36L1, ZMIZ1	148, 167, 169
DM type I (6)	BACH2, CLEC16A, IL2RA, IL7RA, IRF8*, TYK2	157, 170-176
Primary Biliary Cirrhosis (6)	CLEC16A, CXCR5, IL7RA, IL12A, NFKB1, TNFRSF1a	177-180
RA (5)	BATF, CD40, IL2RA, IRF8, MMEL1	181-183
Psoriasis (3)	IL12B, TYK2, ZMIZ1	168, 184-188
Lupus (2)	BACH2, CLEC16A	189
Graves (1)	BACH2	190
DM type II (1)	HHEX	191-199
ALS (1)	ZNF746	200
AD (0)	-	-
Parkinson's disease (0)	-	-

Note, only GWAS or meta-analyses studying at least 100.000 SNP are included in this table. The p-value for association should be at $<1*10^{-5}$. SNP identified within genes or the gene region around the SNP according to the UCSC genome browser were included in this table.
* IRF8 associated with the development of diabetic retinopathy in type I DM patients rather than with the development of DM type I.

Epigenetics

Several mechanisms lead to epigenetic modifications, like DNA methylation, histone modification, non-coding RNA and nucleosome positioning. The interplay between these factors leads to alterations of the chromatin structure and thereby altering the accessibility of the DNA to be transcribed into RNA and thus eventually protein. Importantly, epigenetic alterations are reversible and cell (type) specific [201]. For example, in Th1 cells the promoter of IFN-γ is demethylated, whereas in Treg the FoxP3 promoter must be fully demethylated for stable expression of FoxP3.

In MS, relatively little is known regarding epigenetic modifications compared with controls. One study compared the transciptome and epigenetic modification of CD4+ T-helper cells obtained from three pairs of disconcordant twins and unrelated controls. Surprisingly, they found no significant differences between the affected twin and the non-affected twin. However, it should be noted that this first and very interesting study might be underpowered to detect differences given the very strict quality controls and corrections for multiple testing necessary to interpret the results [202]. Another study used post-mortem NAWM from four MS patients and four non-neurological controls (NDC) for a chromatin immunoprecipitation (ChiP) assay. The findings from this pilot phase were subsequently validated in 19 other MS and 15 NDC using quantitative PCR, immunohistochemistry and blotting techniques to quantify protein levels, and also to compare different types of MS lesions. Interestingly, they found increased levels of deacetylation in early MS lesions whereas marked acetylation was observed in chronic lesions, indicating that during disease progression more transcriptional inhibitors of oligodendrocyte differentiation genes are activated [203]. Moreover, histone deacetylase 1 (HDAC1), an important repressor of transcription, is found in damaged axons in the brain lesions of MS patients [204]. In EAE, treatment with the histone deacetylase inhibitor trichostatin A reduces spinal cord inflammation, demyelination, neuronal and axonal loss and ameliorates disability in the relapsing phase of this MS-model [205]. Moreover, increased citrullinated myelin basic protein (MBP) is found in the NAWM of MS patients compared with other neurodegenerative diseases and non-neurological controls. This citrullinated MBP is less stable and might contribute to the observed myelin loss in MS brain. Citrullination of proteins is mediated by the family of peptidylarginine deiminases (PAD) and it was shown that PAD2 expression is increased in MS brain. In the NAWM of MS brains, there is a hypomethylation of PAD2 promoter, possibly leading to aberrant PAD2 expression. However, in the thymus of the same patients, no alterations in methylation of the PAD2 promoter was found [206]. Additional to epigenetic alterations observed in different types of tissue in MS patients, it will be of interest to assess how environmental factors, like EBV and vitamin D alter the epigenome in different cell types and how epigenetic changes might contribute to the risk of developing MS.

The increase in MS susceptibility mainly occurring in females might at least partially be explained by epigenetics. It is well known that epigenetic modifications can be inherited maternally. The classical epigenetic modification in females is X-chrosomal inactivation. Currently, studying X-chromosomal genetics is very challenging, because of the complex architecture of the X-chromosome.

miRNA

As histone modifications and DNA methylation is regulating the availability of DNA to be transcribed into messenger RNA (mRNA), micro RNA (miRNA) regulates the level of gene transcription at the post-transcriptional level. miRNA usually binds to the 3' untranslated regions (UTR) of mRNA and this binding might either lead to degradation or transcriptional repression, which depends on the level of sequence complementary [207]. A single miRNA can target hundreds of mRNAs and most of the currently known interactions between miRNA and mRNA are based on computational analysis and have not been experimentally validated yet [208].

Numerous studies have been performed to assess whether miRNA are up- or downregulated in MS patients. Similar to modifications in histones and methylation status of the DNA, it should be noted that miRNA expression profiles are cell type and tissue specific. Most of the miRNA studies performed in MS patients have focused on peripheral blood, either in whole blood samples or on sorted T-cell subsets. A few studies have investigated the expression of miRNA in post-mortem white matter brain materials of MS patients, whereas to our knowledge currently no study exist on grey matter brain lesions.

In total, 18 miRNA are found in at least two studies with MS patients or within EAE experiments, mostly upregulated in peripheral blood of MS patients (reviewed in Junker et al [209]). Using another approach, via a search using the miR2Disease database, Angerstein and colleagues [208] found 16 miRNA differentially expressed in MS patients in at least four independent published studies. Five of these miRNA were also previously found in at least two MS or EAE studies. The discrepancy can be explained by the fact that the miR2Disease database does not contain the most recent miRNA profiling studies in MS patients. Several miRNA have consistently been shown to be upregulated in peripheral blood of MS patients. These include miR142-3p, miR146-5p, miR155-5p and miR326. Interestingly, miR155-5p has also consistently been shown to be upregulated in MS brain. Experimentally validating the target genes of these miRNA will help to further understand the underlying mechanism a possibly allow us to further understand the pathogenesis of MS. It would be interesting to assess whether these miRNA affects the expression of genes associated with an increased risk to develop MS.

Gene-environment interactions

Interactions between the environment and genes may also occur, although relatively little is known about these interactions. An interesting study assessed how vitamin D might alter the epigenetic landscape. It was determined which genes are affected by vitamin D treatment in vitro and these were cross-checked against a list of genes associated with autoimmune diseases. Several MS associated genes, including HLA-II were found to have a vitamin D binding element [210, 211]. Thus, low vitamin D levels might alter the transcription of these genes in MS and might thereby be involved in the immunopathogenesis of MS.

Differences in exposure to environmental factors likely alter the expression of certain (MS risk) genes via epigenetic mechanisms. Differences in epigenetics might contribute to the increase in

the female to male ratio in MS [9]. Additionally, maternal transmission of HLA-II alleles observed in MS patients might be due to epigenetical modification or gene-environment interactions. This maternal transmission of HLA-II alleles may contribute to the increased prevalence of MS in females [212].

Functional studies necessary to understand the exact genetic background of MS

With increasing knowledge about genetic variants associated with MS, it is essential to further understand why and ***how*** these polymorphisms ***alter*** the underlying pathways and ***how*** this contributes to development of immunopathology of the CNS (functional immunogenetics). More insight into these processes will facilitate our understanding of the biology of the disease and this might eventually facilitate new drug targets, which will be more specific and hopefully more effective with less side effects. It is important to consider how to design and conduct functional immunogenetic studies.

Considerations for functional immunogenetics studies

Most of the polymorphisms associated with MS are relatively common, with frequencies ranging from 30-70% in the general, non-affected population, with slightly higher prevalence in MS patients. A large collection of samples (biobanking) is an absolute necessity to perform functional immunogenetical studies, because the number of homozygous allele carriers can be rather low leading to statistical power problems to assess functional outcomes reliable. Given the prevalence of a number of SNP, these homozygous carriers are often relatively small groups.

In these biobanks, besides DNA samples, also plasma, serum and ideally CSF samples in combination with peripheral blood mononuclear cells (PBMC) are needed to perform functional cellular experiments. Additionally, large collections of post-mortem material with a DNA sample of the donor are necessary to study the neuronal genes in more detail. Importantly, also a large number of samples of healthy controls is mandatory. In addition to all these samples, also high quality (para) clinical data is necessary to be able to correlate clinical outcomes to the results of functional assays.

Special attention is necessary for the selection of healthy controls. The choice of the controls is not trivial, as the results may become skewed with an inappropriate choice of controls. This might give rise to both false positive as well as false negative results. Matching criteria for the inclusion of controls should absolutely include age, gender and genotype. The biological relationship to the patient should also be taken into consideration for several reasons. First, the risk to develop MS for a healthy control, which is a family member of a MS patient, is slightly though significantly increased, based on the degree of genetic sharing between the control and the patient. Second, inclusion of family members as healthy controls might give rise to misclassification of outcome, as they might develop MS as well at a later stage. This will only be the case for an absolute minority of the controls. Because the differences in outcome may be very subtle, there is a very small chance that this will skew the results to false negative conclusions. Third, depending on the age of controls, especially for younger patients and controls, the environment might be shared in the childhood. This might

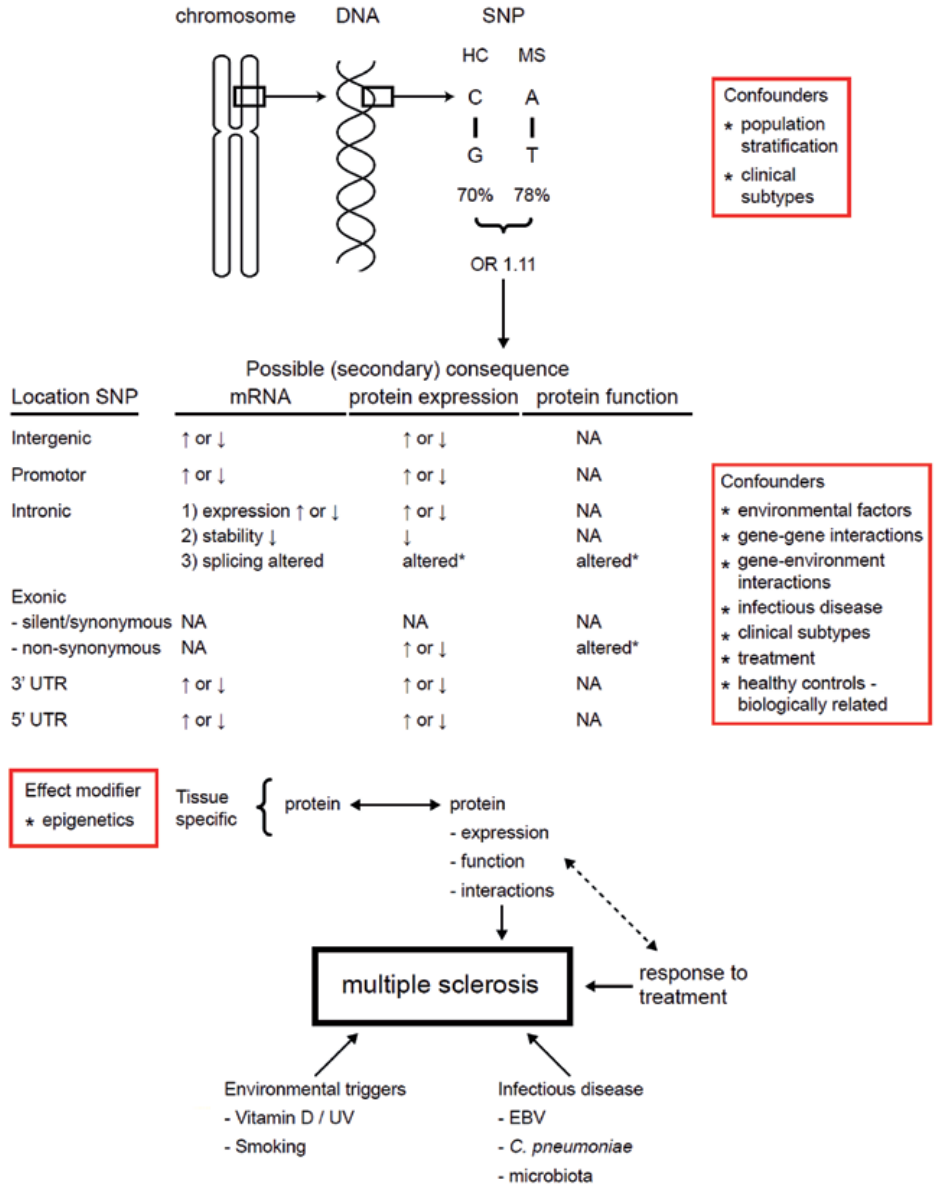
skew the results of expression studies, as environmental influences on epigenetics might alter the expression of certain genes or proteins. Additional (historical) clinical information of the healthy controls, like a history of neurological symptoms, suggestive of MS or comorbidities with other autoimmune diseases or the use of immunomodulatory treatments is important as these factors might affect functional outcomes in an immunogenetic study.

After selection of an appropriate group of patients and controls, the choice of an outcome measure becomes important. Polymorphisms might affect the amino acid composition of a protein and thereby possibly the function or the stability of the protein, or mRNA expression, either directly or via altering the stability of the mRNA leading to altered protein expression. Thus the choice which outcome parameter will be measured in relation to a certain SNP might affect the outcome of the study.

Additional considerations affecting the results, even when the appropriate outcome measurement has been selected, are confounding factors, as well as effect modifiers. Environmental factors modifying epigenetics are possible confounders (Figure 3). For instance, assessing how the vitamin D-related SNP (CYP27B1 and CYP24A1) alter the serum amount of active vitamin D and the effect on the expression of HLA-DR on immune cells. Finding an association between the SNP and the expression of these two parameters would lead to the conclusion that they might have a causal relationship. However, sunlight exposure alters the availability of the bioactive form of vitamin D, the 1,25 dihydroxy vitamin D3. Active vitamin D modulates gene expression of HLA-DR via vitamin D regulatory elements. It is likely that MS patients are less exposed to sunlight compared with healthy controls, because they generally have impaired mobility and some patients MS have heat-related complaints (Uhthoff phenomenon) and hence avoid the sun. The amount of active vitamin D may be influenced by these two polymorphisms or by a lower exposure to sunlight. Therefore, the association between these two SNP and HLA-DR expression may be confounded by the amount of vitamin D due to sunlight exposure. This kind of epidemiological difficulties in establishing an association is termed reverse causation. Correcting for such a factor is hard, because the amount of sunlight exposure somebody has received is difficult to measure reliably and is highly prone to recall bias.

Therefore, one might argue that functional immunogenetic studies should be performed in healthy controls only, since these confounding factors might be less frequently observed in controls. However, as exemplified in **chapter 2.1 and 3** of this thesis, this approach would not have detected significant differences between patients and controls in a biological pathway, as only the soluble form, but not the membrane-bound form of the IL-7Rα is altered by the SNP. Differences between MS and HC would not be detected when only HC would have been studied in relationship to the receptor expression stratified according to the risk SNP. If the outcome of functional immunogenetic studies is to be used to gain more insights in the immunopathogenesis of MS, the inclusion of patient samples is necessary. The inclusion of patient samples is also necessary for the development of new therapies based on identified aberrancies in the underlying pathway.

Figure 3. Considerations for functional immunogenetics studies



Possible consequences of SNP and their contribution to the development of MS. Confounders and effect modifiers in functional immunogenetics studies are indicated. * Altered splicing and a non-synonymous SNP can affect the amount of protein, the ratio between the soluble and membrane-bound form of a protein, or the function of the protein

Scope of the thesis

The vast majority of the 57 MS-associated risk SNP is involved in leukocyte biology. However, ***how*** these polymorphisms ***functionally*** contribute to the immunopathogenic mechanisms and to the increased risk to develop MS is currently poorly understood. Therefore, functional immunogenetic studies are necessary to determine the consequences of these SNP.

One of the first identified SNP in MS is the IL-7Rα. The IL-7Rα is an important factor for the survival of developing T- and B-cells and the homeostasis of the T-cell pool in the periphery [99]. Because of the important function of the IL-7Rα in lymphocyte biology, it is a likely candidate to be involved in autoimmune diseases. This non-synonymous SNP is located in exon 6, encoding the transmembrane domain. Based on a cell line transfected with a Δ6 IL-7Rα construct, it was hypothesised that this SNP causes more splicing of exon 6, leading to increased production of the soluble receptor [78]. The IL-7Rα in humans is mainly expressed by mature T-cells, therefore, we hypothesised that this SNP would alter the expression and the function of the IL-7Rα on T-cell subsets (**chapters 2.1 and 2.2**). Additionally, we asked if the increased production of the soluble IL-7Rα caused by the SNP also alters the level of its ligand IL-7 in the serum (**chapter 3**).

Another important factor for the function of T-cells is antigenpresentation. A SNP in CLEC16A, which is a C-type lectin, is associated with several autoimmune diseases. Ema, the ***Drosophila*** counterpart of CLEC16A, is important in endosomal maturation [213]. Late endosomes are important for proper loading of antigens in the peptide binding groove of HLA class II molecules. Therefore, we hypothesised that CLEC16A is involved in antigen-presentation via the regulation of endosomal maturation (**chapter 4**).

In addition to genetic factors also environmental factors contribute to the development of MS. The strongest environmental risk factor to develop MS is EBV. Virtually all MS patients have immunoglobulins against selected EBV peptides, whereas this percentage in the general population is around 90% [68]. Additionally, also the IgG levels against these EBV peptides are increased in MS patients compared with HC. The majority of MS-associated SNP have functions in the adaptive immune response. Therefore, we hypothesised that some of the immune related MS risk SNP are associated with increased IgG levels against EBV (**chapter 5**).

Finally, in addition to inflammation, neurodegeneration is important in the progression of MS, but the mechanisms behind this are poorly understood. Whether this is a consequence of inflammation or an independent process is currently unknown. Approximately five MS risk SNP have a presumed function in the CNS. Several SNP in kinesins, which are important neuronal transporters have been associated with MS. Only kif21b was validated in several independent studies. Therefore we selected kif21b, and hypothesised that it is involved in neurodegeneration in MS (**chapter 6**).

The general discussion of this thesis is divided into three parts. The first part reflects on MS findings described in this thesis and places them into the context of aberrancies observed in other (autoimmune) diseases (**chapter 7.1**). Additionally, a critical appraisal regarding the findings in genetic studies in MS as well as other immunogenetic studies in MS is presented in **chapter 7.2**. Lastly, future perspectives in the fields of clinical immunology and genetics of MS are reviewed in **chapter 7.3**.

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Chapter 2.1

The IL-7Rα pathway is quantitatively and functionally altered in CD8 T cells in multiple sclerosis.

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Abstract

The IL-7Rα single nucleotide polymorphism (SNP) rs6897932 is associated with an increased risk for multiple sclerosis (MS). The IL-7Rα is a promising candidate to be involved in autoimmunity, since it regulates T-cell homeostasis, proliferation and anti-apoptotic signalling. However, the exact underlying mechanisms in the pathogenesis of MS are poorly understood.

We investigated whether CD4 and CD8 lymphocyte subsets differed in IL-7Rα expression and functionality in 78 MS patients compared to 59 healthy controls (HC). A significantly higher frequency of IL-7Rα$^+$ CD8 effector memory (CD8EM) was found in MS. Moreover, IL-7Rα membrane expression was significantly increased in MS in naive and memory CD8 (all $p<0.05$) with a similar trend in CD8EM ($p=0.055$). No correlation was found between the expression level or frequency of IL-7Rα$^+$CD8$^+$ and rs6897932 risk allele carriership. Upon IL-7 stimulation, MS patients had stronger STAT5 activation in CD8EM compared to HC. IL-7 stimulation had a differential effect on both mRNA and protein expression of granzyme A and granzyme B between MS and HC. Stainings of different lesions in postmortem MS brain material showed expression of IL-7 and CD8$^+$IL-7Rα$^+$ in pre-active, but not in active demyelinating MS lesions, indicating involvement of IL-7Rα$^+$ lymphocytes in lesion development.

The intra-lesional production of IL-7 in combination with the lower threshold for IL-7-induced cytotoxicity in MS may enhance the pathogenicity of these CD8 T-cells. This is of special interest in light of the established demyelinating and cytotoxic actions of granzyme A.

Introduction

Multiple sclerosis (MS) is a complex disease, presumed to be autoimmune mediated. The exact etiology is unknown, but a combination of genetic and environmental factors including infections is important in the development of the disease [1]. Extensive research has focused on the role of CD4$^+$ T-cell subsets, notably Th1 and Th17 cells. However, recently increasing evidence for an important role of antigen specific CD8$^+$ cytotoxic T-cells has emerged [2, 3].

For several decades, the only genetic association with an increased risk to develop MS was the class II HLA-allele DRB1*15:01 [4]. Recently, a protective effect of the HLA class I allele HLA A*02:01 has been shown and some additional HLA class I protective alleles have been suggested [5]. Although many genetic studies have been performed, very few non-HLA genetic factors were found to be associated with MS. However, the International Multiple Sclerosis Genetics Consortium (IMSGC) published the results of the first genome wide association (GWAS) study in MS patients. The IMSGC validated the HLA-II association and in addition identified several single nucleotide polymorphisms (SNP) in genes associated with MS with modest odds ratios compared to the HLA-II association. Interestingly, most of the MS risk SNP are involved in T-cell homeostasis and differentiation [5, 6].

One of the most interesting risk SNP identified was rs6897932, located in the IL-7Rα, also known as CD127 [5-8]. CD127 is involved in homeostasis and longevity of T-lymphocytes [9]. Since auto-reactive T-cells are thought to play an important role in MS, a genetic variation within this important survival factor can be important for the disease [10]. Moreover, early reports on the function of IL-7 implicated it to be involved in cytotoxicity [11], although the exact mechanisms (e.g. the specific cytotoxic pathway) have not been studied thoroughly.

The IL-7Rα risk SNP rs6897932 is located in exon 6, encoding the transmembrane domain of the receptor. The SNP is presumed to cause a splice variant, which leads to increased levels of the soluble form of the receptor [8]. Interestingly, this SNP is also significantly associated with type I diabetes mellitus [12] and rheumatoid arthritis [13], other auto-immune mediated diseases where auto-reactive lymphocytes play an important role. Additionally, these diseases have a considerable overlap in genetic associations with MS [14]. Currently, no studies are available about putative cellular differences in the IL-7Rα axis and downstream signalling aberrations in MS.

Our current study focused on the role of the IL-7Rα expression on lymphocytes of MS patients. We show that the frequency of IL-7Rα$^+$ CD8 effector memory (CD8EM) T-cells is significantly higher in MS patients. Moreover, IL-7 stimulation resulted in an enhanced phosphorylation of STAT5 in CD8EM, which in MS patients leads to dysregulated changes in the expression of the cytotoxic molecules granzyme A and B, which have been implicated for their demyelinating effects. Our results can not be explained by the genetic association solely and shed new light on the importance of the IL-7Rα pathway in MS patients.

Materials and methods

Patients and controls

Fifty-one consecutive relapsing-remitting MS patients, diagnosed according to the McDonald criteria [15] and seven patients with clinically isolated syndrome, aged between 18-75 years and seen on regular basis in the MS Centre ErasMS in Rotterdam were included in the study. Exclusion criteria were an exacerbation and/or the use of methylprednisolone three months prior to sampling. In the first phase, the use of immunomodulating therapy was an additional exclusion criterion. In the second phase of this study, 15 patients treated with interferon β-1a and one with Glatiramer acetate were included. In phase three, also ten primary progressive and ten secondary progressive patients were included. Healthy controls, aged 18-75 years old, were relatives of the MS patients who accompanied a patient to the outpatient clinic in our hospital. Exclusion criteria were 1) diagnosis of MS or 2) prior symptoms suggestive of CNS demyelination, such as optic neuritis, myelitis and 3) use of any immunomodulating therapy for an autoimmune disease. This study was approved by the Medical Ethical Committee of the Erasmus MC and written informed consent was obtained from all patients and controls.

Flowcytometry for IL-7Rα expression on lymphocyte subsets

PBMC were collected in CPT-tubes and processed according to the manufacturers protocol (BD Biosciences). After collection, the PBMC were cryopreserved and stored in liquid nitrogen. For flowcytometric analysis 1*10E6 lymphocytes were stained with CD3 FITC, CD4 PE-Cy7, CD8 APC-Cy7, CD27 APC, CD45RA PerCP-Cy5.5 (eBioscience) and CD127 PE (eBioscience) for 20 min.

Cell stimulation experiments

For flowcytometry assay 1*10E6 PBMC and for Western blot 2*10E5 PBMC were cultured in RPMI$^+$ serum-free medium with or without 5 ng/ml IL-7 (Peprotech) or IFN-γ 50 U/ml (Boehringer Ingelheim) at 37°C with 5% CO_2 for 15 min. For q-PCR 1*10E5 T-cells or PBMC per well were cultured in RPMI$^+$ serum-free medium with or without 5 ng/ml IL-7 (Peprotech) or IFN- γ 50 U/ml (Boehringer Ingelheim) at 37°C with 5% CO_2 for 24 h. The optimal concentration of IL-7 was determined with titration experiments (see Supplementary Figure 1). In the validation experiments for Western blotting and q-PCR, cells were pre-incubated with 25 μM of the JAK inhibitor AG-490 (Sigma-Aldrich) for 2 h and thereafter co-incubated with IL-7 or IFN- γ.

pSTAT1 and pSTAT5 expression by lymphocyte subsets upon IL-7 stimulation

Stimulated and unstimulated cells were fixed with Cytofix at 37°C for 10 min, permeabilized with Perm III Buffer for 30 min on ice and then stained with CD3 PerCP, CD4 Amcyan, CD8 FITC, CD27 APC, CD45RA PE-Cy7, pSTAT1 Pacific Blue and pSTAT5 PE for 30 min at 4°C. Since IL-7 stimulation leads to phosphorylation of STAT5 and not STAT1, pSTAT1 was used as a negative control for IL-7 stimulation.

Moreover, since IFN-γ stimulation leads to phosphorylation of STAT1, but not STAT5, IFN-γ was used as negative control for pSTAT5 to ascertain the specificity of the effects. The differences in percentage and MFI of pSTAT1$^+$ and pSTAT5$^+$ cells between the unstimulated and the 15 min stimulation condition were calculated and compared between MS patients and controls.

Intracellular expression of granzyme A and B upon IL-7 stimulation

Baseline expression and expression of granzyme A and B upon 22 hours of IL-7 stimulation was assessed. PBMC were extracellular stained for CD3 V500, CD8 PE-Cy7, CD27 APC-H7, CD45RA PerCP-Cy5.5 (eBioscience), CD127 eFluor450 (eBioscience) for 30 minutes at 4°C. Next, the cells were fixed and permeabilized with FOXP3 Staining buffer set (eBioscience) and intracellular stained for granzyme A PE (R&D Systems) and granzyme B AlexaFluor 647 (R&D Systems) for one hour at 4°C. Unstained cells and isotype controls were used as negative controls.

All antibodies and buffers for flowcytometric analyses were obtained from BD Biosciences unless stated otherwise. Flowcytometry was performed on an LSRII flowcytometer (BD Biosciences). Data analysis was performed with Facs Diva software, version 6.1 (BD Biosciences).

Confirmation of pSTAT1 and pSTAT5 expression with Western blot

For analysis of STAT1 and STAT5 protein expression and phosphorylation of STAT1 and STAT5, PBMC were lysed in 50 μl radioimmunoprecipitation assay buffer (50 mM Tris-HCL, 150mM NaCl, 1% NP-40, 0.1% SDS, 0.5% deoxycholic acid) supplemented with phosphatase and protease inhibitors (Roche). Insoluble material was removed by centrifugation at 10.000 x g for 10 min at 4°C and the soluble fraction was assayed for total protein. Briefly, proteins were separated on a 10% SDS polyacrylamide gel and transferred onto a polyvinylidene fluoride membrane (Millipore). The membranes were incubated with antibodies specific for STAT1, pSTAT1 (pY701), STAT5 and pSTAT5 (pY694) (BD Biosciences). Protein loading was analyzed by reprobing blots with an antibody against β-actin (Abcam). Detection of immunoreactive bands was performed using Western Lightning Plus-Enhanced Chemiluminescence Substrate (Perkin Elmer). Quantification was performed using ImageJ software version 1.44d (http://rsb.info.nih.gov/ij/).

IL-7 proliferation assay

1*10E5 PBMC were cultured in RPMI$^+$ medium with 10% FCS and 10% penicillin/ streptamycin at 37°C with 5% CO_2 for 72 h in three conditions: 1) unstimulated, 2) with 5 ng/ml IL-7 and 3) with 10 ng/ml PHA (Sigma-Aldrich) as positive control. After 3 days, proliferation was determined by incorporation of 0.5 μCi/well of ^{3}H-thymidine (GE Healthcare) for 18 h. All culture conditions were performed in triplo.

Gene expression

Total RNA was extracted from PBMC or sorted T-cells using the GenElute Mammalian Total RNA kit (Sigma-Aldrich). RNA samples were treated with DNAse I (Invitrogen) to remove any contaminating DNA. Using 1 µg of the total RNA as template, copy DNA (cDNA) was prepared using Superscript II (Invitrogen). Target gene mRNA expression was determined by real-time quantitative reverse transcription PCR using TaqMan technology on a 7900HT PCR machine (Applied Biosystems) with an initial step of 2 min at 50 ºC and 10 min at 95 ºC, followed by 40 cycles of 15 seconds at 95 ºC and 1 min at 60 ºC. Expression levels were calculated with the obtained Ct values compared with standard curves of the gene of interest. Target gene expression levels were corrected for 18S RNA levels. Sequences and probes were selected using the universal probe Libary Assay Design Center from Roche. Sequences of the PCR primers are shown in Supplementary Table 1 and fluorogenic probes were obtained from Roche. All q-PCR reactions were performed in duplo.

Magnetic cell sorting (MACS)

To assure that the results obtained from the q-PCR were T-cell specific, T-cells were negatively isolated with MACS beads (Miltenyi) according to the manufacturers' protocol. Purity of the cells was routinely >95% as assessed by flowcytometry with staining for CD3 FITC, CD4 PE-Cy7, CD8 APC-Cy7, CD14 APC, CD19 PerCP-Cy5.5 and CD56 PE on positive and negative sorted fractions.

Genotyping

rs6897932 genotyping was performed as previously described with either a Sequenom platform [16] or Taqman assay [17]. Both MS patients (p=0.98) and healthy controls (p=0.89) were in Hardy Weinberg equilibrium.

Collection of autopsy tissue and immunohistochemistry

Human autopsy brain tissues from five MS patients and five non-demented controls were obtained from The Netherlands Brain Bank (NBB), Netherlands Institute for Neuroscience, Amsterdam. All Material has been collected from donors from whom a written informed consent for brain autopsy and the use of the material and clinical information for research purposes had been obtained by the NBB.

The immunohistochemical methods used in this study are described in detail before [18]. In brief, 6 µm frozen sections of human brain white matter were thaw-mounted on gelatin-coated slides and kept overnight at room temperature (RT) in humidified atmosphere. After air-drying for 1 h, slides were fixed at room temperature in fresh acetone containing 0.02% (v/v) H_2O_2 for blocking endogenous activity. After washing in PBS, slides were incubated with optimally diluted primary antibodies overnight at 4°C in humidified atmosphere. Incubations with horse radish peroxidase (HRP)-labeled rabbit anti-mouse-Ig (Dako) or in case of a 3-step staining with secondary biotin-labeled rabbit anti mouse-Ig (Dako) and tertiary HRP-labeled avidin-biotin complex (ABC/HRP; Dako) were performed for 1 h at RT. HRP activity was revealed by incubation with 3-amino-9-ethyl-carbazole (AEC; Sigma) for 10 min at RT, leading to

a bright red precipitate. Nuclei were counterstained by hematoxylin (Merck). Slides were embedded with glycerol-gelatin. Reactive human tonsil sections were included in each staining procedure as positive control tissue. Incubation with irrelevant isotype control antibodies and omission of primary antibody were used as negative control stainings.

For detection of IL-7, Tyramide Signal Amplification (TSA) with HRP-streptavidin and biotin-XX tyramide (Molecular Probes) was used, according to the manufacturer's instruction.

The antibodies used in this study were directed against IL-7 (Biosource), IL-7Rα (R&D Systems), CD4, CD8, CD68 and HLA-ABC antigen (all from Dako). Isotype controls used were mouse IgG1 and mouse IgG2a (both from R&D Systems).

Double staining of IL-7Rα with CD8 or CD68 was performed as described before [19]. For double staining of IL-7Rα with CD8, IL-7Rα was first shown as described above, only with the difference that it was visualized with diaminobenzidine (DAB; Sigma). After HRP revelation slides were blocked with 10% normal mouse serum (Sanquin) for 15 min. CD8 antibody was then incubated for 1 h at RT, followed by incubation with alkaline phosphatase (AP)-labeled goat anti-mouse-IgG1 at RT for 1 h. AP activity was revealed with naphthol-AS-MX phosphate (Sigma) and Fast Blue BB base (Sigma) at 37°C for 30 min, resulting in a blue precipitate.

For double staining of IL-7Rα and CD68, IL-7Rα was detected in blue and therefore AP-labeled goat anti-mouse-IgG (Dako) and AP-labeled rabbit anti-goat-IgG (Southern Biotech Associates) were used. After blocking with 10% normal mouse serum for 15 min, CD68 antibody (Dako) was detected in red, by using the Animal Research Kit (ARK)-biotin (Dako). Staining for AP activity was performed prior to staining for HRP activity (with AEC). As a control for all double stainings, single stainings were performed in parallel with all the incubation steps of the double staining procedure, but omitting the first antibody incubation.

Staging of MS lesions

MS brain lesions were staged on the basis of internationally accepted inflammation and demyelination criteria described earlier [20, 21] using three different markers, i.e. acid phosphatase, HLA-DP,DQ,DR and neutral lipids. To detect infiltrating monocytes, macrophages and microglia cells, acid phosphatase, a lysosomal enzyme, is a useful marker. Activated cells can be detected with an antibody against HLA-DP,DQ,DR (Dako). Myelin breakdown products, reflecting active demyelination, were detected with oil-red O (ORO; Sigma), which stains neutral lipids

Statistical analysis

Comparisons between MS patients and HC were performed using Mann Whitney U-test. q-PCR analyses were tested with Wilcoxon signed rank test. When other statistical analyses were used, this is indicated. All statistical analyses were performed with SPSS version 17.0 and p-values below 0.05 were considered statistically significant. P-values are denoted in the figures as: * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

Results

Clinical and demographic characteristics

Briefly, 71 included patients fulfilling the McDonald criteria for MS [15] and seven clinically isolated syndrome patients (CIS) with a high risk to develop MS were included. Four of the seven CIS patients developed RRMS, the other three CIS patients remained free of neurological complaints after a follow up of at least 24 months (range 24-54 months). Detailed information on all patients and controls are depicted in Table 1.

Table 1. Clinical characteristics of patients included in IL-7Rα expression and function study in PBMC

	HC (n=59)	CIS* (n=7)	RRMS** (n=51)	SPMS*** (n=10)	PPMS (n=10)
Age at onset (SD)	NA	36 (12)	31 (11)	29 (8)	41 (9)
Age at sampling (SD)	39 (13)	39 (11)	41 (10)	51 (9)	53 (11)
Female (%)	64	100	78	90	70
Disease duration, years (SD)	NA	NA	8.8 (5.1)	21.7 (10)	12.4 (8.1)
Presenting symptoms (n)					
Optic nerve	NA	1	17	4	1
Spinal cord		1	15	2	4
Brainstem/cerebellum		1	10	1	1
Cerebrum		2	4	0	1
Multifocal		2	5	3	3

* None of the samples of the CIS patients were taken during the acute phase of the CIS attack. Four of the seven CIS patients eventually developed RRMS, the remaining CIS patients all fulfilled the criteria for dissemination in space, but not dissemination in time. The remaining CIS patients were followed for at least 24 months (range 24-59)

** 15 patients treated with immunomodulating therapy (14 with interferon β-1a; one with glatiramer acetate)

*** one patient treated with interferon β-1a

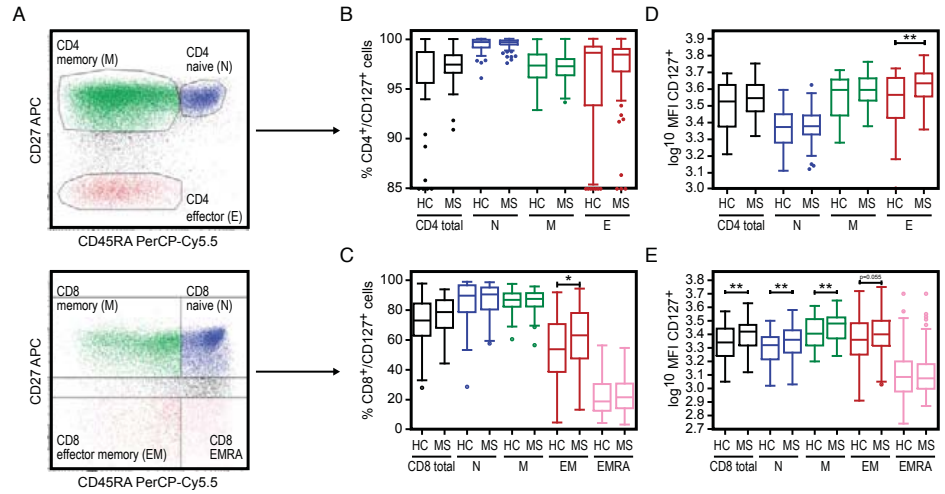
Increased CD127 expression on CD8 lymphocyte subsets in MS patients

T-lymphocyte subsets were gated according to the classical phenotypical analysis [22] (Fig. 1A). Subsequently, the frequency of CD127 positive cells within each subset and expression level of CD127 per cell were assessed. The percentage of CD127$^+$ T-cells within the different CD4$^+$ T-cell populations between MS patients and healthy controls (HC) was similar (Fig. 1B). However, the number of CD127 molecules per cell on the cell surface (mean fluorescence intensity, MFI) on CD4$^+$ effector T-cells was significantly increased (Fig. 1D) in MS patients compared to HC (p=0.002). Interestingly, in the CD8$^+$ T-cell pool (Fig. 1C), a significantly higher percentage of CD127$^+$ CD8$^+$ effector memory T-cells (CD8 EM) was found in MS patients (p=0.02). Also the MFI reflecting the expression of CD127 was increased in all CD8$^+$ lymphocyte subsets (all p<0.05) with a similar trend for the CD8EM (p=0.055). No differences in CD127 expression were found in the CD8EMRA population in MS patients (Fig. 1E).

The frequency and expression of CD127$^+$ in both CD4 and CD8 T-cells were comparable between the different MS disease courses. In addition, no correlation was found between the expression of CD127 and disease duration (linear regression, p=0.9). Moreover, no differences were found between untreated and interferon β-1a treated patients (Mann Whitney U-test, p=0.43). Therefore, all data were pooled and shown together.

As observed by others, both CD4$^+$ and CD8$^+$ memory T-cell subsets were increased in frequency (resp. p=0.01 and p=0.02) in MS patients compared to HC (data not shown).

Figure 1. CD127 expression by CD4 and CD8 T-cell subsets in MS and HC

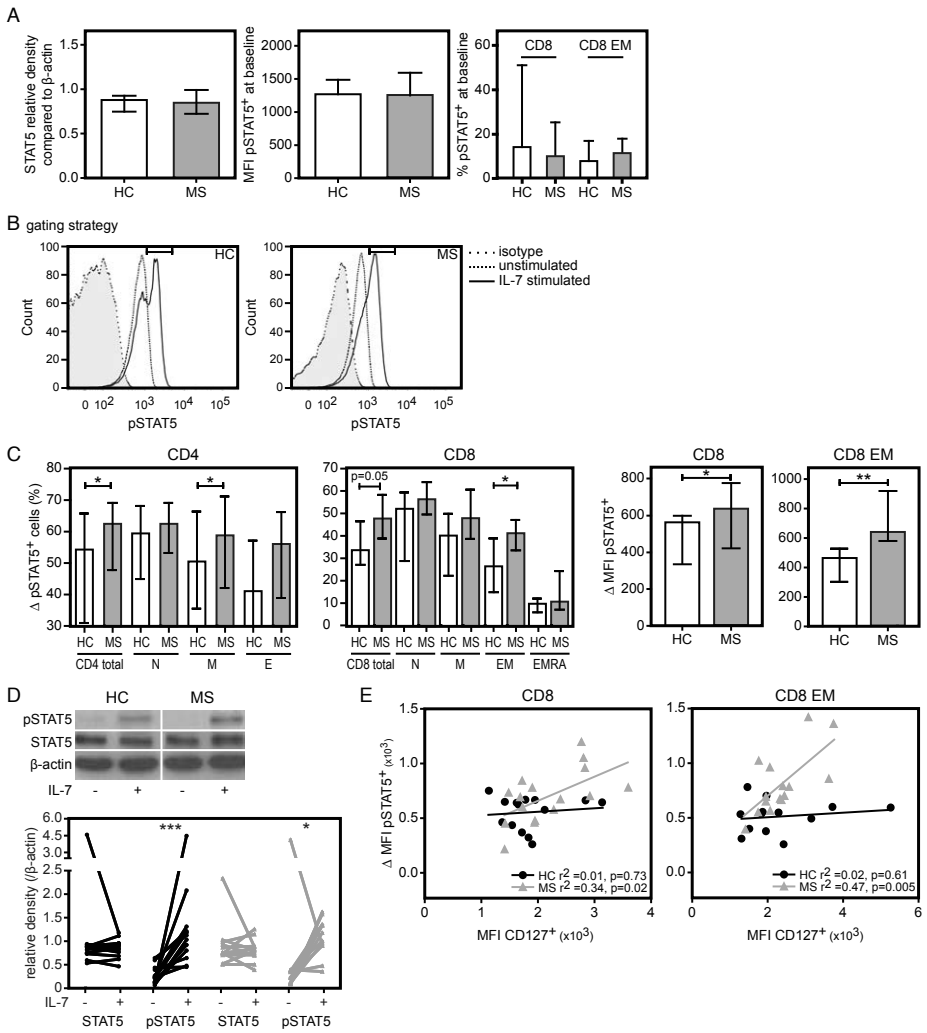


CD4 (upper row) and CD8 (lower row) T-cells **A)** gated according to functionally different subsets were stained for CD127. **B-C)** Frequencies of CD127$^+$ cells within the represented subset were compared between 77 MS patients and 58 HC. **D-E)** Level of CD127 expression (mean fluorescence intensity, MFI) corrected for background signal was compared between MS and HC. In the figures, N denotes naive, M memory, E effector (CD4 only), EM effector memory (CD8 only), EMRA effector memory CD45RA$^+$ (CD8 only)

IL-7 stimulation leads to a higher increase of phosphorylated STAT5 in CD8 T-cells of MS patients

Next, we questioned whether the significantly increased CD127 expression would lead to an increased phosporylation of STAT5 (pSTAT5), the downstream target of CD127. At baseline, no differences in unphosphorylated or phosphorylated STAT5 were observed between age-matched MS and HC (Fig. 2A). After stimulation of PBMC MS patients and HC with 5 ng/ml IL-7 for 15 min, the increase of pSTAT5 compared to the unstimulated cells was measured using 7 color flowcytometry with the illustrated gating strategy (Fig. 1A and 2B). In MS patients, a significantly higher percentage of pSTAT5$^+$ CD4$^+$ memory T-cells compared to HC was found upon IL-7 stimulation. Furthermore, a higher increase in pSTAT5 in total CD8$^+$ T-cells (p=0.05) and CD8EM T-cells of MS patients was observed (p=0.02).

Figure 2. Higher increase of IL-7-induced phosphorylated STAT5 in MS



The signalling cascade downstream of the IL-7Rα was investigated. **A)** Baseline levels of unphosphorylated STAT5 (both groups n=10) were determined by Western blot quantification. Baseline phosphorylated STAT5 (both groups n=15) was determined by flowcytometry. **B)** Representative CD8⁺ pSTAT5 staining from one MS patient and one HC is shown. Dashed line is unstimulated condition, continuous line is 5 ng/ml IL-7 stimulation for 15 min and grey filled area is isotype control. **C)** Increase in the percentage pSTAT5⁺ cells and the delta MFI was calculated as the difference between unstimulated and IL-7 stimulation (HC n=15, MS n=16). N denotes naive, M memory, E effector (CD4 only), EM effector memory (CD8 only), EMRA effector memory CD45RA⁺ (CD8 only). **D)** Representative Western blot of STAT5 and pSTAT5 of one MS patient and one HC confirmed the flowcytometric findings. In the lower part of the figure, quantification of the Western blots is shown. **E)** Linear regression analysis for the correlation between the expression level of CD127 and the delta pSTAT5 upon IL-7 stimulation in total CD8⁺ T-cells and CD8EM T-cells from MS patients and HC.

A similar trend was found for CD8 memory T-cells, although this did not reach statistical significance (Fig. 2C). These findings were confirmed by Western Blotting, one representative blot is shown as well as quantification for 12 HC and 12 MS patients (Fig. 2D). As expected, IFN-γ stimulation which phosphorylates STAT1 and not STAT5 did not increase pSTAT5 levels. Additionally, no upregulation of pSTAT1 was observed upon IL-7 stimulation (data not shown). Interestingly, in MS patients a significant correlation was found between the MFI of CD127 and the increased MFI of pSTAT5 upon IL-7 stimulation. In contrast, no correlation between these two parameters was found in healthy controls (Fig. 2E).

No differences in IL-7 induced proliferation

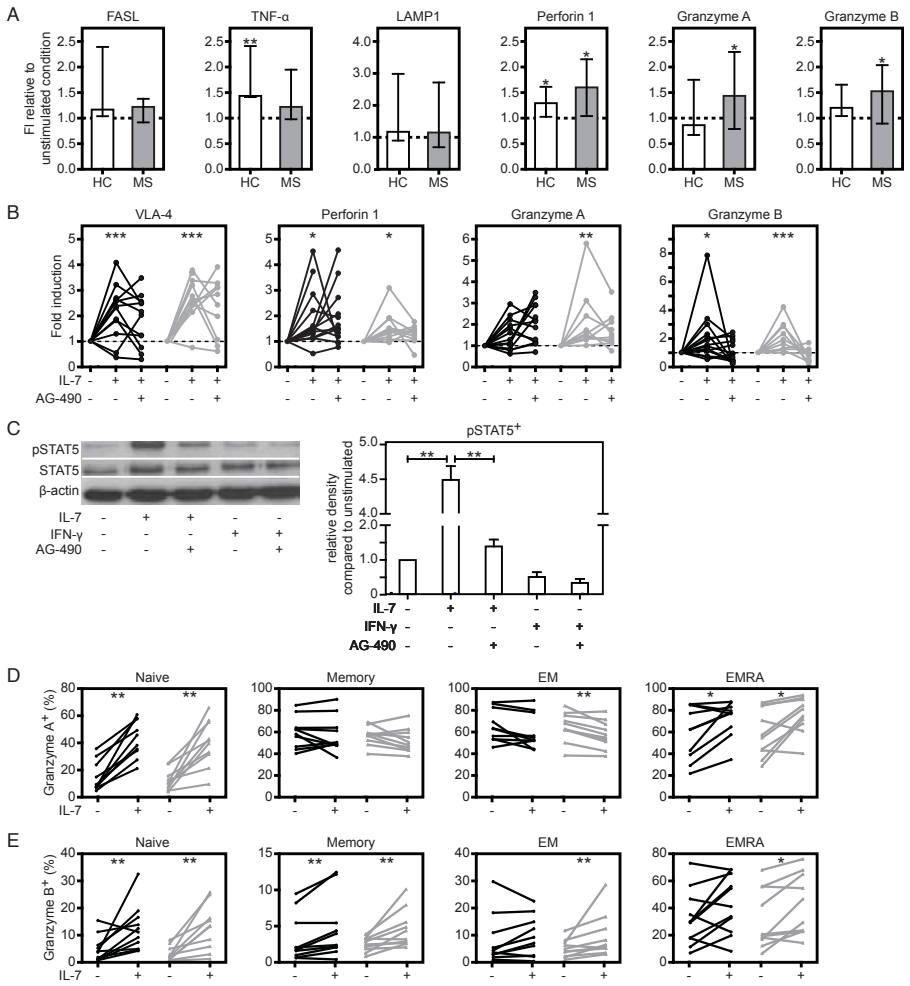
In view of the role of IL-7 in homeostasis, proliferation and anti-apoptotic signalling in T-cells and its increased receptor expression on the majority of CD8 T-cell subsets in MS patients, we next questioned whether this increased expression influenced the proliferative response in MS patients. No significant difference in IL-7 induced proliferation in PBMC was found between 25 MS patients and 24 healthy controls (Supplementary Figure 2).

Differential upregulation of cytotoxicity related genes upon IL-7 stimulation in MS patients

One of the earliest reports on the function of IL-7 implicated it as an important factor for cytotoxicity, although the exact mechanism of action was not investigated. Since most differences in the IL-7Rα pathway in MS patients were observed in CD8⁺ T-cells, we investigated the effect of IL-7 stimulation in PBMC on genes involved in cytotoxicity. LAMP1 (CD107a) a marker for degranulation and FASL (CD178) were not affected by IL-7 stimulation, whereas perforin 1 was comparably upregulated in MS and HC. Granzyme B was also upregulated in both MS and HC upon IL-7 stimulation. Interestingly, granzyme A was only upregulated in MS patients and not in HC. Finally, we tested TNF-α and observed a significant increase in TNF-α mRNA in HC and the same trend in MS, although not significant (Fig 3A).

The observed mRNA upregulation of the cytotoxic molecules was modest. To further validate and more specifically study cytotoxic gene expression and regulation, we used a secondary cohort of patients and controls. In this validation study, we used a Jak inhibitor to block the downstream effects of IL-7 on STAT5 phosporylation. All changes in gene expression were validated in this independent group of patients and controls. Moreover, the combination of the Jak inhibitor with IL-7 stimulation resulted in a partial downregulation of genes induced by IL-7 stimulation (Fig. 3B). As expected, the Jak inhibitor also led to a partial inhibition of phosphorylation of STAT5 (Fig. 3C). To further ascertain that IL-7 induces the expression of granzyme A and B, we stimulated PBMC with IL-7 for 22 hours and determined protein expression of granzyme A and B and compared this with baseline expression in functional CD8 T-cells subsets co-expressing an IL-7Rα⁺. For granzyme A in MS, a four to five fold increase in protein expression was found in naive CD8 T-cells, whereas in CD8 EMRA T-cells a slight increase was observed. A significant, though very slight decrease in granzyme A expression

Figure 3. Differential changes in IL-7-induced gene expression in MS and HC



Gene expression of an activation marker and molecules involved in cytotoxicity upon 24 h of 5 ng/ml IL-7 stimulation in PBMC. **A)** Fold changes of molecules involved in the three major cytotoxic pathways. Number of MS patients ranged from eight to 14 and of HC from seven to 14, dependent on the gene studied. Note that comparisons in figures A and B are within the groups compared to baseline. **B)** Genes with a modest significant increase upon IL-7 stimulation were tested in an independent group of eight to ten patients and 12 to 15 healthy controls for upregulation upon IL-7 stimulation. Additionally, changes in VLA-4 expression upon IL-7 stimulation were tested. Moreover, to show that the effect was IL-7 pSTAT5 dependent, a JAK inhibitor (25 μM AG-490 with pre-incubation of 2 h before IL-7 was added) was used to inhibit the pSTAT5 signal. **C)** Representative Western blot for (phosphorylated) STAT5 upon IL-7 stimulation with simultaneous addition of a JAK inhibitor. Quantification was performed with densitometry. IFN-γ served as a negative control for the upregulation of pSTAT5. **D-E)** Protein expression of granzyme A and granzyme B (resp.) was assessed before and after IL-7 stimulation by intracellular FACS in 10 HC and 10 MS patients and compared with a Wilcoxon matched-pairs signed rank test.

in MS patients was observed in CD8 EM T-cells (Fig. 3D), which might be a result of activation and subsequent degranulation, a phenomenon that has previously been shown for granzyme B [23]. In HC, only in naive and CD8 EMRA cells, a significant increase in granzyme A was observed. Granzyme B increased in MS patients in all subsets analyzed, most pronounced in the naive CD8 T-cells. In HC, the increase in granzyme B was restricted to the naive and memory CD8 subsets (Fig. 3E).

Next, we reasoned that lymphocyte trafficking, including $CD8^+$ effector memory T-cells, into the CNS [24] is mediated by very late antigen 4 (VLA-4). This trafficking is a major process in MS [25]. A recent study showed that IL-4 mediated STAT6 phosphorylation inhibited VLA-4 expression [26]. Since STAT6 is negatively regulated by STAT5 via SOCS1 and we observed a significant increased pSTAT5 upon IL-7 stimulation, we hypothesized that IL-7 might induce VLA-4 expression. Indeed, upon 24 h of IL-7 stimulation, both MS patients and HC upregulated expression of VLA-4 gene (CD49D subunit) as shown in Figure 3B.

To demonstrate that the observed effects were T-cell specific, we sorted $CD3^+$ T-cells which were subsequently stimulated according to the same protocol. A similar upregulation of genes involved in cytotoxicity was observed (data not shown). Since IL-7 can induce the production of IFN-γ, we finally investigated if the observed effects could be IFN-γ mediated instead of IL-7. PBMC and sorted $CD3^+$ T-cells were stimulated with IFN-γ, which did not lead to changes in the expression of cytotoxic genes (data not shown), confirming that the effects were indeed IL-7 mediated.

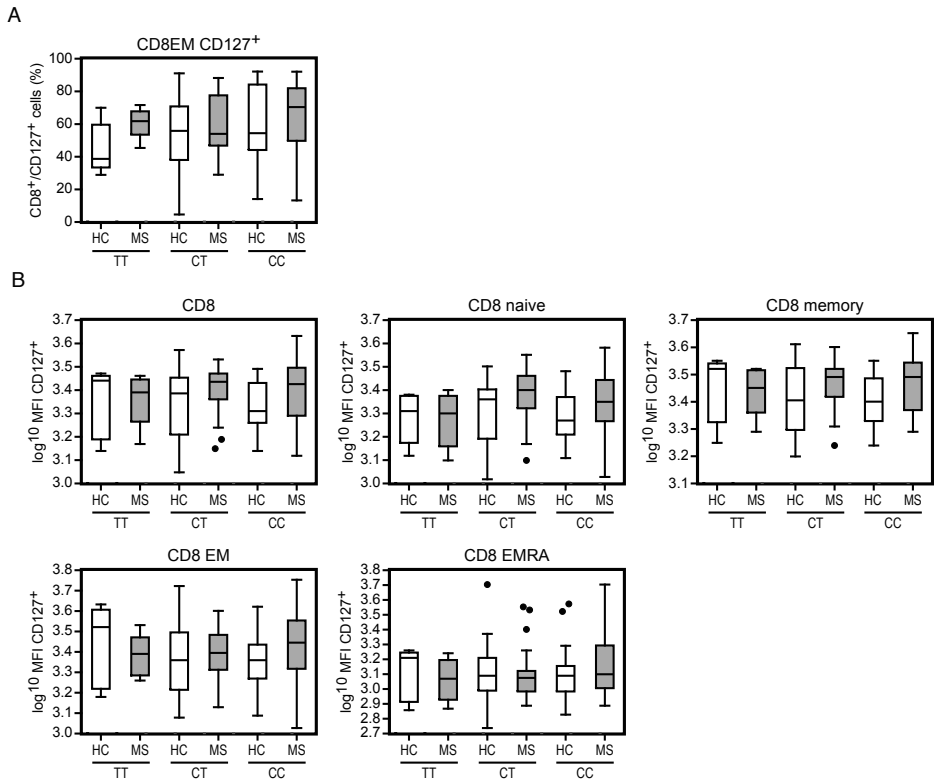
No association between the IL-7Rα risk SNP rs6897932 and increased expression of IL-7Rα

MS patients have a higher frequency of the risk allele of the SNP rs6897932. In this study we observed both a higher percentage of $CD127^+CD8$EM T-cells as well as higher expression of CD127 on most $CD8^+$ T-cell subsets. This prompted us to investigate whether rs6897932 was associated with the increased CD127 expression on CD8 T-cells in MS patients. No significant differences were found when CD127 expression was stratified according to rs6897932 homozygous risk carriership [CC] (HC n=25, MS n=38) compared to heterozygous risk carriership [CT] (HC n=26, MS n=26) and homozygous non-risk carriership [TT] (HC n=5, MS n=5) for both the frequency of $CD127^+CD8$EM (Fig. 4A) and expression of $CD127^+$ on $CD8^+$ T-cell subsets (Fig. 4B). CD127 expression on the remaining $CD8^+$ and $CD4^+$ subsets stratified to rs6897932 can be found in Supplementary Figure 3. Pooled analysis of both MS patients and healthy control showed no significant differences between the different genotypes, indicating that a power problem was highly unlikely to have occurred (data not shown).

$CD8^+IL\text{-}7R\alpha^+$ T-cells are mainly localised in pre-active MS lesions

Using immunohistochemistry, we addressed the question whether $CD8^+$ T-cells co-expressing the IL-7Rα are present in pre-active and active lesions in the brain of MS patients (for clinical details of the MS patients and NDC, see table 2). Interestingly, only in pre-active lesions in the perivascular space $CD8^+IL\text{-}7R\alpha^+$ T-cells could be detected. In active lesions, only $CD8^+$ T-cells lacking an IL-7Rα were found in white matter tissue of five MS patients.

Figure 4. CD127 expression stratified according to MS risk SNP carriership



Stratification according to rs6897932 SNP genotype. CC denotes homozygous risk genotype (HC n=25, MS n=38), CT heterozygous risk genotype (HC n=26, MS n=26) and TT homozygous non-risk genotype (HC n=5, MS n=5). **A)** Percentage of CD127$^+$ CD8EM stratified according to genotype. **B)** MFI corrected for background signal of CD127$^+$ cells for all CD8 subsets.

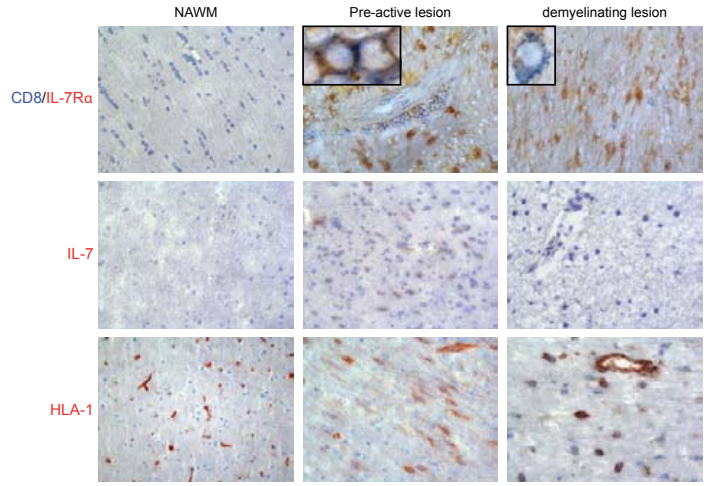
Expression of IL-7 could only be detected in pre-active MS lesions and not in active demyelinating lesions. As expected, no CD8$^+$ T-cells nor IL-7 expression could be detected in normal appearing white matter. Interestingly, also a number of microglia cells (based on morphology and CD68 expression, data not shown) co-expressed the IL-7Rα and these microglia cells were observed in both pre-active and active lesions. HLA class-I expression was predominantly found in pre-active lesions with the highest intensity compared to active lesions. In active demyelinating lesions and normal appearing white matter, only the blood vessel endothelium and some microglia cells expressed HLA class I (Figure 5). In brain materials from five non-demented controls (NDC), no CD8 T-cells were found. In a single NDC brain sample could IL-7 be detected. As expected, in NDC HLA-I could only be detected on endothelial cells of blood vessels (data not shown).

Table 2. Clinical characteristics of patients included in post-mortem IL-7/IL-7Rα expression in white matter study

	Age at death	Gender	Age at onset	Presenting symptom	MS disease form	Cause of death
NDC1	68	F	NA	NA	NA	Metastasised mamma carcinoma
NDC2	76	F				Ischemic CVA
NDC3	78	F				Pulmonary emphysema and cardiac insufficiency
NDC4	91	F				Myocardial infarction
NDC5	73	M				Colon carcinoma with liver metastases, without brain metastases
NDC6	73	M				Pulmonary embolism
MS1	40	F	26	Optic neuritis	SP	Dehydration
MS2	59	F	38	Optic neuritis	SP	Respiratory insufficiency
MS3	71	F	43	Optic neuritis	SP	Respiratory insufficiency
MS4	46	M	23	Optic neuritis	SP	Pneumonia
MS5	72	M	50	Optic neuritis	SP	Carcinoma of bladder and intestine without brain metastases

NA, not applicable

Figure 5. CD8 T-cells in pre-active white matter lesions express IL-7Rα



Double staining to detect CD8 T-cells co-expressing IL-7Rα, single staining for IL-7 and HLA class I expression was performed in pre-active, active demyelinating lesions and normal appearing white matter (NAWM). Figures are representative stainings from five patients. In columns, the different lesion types. Upper row, double staining with CD8 (blue) and IL-7Rα (red), middle row IL-7 (red) single staining and lower row HLA class I (red) single staining. Nuclei were counterstained with hematoxylin. Original magnification 430x.

Discussion

Classically, MS has been presumed to be a CD4 mediated disease, though the role of the different CD4 effector T-cells, eg. T-helper1 and T-helper17 cells is currently heavily debated [27]. In contrast, the number of articles about the role of $CD8^+$ T-cells in MS is relatively limited, although most studies report that $CD8^+$ T-cells outnumber $CD4^+$ T-cells in MS lesions [28]. Of note is the presence of clonally expanded CD8 T-cells in post-mortem tissue of active MS lesions [29] and the demonstration of oligoclonally expanded CD8 T-cells in the CSF of MS patients [30].

The homeostasis of CD8 T-cells is maintained by signals from the IL-7Rα, amongst others (10). Moreover, a functional role of IL-7Rα in MS pathogenesis is implied by the fact that a genetic variant enhances the risk for MS (5-8) and this makes it an interesting candidate for further study.

Hence, we addressed the question whether IL-7Rα expression and functioning in both $CD4^+$ and $CD8^+$ T-cells subsets is altered in MS patients. We demonstrate that the frequency of $CD127^+$ CD8EM and the expression levels of CD127 on most $CD8^+$ subsets is increased in MS patients. Upon IL-7 stimulation, a differential effect on granzyme A and B expression in MS patients was found and these effects may further enhance the cytotoxicity of these CD8EM, which are candidates to function as antigen-specific $CD8^+$ T-cells, because they have an effector memory phenotype [31].

Recently, there is accumulating attention for a regulatory subset of CD8 T-cells that co-express CD25 and FOXP3. The question rises whether the differences observed here are caused by differences in frequency of this specific subset. It should be noted however that no numerical differences in CD8 Tregs between MS patients and HC have been described [32], nor functional defects in this subset [33]. Although we previously found a significant increased frequency of CD8 Tregs co-expressing the IL-7Rα in MS patients [34], this increase was only around 5% of the total increase in IL-7Rα expression on CD8 T-cells observed in this study. Therefore an increase of $IL\text{-}7R\alpha^+$ CD8 Tregs cannot explain the abundance in IL-7Rα expression in CD8EM T-cells observed here.

In the periphery, IL-7 is expressed on stromal cells in the bone marrow and in lymphoid tissues [35]. It is thought that autoreactive T-cells in MS patients are activated in secondary lymphoid organs and then migrate across the blood brain barrier into the brain [36]. This potential transmigration is underlined by our observation that IL-7 induces VLA-4, an important marker for T-cell activation and transmigration into the brain by binding to VCAM-1 on activated brain endothelial cells and epithelial cells of the choroid plexus [37]. Natalizumab (Tysabri), a blocking monoclonal blocking antibody against VLA-4 is an approved treatment for MS [38]. Moreover, CD8EM are T-cells with the capacity to migrate to nonlymphoid tissues [39]. In the case of MS, they potentially migrate into the CNS, were they re-encounter IL-7 (Fig. 5), produced by reactive astrocytes in MS lesions [40]. This intrathecally-produced IL-7 might further activate and increase the cytotoxic potential of these T-cells by the upregulation of genes involved in cytotoxicity, as we have shown here in vitro (Fig. 3A-B). Moreover, it has been shown that both granzyme A and B are upregulated during a relapse compared with patients in remission and controls [41], further emphasizing the importance of these molecules in the MS disease course.

Granzyme A is of particular interest, because in contrast to granzyme B, it is capable of causing direct myelin damage via degradation of myelin basic protein (MBP) [42]. Moreover, oligodendrocytes are sensitive to granzyme-perforin mediated killing, whereas other neural cells are less sensitive to granzyme mediated effects [43]. Our finding that granzyme A is not upregulated in healthy controls is in concordance with a previous report [44]. It is particularly interesting that this pathway is dysregulated in MS patients.

Importantly, the observed differences can not be attributed to the genetic association in the IL-7Rα. No correlation with the rs6897932 SNP and expression of IL-7Rα was detected. Other factors must contribute to the increased expression of the IL-7Rα and dysregulation of this pathway in MS. It is conceivable that rare genetic polymorphisms with high odds ratio which are currently not detected in GWAS [45] contribute to this phenomenon, but also environmental factors can be important. One interesting environmental factor might be EBV, because infectious mononucleosis is a well known risk factor for MS [46]. An EBV infection alters the expression of the IL-7Rα and IL-15R, an IL-7Rα family member. During acute infection, almost all $CD8^+$ T-cells loose their IL-7Rα expression, which quickly recovers after infection [47]. Moreover, antigen-specific CD8EM can persist in inflamed tissue [48] for a long time after clearance of viral antigens. The observed pattern of expression in the different types of lesions of MS patients suggests that in the pre-active lesions all requirements are locally met to initiate a strong anti-viral immune response with cytotoxic potential. In contrast, fully activated $CD8^+$ T-cells present in active demyelinating lesions do not require IL-7 and therefore lack IL-7Rα expression. Alternatively, it can not be excluded that the $CD8^+$ T-cells expressing IL-7Rα have bound IL-7 prohibiting detection.

In our study, we initially included only untreated relapsing-remitting MS patients. The main advantage of this approach is that the results are not be influenced by the use of immunomodulating therapy. Conversely, this approach might have introduced a potential bias, because untreated patients are generally more mildly affected and differences between relapsing-remitting and primary progressive patients can not be assessed. We are confident that the severity bias has been eliminated by the inclusion of 15 treated patients in the second phase of this study, confirming the initial findings. Moreover, the inclusion of both primary and secondary progressive MS patients has likely prevented the second disadvantage, especially because no differences between subgroups were detected.

The IL-7Rα/IL-7 pathway is also implicated in other autoimmune diseases. For example, IL-7Rα expression by lymphoid cells in the joint in rheumatoid arthritis (RA) patients is increased compared with osteoarthritis patients [49]. Treatment response to methotrexate in RA patients correlated with decreased IL-7 serum level, but not with TNF-α levels [50]. In a mouse model for RA, anti-IL-7 treatment completely inhibited autoimmune arthritis [51]. Moreover, IL-7 expression is increased in the inflamed salivary glands in primary Sjögren's syndrome compared with non-Sjögrens sicca syndrome patients [52].

In conclusion, MS is associated with increased expression of IL-7Rα on most $CD8^+$ T-cell subsets and with higher frequencies of CD8EM cells co-expressing IL-7Rα. Functional experiments showed a lower threshold for IL-7 induced $CD8^+$ cytotoxicity in MS patients. We also confirmed earlier reports

that IL-7 is produced in the lesions of MS patients, strongly suggesting that IL-7 and its receptor are a functionally relevant couple at the site of the pathology. Differences between patients and controls could not be explained by the rs6897932 MS risk SNP. A recent study showed that IL-7Rα knock out mice are almost completely protected against experimental autoimmune encephalomyelitis (EAE) [53], the experimental model of MS. Taken together, the IL-7 / IL-7Rα pathway appears an interesting target for therapeutical interventions in MS.

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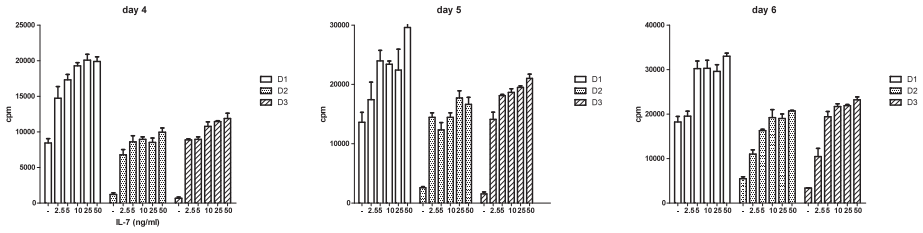
Supplementary materials

Supplementary Table 1. Taqman primers

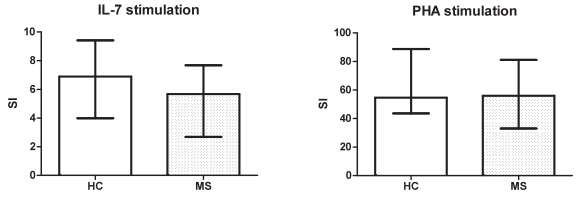
Gene	Forward primer sequence	Reverse primer sequence
FASL (CD178)	GCTGGCAGAACTCCGAGA	TTTTCAGGGGGTGGACTG
TNF-α	GCCCAGGCAGTCAGATCATC	GGGTTTGCTACAACATGGGCT
LAMP1 (CD107a)	GTGGGTCCAGGCTTTCAA	AGCATGCTGTTCTCGTCCA
Perforin	CCGCTTCTCTATACGGGATTC	GCAGCAGCAGGAGAAGGAT
Granzyme A	TTAACCCTGTGATTGGAATGAAT	AGGGCTTCCAGAATCTCCAT
Granzyme B	CGGTGGCTTCCTGATACAA	CCCCAAGGTGACATTTATGG
VLA-4 (CD49D)	GGAATATCCAGTTTTTACACAAAGG	AGAGAGCCAGTCCAGTAAGATGA
18S	Commercial kit Applied Biosystems	Commercial kit Applied Biosystems

Supplementary Figure 1. IL-7 titration experiment



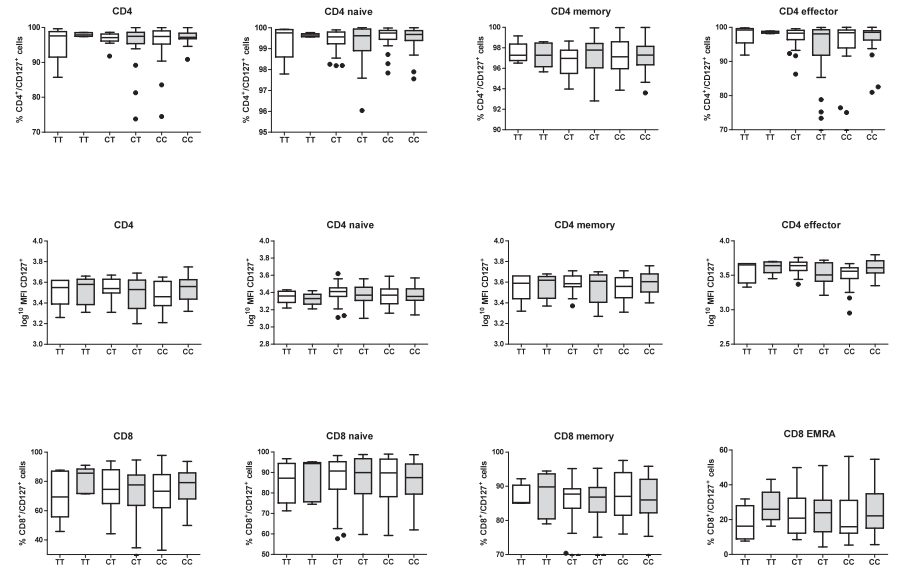
Determination of the optimal IL-7 dose and stimulation duration in 3 independent healthy donors. Read out is incorporation of ^{3}H-thymidine after 18 h.

Supplementary Figure 2. Proliferative response upon IL-7 stimulation



1*10E5 PBMC of 25 MS patients and 24 HC were cultured in triplo for 3 days with or without IL-7 or PHA. ^{3}H-thymidine incorporation was measured on day three. Stimulation index was calculated using the unstimulated condition as the reference situation.

Supplementary Figure 3. No correlation between the rs6897932 [C] genotype and CD127 expression



Upper row percentage of CD127^{+} CD4 T-cells, middle row MFI of CD127^{+} CD4 T-cells and lower row the remaining percentages of CD127^{+} CD8 T-cells. White bars are healthy controls, grey bars are MS patients.

Chapter 2.2

Role of CD8 regulatory T-cells in multiple sclerosis.

K.L. Kreft, E. Verbraak, A.F. Wierenga-Wolf, J.D. Laman and R.Q. Hintzen

Annals of Neurology 2011;69:593

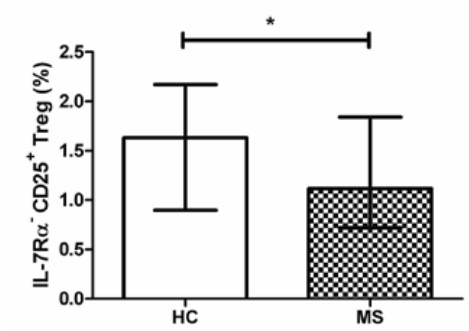
Letter to the editor.

We were positively intrigued by the article of Correale and Villa [1] about CD8 regulatory T-cells (Treg) in MS patients. Several interesting issues were elucidated in this study. First, no numerical differences in $CD25^+FOXP3^+$ $CD8^+$ T-cells were found in this small study with ten MS patients and ten healthy controls (HC). This is in line with another recent study [2] and also with our findings in a larger cohort of 32 stable MS patients and 37 healthy controls (unpublished).

Second, no functional differences could be found in the suppressive capacity of $CD8^+CD25^+FOXP3^+$ T-cells derived from peripheral blood of MS patients compared to HC. [1] It is of note that in the $CD4^+$ Treg subset, the $CD25^+FOXP3^+$ cells consist of at least two distinct populations: cells co-expressing the IL-7R (CD127) versus IL-7R negative cells. Importantly, FOXP3 acts as a suppressor of the promoter of the IL-7R gene. [3] It has hence been suggested that functionally active FoxP3 suppresses IL-7R expression and that IL-7R expression therefore is a proxy of the suppressive capacity of Tregs. [4] Accordingly, IL-7R negative regulatory T-cells are considered to be the most effectively suppressive Tregs.

Within our $CD25^+FOXP3^+$ CD8 T-cells, we found a significantly decreased percentage of $IL\text{-}7R^-$ $CD25^+FOXP3^+$ CD8 T-cells in MS patients compared to HC (Fig. 1). In parallel, a significantly increased frequency of $IL\text{-}7R^+$ regulatory CD8 T-cells in MS patients was detected.

Figure 1. $IL\text{-}7R\alpha^-$ CD8 Treg are decreased in MS patients



CD8 Tregs were gated according to $CD3^+$ $CD8^+$ $CD25^{+/bright}$ $FOXP3^+$ and $CD127^-$ and compared between 37 HC and 32 MS patients. Cells were fixed and permeabilised using the FOXP3 staining set (eBioscience) according to the manufactures' protocol. All antibodies were obtained from BD Biosciences, except the FOXP3 antibody which was obtained from eBioscience. Cells were measured on an LSRII and analysed using Facs Diva software 6.1 (BD). Statistical analysis was performed using a Mann Withney U-test in SPSS version 17 (SPSS).

As expected, in view of the suppressor action of FOXP3 on the IL-7R promoter, the level of FOXP3 expression was significantly higher in $IL\text{-}7R^-CD25^+FOXP3^+$ compared to $IL\text{-}7R^+CD25^+FOXP3^+$ (data not shown).

In conclusion, the balance between CD8 regulatory T-cells with a potentially high, and those with a potentially low suppressive capacity based on IL-7R expression is disturbed in MS patients. Therefore, we suggest to stratify CD8 Treg according to IL-7R expression and it would be of interest to assess if differential IL-7R expression on the T-cell clones used by Correale and Villa is related with their suppressor function.

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Chapter 3

Decreased systemic IL-7 and soluble IL-7Ralpha in multiple sclerosis patients.

K.L. Kreft, E. Verbraak, A.F. Wierenga-Wolf, M. van Meurs, B.A. Oostra, J.D. Laman and R.Q. Hintzen

Genes and Immunity 2012; 13(7):587-92

Abstract

Polymorphisms (SNP) in the IL-7Rα/IL-7 pathway are associated with an increased risk to develop multiple sclerosis (MS). The rs6897932 SNP in the IL-7Rα leads to increased soluble IL-7Rα production. Given the functional interaction between sIL-7Rα, membrane bound IL-7Rα and IL-7, we assessed IL-7, mIL-7Rα and sIL-7Rα levels in MS patients and healthy controls (HC).

128 MS patients had significantly lower sIL-7Rα levels compared to 73 HC. The levels of sIL-7Rα increased dose-dependent upon rs6897932 [C] risk allele carriership in both HC and MS. Next, we hypothesized that lower sIL-7Rα could result in a higher mIL-7Rα to soluble IL-7Rα ratio. Indeed, 52 MS patients had significantly increased mIL-7Rα to sIL-7Rα ratio for both CD4 and CD8 T-cells compared with 44 HC. Given the supposed role of IL-7 in autoimmunity, we determined whether sIL-7Rα influences IL-7 levels. IL-7 levels were significantly decreased in 40 MS patients compared to 40 HC. In conclusion, MS patients had lower free IL-7, higher membrane to soluble IL-7Rα ratio. The soluble IL-7Rα levels correlate with the rs6897932 [C] risk allele carriership. The skew at the IL-7 and IL-7Rα level may influence responsiveness of IL-7Rα$^+$ cells.

Introduction

One of the first non-HLA genetic polymorphisms found to be associated with an increased risk to develop multiple sclerosis (MS) is the IL-7Rα (rs6897932) [1-3]. Recently, also a detailed genetical analysis found associations with MS risk at different levels in the entire IL-7Rα pathway, for example in the IL-7 gene itself and in the downstream signaling molecules TYK2 and SOCS1 involved in cytokine signaling and secretion [4].

The IL-7Rα/IL-7 axis is an interesting candidate in autoimmunity, because it is an important survival factor for CD4 and CD8 T-cells, and is involved in early B-cell development [5]. Interestingly, IL-7Rα is genetically implicated in other autoimmune diseases, like rheumatoid arthritis (RA) [6] and type I diabetes mellitus (DM I) [7]. Increased expression of the IL-7Rα on T- and B-lymphocytes and macrophages in rheumatoid arthritis and undifferentiated arthritis compared with osteoarthritis was found in the inflamed joint [8].

A transfection study showed that the MS risk SNP rs6897932 [C] caused more exon 6 mRNA splicing, which encodes the transmembrane domain of the receptor [3], potentially leading to altered soluble levels of the receptor. A recent study correlated rs6897932 [C] with sIL-7Rα on protein level, but could not detect differences between MS patients and controls [9]. However, this study did not investigate whether sIL-7Rα levels correlate with altered membrane bound IL-7Rα levels. A single study showed higher IL-7 mRNA levels in the mononuclear cells in CSF of MS patients compared with other neurological diseases without oligoclonal bands [2]. On the protein level, only one study with MS patients in remission and during active disease exists [10]. However, this study did not investigate IL-7 in the context of sIL-7Rα.

The aim of the current study was to assess in a larger and well specified cohort of MS patients whether differences between MS patients and HC occur and whether sIL-7Rα levels are genetically driven by the rs6897932 [C] polymorphism. Moreover, we assessed whether levels of soluble and membrane bound IL-7Rα levels are correlated. Since sIL-7Rα might alter unbound IL-7 levels, we determined IL-7 levels to investigate aberrancies in the IL-7/IL-7Rα axis in MS patients.

Materials and methods

Patients and controls

Consecutive MS patients diagnosed according to the McDonald criteria for multiple sclerosis [11] were included in this study. A relapse or the usage of methylprednisolone three months prior to sampling were exclusion criteria. Healthy controls were relatives of the MS patients. Healthy controls with prior neurological symptoms suggestive for MS, such as optic neuritis or myelitis or the diagnosis of MS or the usage of any immunomodulating therapy for other autoimmune diseases were exclusion criteria. This study was approved by the Medical Ethical Committee of the Erasmus MC and written informed consent was obtained from all patients and controls.

sIL-7Rα ELISA

Plasma samples were collected using CPT Heparin Tubes (BD) and processed according to the manufacturers protocol. The ELISA for sIL-7Rα was used as previously described [12]. Briefly, a capture antibody (monoclonal mouse anti-human IL-7Rα antibody, clone 40131, R&D Systems) was coated overnight onto Nunc Maxisorb 96 wells plates. 100 µl plasma was incubated at room temperature for 1 h under constant shaking and a detection antibody directly biotinylated was subsequently added for 1 h (biotinylated goat anti-human IL-7Rα antibody, R&D Systems), followed by TMB substrate reaction detected at 450 nm wavelength and corrected for a 605 nm wavelength. Recombinant human IL-7Rα (R&D systems) was used to make a standard curve and the detection limits of this ELISA were 21 and 300 ng/ml. To assess whether the results of the sIL-7Rα ELISA are influenced by IL-7 binding to the sIL-7Rα, plasma samples were pre-incubated with 50 pg/ml of IL-7 (Peprotech) overnight at room temperature. Moreover, a linear dilution of IL-7 was used to assess whether high concentrations of IL-7 are able to block the sIL-7Rα detection in the ELISA. After the incubation of recombinant proteins, sIL-7Rα was assessed as described and compared with the same plasma sample without addition of IL-7.

IL-7 ELISA

Serum samples of 40 MS patients and 40 HC were drawn using coagulation tubes (BD) and samples were processed according to the manufacturers' protocol and stored at -80 °C until analysis. 250 µl of serum was analyzed for IL-7 according to the manufacturers' protocol (Quantikine HS IL-7 ELISA, R&D Systems). The detection range was between 0.22-14.79 pg/ml. We determined whether the detection of IL-7 in this ELISA is influenced by binding of IL-7. Therefore serum was pre-incubated with 250 ng/ml recombinant sIL-7Rα overnight and IL-7 levels were determined as described in 'untreated' and pre-incubated serum. Moreover, a linear dilution of the sIL-7Rα in serum samples was used to determine whether high concentrations of sIL-7Rα would inhibit the detection of IL-7.

Qualitative ELISA to detect complexes of sIL-7Rα and IL-7

Plasma and serum samples were assessed for the presence or absence of complexes between the sIL-7Rα and IL-7. The capture antibody against the IL-7Rα (monoclonal mouse anti-human IL-7Rα antibody, clone 40131, R&D Systems) was coated overnight onto Nunc Maxisorb 96 wells plates. Plasma and serum was incubated, constant shaking, at room temperature for 1 h. Subsequently, a directly biotinylated antibody against IL-7 (biotinylated goat anti-human IL-7 antibody, R&D Systems) was used as detection antibody, followed by a TMB substrate reaction detected at 450 nm wavelength and corrected for the 605 nm wavelength. As positive control, recombinant human sIL-7Rα (50 ng/ml) and IL-7 (5 nM) was incubated at room temperature overnight to form complexes.

Flowcytometry

Peripheral blood mononuclear cells (PBMC) were isolated from CPT tubes (BD), liquid nitrogen frozen until flowcytometric analysis. 1*10E6 PBMC of 44 HC and 52 MS patients were stained with mouse

monoclonal anti-human antibodies against CD3 FITC (clone SK7), CD4 PE-Cy7 (clone SK3), CD8 APC-Cy7 (clone SK1), CD27 APC (clone L128), CD45RA PerCp-Cy5.5 (clone HI100) and CD127 PE (IL-7Rα, clone eBioRDR5). All antibodies were obtained from BD Biosciences, except CD45RA PerCP-Cy5.5 and CD127 PE (eBioscience). Samples were measured on an bead-calibrated LSR II (BD Biosciences) and data was analyzed with Facs Diva 6.1 software.

Genotyping

Genotype data was obtained using either Illumina Affymetrix 650k or Taqman genotype assay (assay id C_2025977_10). All assays were performed according to the manufacturers' protocol. In all assays, the standard call rate was >95%. All water controls were successful, the CEPH-A and CEPH-B were concordant and all duplicates were concordant. Both cases and controls were in Hardy Weinberg equilibrium (resp. p=0.98 and 0.89).

Statistical analysis

Differences were tested using a Mann-Whitney U-test (comparing two groups) or non-parametric one-way ANOVA (comparing multiple groups) using SPSS version 17. For the inhibition ELISA for IL-7 and sIL-7Rα, a paired t-test was used. p-values <0.05 were considered significant and are denoted in the figures as * p<0.05, ** p<0.01, *** p<0.001.

Results and discussion

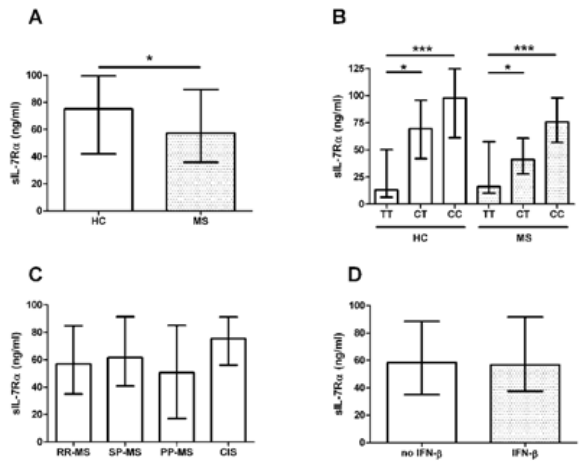
We assessed the concentration of soluble IL-7Rα in plasma of MS patients (n=128) and HC (n=73, table. 1). MS patients had significantly lower levels of sIL-7Rα compared with HC (median MS 57.3 ng/ml, interquartile range (IQR) 35.9-89.4 ng/ml vs HC 75.2 ng/ml, IQR 42.1-99.5 ng/ml, p=0.04), Fig. 1A. This is an unexpected finding, because the frequency of the rs6897932 [C] SNP is increased in MS patients, which supposedly causes increased splicing of exon 6 of the IL-7Rα. To test whether this SNP correlates with sIL-7Rα level, we stratified these results according to rs6897932 [C] risk carriership. In both MS patients and healthy controls, the C-allele risk carriership indeed strongly correlates with higher levels of the sIL-7Rα. A dose-dependent increase of the sIL-7Rα was found in C-allele risk carriers (Fig. 1B). Homozygous C risk carriers had significantly (p<0.0001) more sIL-7Rα (median 75.6 ng/ml, IQR 56.7-97.9 ng/ml, n=59) compared with 41.2 ng/ml (IQR 27.9-60.7 ng/ml, n=51) in the heterozygous C risk carriers and 16.0 ng/ml (IQR 10.2-57.47 ng/ml, n=7) in the TT non-risk carriers. In HC, the same association was observed (p<0.0001), confirming a previous study [9]. No significant differences were found between 17 primary-progressive, 23 secondary-progressive, 82 relapsing-remitting MS patients and 6 clinically isolated syndrome patients (Fig. 1C). Additionally, no differences were observed between 40 MS patients treated with immunomodulating therapy and 88 untreated MS patients (Fig. 1D).

Table 1. Demographic characteristic

	CIS [1] (n=6)	RRMS (n=91)	SPMS (n=25)	PPMS (n=19)	HC (n=84)
Age at onset (SD)	33 (8)	32 (10)	31 (8)	40 (11)	NA
Age at sampling (SD)	36 (8)	43 (11)	48 (9)	51 (11)	41 (13)
Disease duration (SD)	NA	6 (6)	13 (9)	8 (11)	NA
Female percentage	100	76	84	53	58
Presenting symptoms (n) Optic nerve Spinal cord Brain stem/cerebellum Cerebrum Multifocal	 1 2 1 1 1	 26 28 20 5 12	 6 10 2 1 6	 3 10 2 3 1	NA
CSF abnormal (oligoclonal bands and/or IgG-index >0.67) n/total	4/5	56/69	11/14	14/16	NA
Use of immunomodulating therapy (n)	0	33	7	NA	NA
rs6897932 (n) TT CT CC	 0 1 5	 5 39 40	 3 7 13	 1 8 8	 9 32 35

[1] Of note, three CIS patients developed RR-MS after sIL-7Rα quantification. The remaining three CIS patients were respectively followed for 3, 24 and 53 months.
NA: not applicable

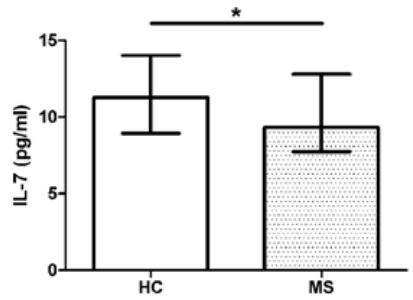
Figure 1. Soluble IL-7Rα levels are decreased in MS patients and strongly correlate with the rs6897932 [C] risk genotype in MS and HC



A) sIL-7Rα levels were compared between 128 MS patients and 73 HC. **B)** sIL-7Rα levels in HC and MS stratified according to rs6897932 [C] risk genotype. **C)** Comparison of sIL-7Rα levels between different MS subtypes. RR-MS n=82, SP-MS n=23, PP-MS n=17, CIS n=6 **D)** sIL-7Rα levels were compared in the plasma of 88 untreated and 40 IFN-β treated MS patients.

A possible consequence of the decreased level of sIL-7Rα is an increased level of circulating free IL-7. To address the question whether IL-7 levels are altered in MS patients, we determined the IL-7 concentration in serum of a subset of MS patients (n=40) and HC (n=40). In the MS group serum IL-7 levels were slightly but significantly decreased (median MS 9.3 pg/ml, IQR 7.8-12.8 pg/ml vs HC 11.3 pg/ml, IQR 8.9-14.0 pg/ml, p=0.03, Fig. 2). This finding appears in contrast with a recent study by Haas and colleagues that described increased levels of systemic IL-7 [10]. This discrepancy may be explained by differences in the level of disease activity (our study included patients in remission only).

Figure 2. IL-7 serum levels are decreased in MS patients



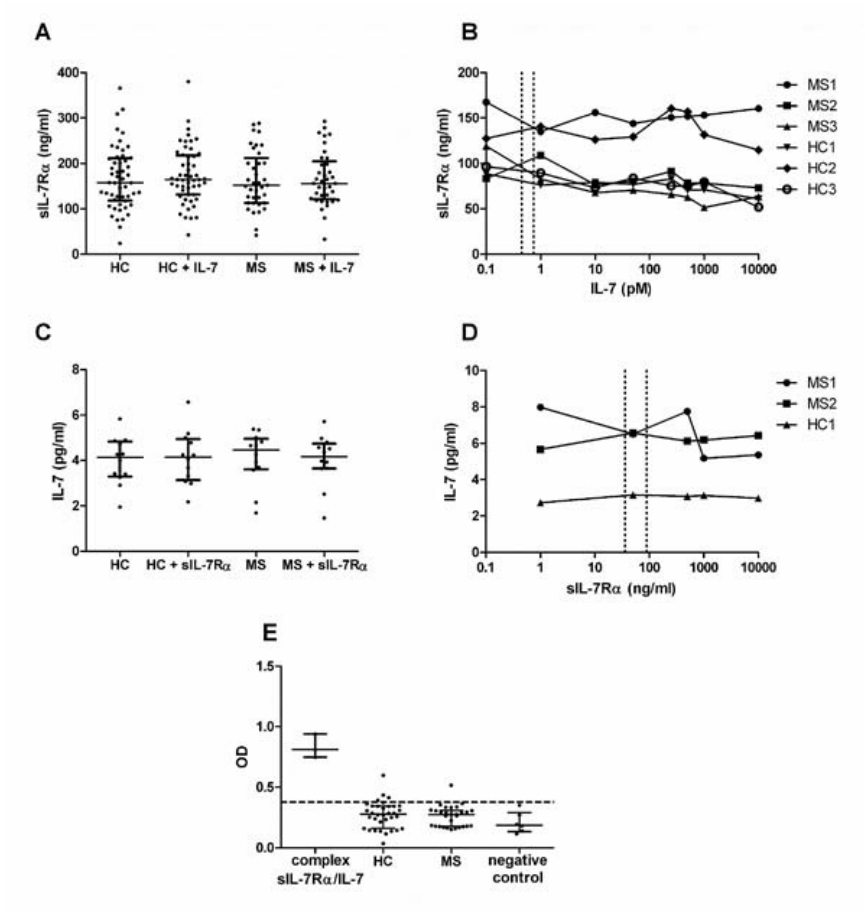
Comparison of systemic IL-7 serum levels between 40 MS patients and 40 healthy controls.

IL-7 is an important cytokine, expressed mainly in the thymus, bone marrow and lymphoid tissues, where it facilitates T-cell development and expansion, thereby contributing to the homeostasis of the peripheral T-cell pool [13]. No studies exist on the expression of IL-7 in the thymus of MS patients versus HC. It can be hypothesized that these lower systemic IL-7 levels also reflect lower thymic IL-7 levels, which might contribute to the lower number of recent thymic emigrants observed in MS [14]. Moreover, IL-7Rα signaling is required for intrathymic regulatory T-cell development [15].

Recently, it was shown that the receptor affinity of the soluble form is comparable with that of the membrane bound IL-7Rα [12] and that the soluble receptor can inhibit IL-7 signaling in CD8 T-cells of HIV patients [16]. Therefore, we investigated whether our results are influenced by complexes between IL-7 and sIL-7Rα. First, we assessed whether the sIL-7Rα ELISA is inhibited by IL-7 binding. Therefore, we pre-incubated the plasma of 36 MS and 52 HC with 50 pg/ml of IL-7 and compared the sIL-7Rα levels determined with the results obtained from untreated plasma. Pre-incubation with IL-7 did not influence the signal in both HC and MS (paired t-test resp. p=0.41 and p=0.89) indicating that the results of the ELISA are not altered by binding of IL-7 to the sIL-7Rα (Fig. 3A). We did not observe a reduction in signals of sIL-7Rα up to 10.000 pM (equals 174.000 pg/ml) of IL-7 (Fig. 3B). Next, we investigated whether the IL-7 ELISA signal is inhibited by binding of the sIL-7Rα to IL-7. Therefore, we pre-incubated serum with 250 ng/ml of recombinant sIL-7Rα overnight. This did not result in an

inhibition of the detection of IL-7 in both HC and MS (paired t-test resp. p=0.52 and p=0.27, Fig. 3C). Concentrations of sIL-7Rα up to 10.000 ng/ml did not have any inhibitory effect on IL-7 detection (Fig. 3D).

Figure 3. Complexes between IL-7 and sIL-7Rα are rarely detectable in MS and HC and are not affecting the detection of IL-7 and sIL-7Rα.



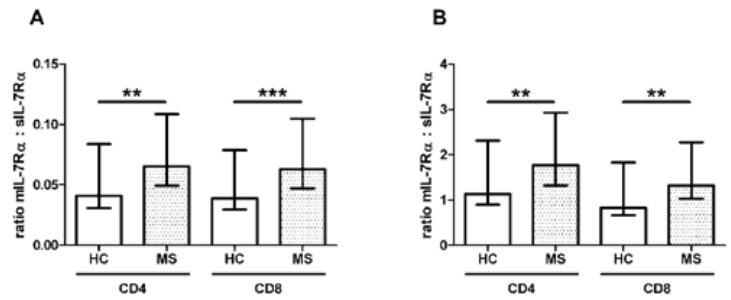
A) sIL-7Rα levels were determined directly ex vivo and after pre-incubation with 50 pM IL-7 (equals 870 pg/ml) in 52 HC and 36 MS patients. **B)** In three MS patients and three age and gender matched HC, IL-7 was titrated into the plasma in a dose-dependent fashion. Physiological levels of IL-7 are indicated between the dashed lines. **C)** IL-7 levels were determined directly ex vivo or after overnight incubation with 500 ng/ml sIL-7Rα in 12 MS patients and 12 age and gender matched HC. **D)** IL-7 detection after a dose dependent addition of the sIL-7Rα overnight in one healthy control and two MS patients. Physiological levels of IL-7 are indicated between the dashed lines. **E)** Complexes of IL-7 bound to the sIL-7Rα were determined in the plasma of 29 MS patients and 34 age and gender matched HC.

Moreover, we assessed whether the IL-7 ELISA is detecting IL-7 bound to the sIL-7Rα. To measure such complexes we set up an ELISA using anti-IL-7 as a primary antibody and anti-sIL-7Rα as a secondary antibody. No complexes were found using this approach in eight MS patients and eight age and gender HC (data not shown). Using the reverse approach, now using anti-sIL-7Rα as a capture antibody and anti-IL-7 as detection antibody, we found comparable results. Only in 1/29 MS patients and 1/34 HC, sIL-7Rα/IL-7 complexes were detected (Fig. 3E, positive control included recombinant IL-7 (35 nM) and sIL-7Rα (50 ng/ml).

These observations indicate that it is not likely that complexes between IL-7 and sIL-7Rα have influenced the ELISA measurements. Comparable conclusions were drawn by Rose et al. [12].

Membrane expression of the IL-7Rα on most CD8 T-cell subsets is significantly increased in MS patients and the frequency of IL-7Rα positive CD8 effector memory subset (CD8EM defined as $CD27^{-}$ $CD45RA^{-}$) is significantly higher in MS patients [17]. Therefore, we investigated whether systemic concentrations of sIL-7Rα correlate with the level of IL-7Rα membrane expression on functional CD4 and CD8 lymphocyte subsets. We found that in 52 MS patients compared with 44 HC the ratio between mean fluorescence intensity (MFI, an estimate for the number of receptors per cell) of IL-7Rα on both CD4 and CD8 T-cells and the sIL-7Rα levels is significantly increased (Fig. 4A and B, $p<0.01$. Equal differences were found in all functional CD4 and CD8 T-cell subsets, all $p<0.01$, data not shown). Previously, we showed that no correlation exist between levels of membrane IL-7Rα expression and the rs6897932 [C] MS risk genotype [16]. Hence, only the levels of the soluble IL-7Rα correlate with the risk genotype (Fig. 1B). It is conceivable that the lower serum concentrations of 'unbound' IL-7 and 'free' sIL-7Rα, in conjunction with the increased membrane bound IL-7Rα found in MS patients lead to an increased IL-7 responsiveness when IL-7 binds to the membrane bound receptor.

Figure 4. Increased ratio of membrane to soluble IL-7Rα in MS patients



For both CD4 and CD8 T-cells, **A)** the MFI of IL-7Rα and **B)** the frequency of IL-7Rα positive cells was determined and the ratio between membrane bound IL-7Rα and sIL-7Rα levels in plasma obtained from the same sample was determined in 44 HC and 52 MS patients.

The increased ratio of membrane to soluble IL-7Rα in MS patients in conjunction with the increased frequency of CD8EM co-expressing the IL-7Rα is of potential interest, because these cells are specialized

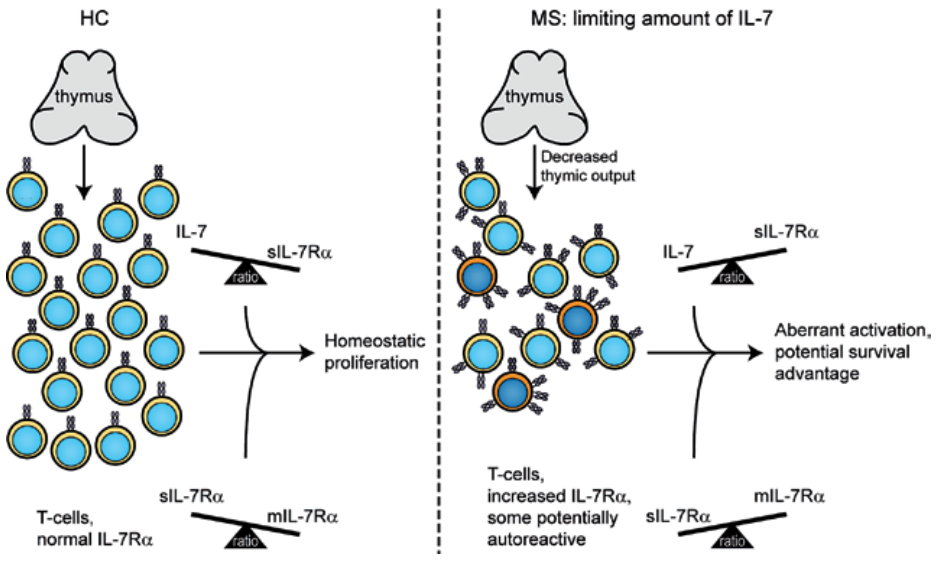
in tissue invasion and homing within inflamed organs [18]. Increased IL-7Rα signaling capacities were observed in CD8 T-cells of MS patients, leading to increased mRNA expression of cytotoxic molecules [17]. Moreover, altered expression of both the membrane and soluble form potentially provides a survival advantage, since membrane bound IL-7Rα signaling prevents activation induced cell death [19].

Reactive astrocytes in MS white matter brain lesions produce IL-7 [20], suggesting that the potential increased IL-7 responsiveness of CD8 T-cells leads to enhanced cytotoxic responses in the brain, potentially inducing tissue damage. Moreover, this locally produced IL-7 in the brain may contribute to local expansion of these T-cells.

Whether IL-7 levels along with membrane and soluble IL-7Rα expression are altered during different phases of disease activity in RR-MS patients (eg. during relapses vs in remissions) deserves to be further studied.

In conclusion, we showed that MS patients have lower systemic levels of IL-7 and sIL-7Rα and increased ratio of membrane bound to soluble IL-7Rα. This might facilitate cellular responses by cells expressing high levels of IL-7Rα, which are potentially auto-reactive lymphocytes (Fig. 5). The sIL-7Rα levels are genetically driven by the rs6897932 [C] MS risk SNP. Targeting the IL-7Rα pathway might be a promising therapeutic intervention in MS as well as other in autoimmune diseases, for example rheumatoid arthritis [21].

Figure 5. Model of altered IL-7/IL-7Rα pathway in MS



Model of the IL-7/sIL-7Rα/mIL-7Rα axis in MS. MS patients have lower levels of IL-7 and sIL-7Rα, but the ratio between these two parameters is altered. Moreover, the ratio between mIL-7Rα and sIL-7Rα in MS is significantly increased, thereby facilitating aberrant activation of potentially auto-reactive T-cells.

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Chapter 4

Multiple sclerosis-associated CLEC16A is a key regulator of late endosomal processing and surface expression of HLA class II in antigen-presenting cells.

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Manuscript submitted

Abstract

Members of the C-type lectin family are key drivers of antigen-presenting cell (APC) responses to antigens. C-type lectin CLEC16A is a risk gene for several autoimmune disorders, including multiple sclerosis (MS), but the underlying mechanism is poorly understood. Here, we report that CLEC16A is coupled to the function of HLA class II (HLA-II), the prime autoimmune disease risk locus. CLEC16A knockdown in different human APC interferes with the biogenesis of HLA-II$^+$ late endosomes compartments, impairing plasma membrane expression of HLA-II. In addition, we show evidence that CLEC16A participates in the late endosomal dynein motor complex, which regulates late endosomal biogenesis. In primary MS tissues, CLEC16A expression levels are significantly elevated. Vitamin D, an important protective environmental factor in MS, downmodulates CLEC16A in parallel with HLA-II in primary APC. These data reveal that CLEC16A is crucial for late endosomal biogenesis and processing of HLA-II and highlight its functional relevance for autoimmune disease.

Introduction

Recent genome-wide association (GWA) studies have shown that single nucleotide polymorphisms (SNP) in the C-type lectin domain family 16, member A (CLEC16A) gene are associated with increased risk to develop multiple sclerosis (MS)[1-3], and several other autoimmune diseases, such as type I diabetes [4], celiac disease [5], primary biliary cirrhosis [6]. Hence, CLEC16A appears to play a critical role in inflammatory pathology.

CLEC16A is a member of the C-type lectin family of receptors, which are broadly expressed by antigen-presenting cells (APC) and share innate immune functions in recognition of pathogens or (altered) self molecules via their carbohydrate recognition domains (CRD) [7, 8]. Certain C-type lectins, such as DC-SIGN, DEC205 and CLEC9A (DNGR-1), have specific internalization motifs to target antigens into the endosomal pathway for HLA-mediated presentation to T cells. Although several C-type lectins are able to stimulate antigen presentation, the underlying mechanisms remain to be fully elucidated.

Currently the function of CLEC16A is enigmatic. It was predicted that CLEC16A contain a CRD that is too short to be functionally active [9, 10], suggesting a non-classical C-type lectin function. The CLEC16A gene, together with a set of other immune genes, is located on the short arm of chromosome 16, adjacent to the class II transactivator (CIITA) [11], the master regulator of HLA class II (HLA-II) transcription. The HLA-II locus is known as the strongest genetic risk factor in MS [3] and other autoimmune diseases . New molecules are being recognized that regulate HLA-II processing and show evidence for a role in autoimmunity, as we recently reported [12].

In a previous ***Drosophila*** study, Ema the orthologue of human CLEC16A, was identified as a molecule involved in endosomal maturation [13], which is a critical step for HLA-II antigen presentation. After synthesis in the endoplasmic reticulum, HLA-II molecules are transported to specialized late endosomes termed MIIC (MHC class II-containing compartments) [14, 15]. In MIIC, HLA-II molecules are loaded with peptides and eventually transported to the plasma membrane. Given the impact of endosomal maturation on HLA-II antigen presentation, we hypothesize that CLEC16A regulates these processes.

In the present report, we provide conclusive evidence for a role of CLEC16A in endosomal processing and surface expression of HLA-II in different types of APC. In addition, expression levels of CLEC16A were significantly increased in MS patients compared to controls. Therefore, the immune function of CLEC16A likely increases the risk for a broad spectrum of autoimmune diseases by enhancing HLA-II antigen presentation to possible pathogenic CD4$^+$ T helper cells.

Methods and materials

Patients and healthy controls

Consecutive MS patients in the Rotterdam MS centre ErasMS, aged between 18 and 65 years, diagnosed with MS according to the McDonald criteria were included [16]. Healthy donors included in this study, aged between 18 and 55 years, were relatives or family members of patients. Exclusion criteria were prior symptoms suggestive for MS or diagnosis with MS and use of immunomodulatory drugs for other autoimmune diseases. This study was approved by the Medical Ethical Committee of the Erasmus MC. All participants gave written informed consent.

Post-mortem brain material

White matter tissues of MS patients and age- and gender-matched non-demented controls were obtained from The Netherlands Brain Bank (NBB) in Amsterdam, The Netherlands. Patient material has been collected from donors who gave written informed consent for brain autopsy and use of this material and clinical information for research purposes. Ethical approval was obtained by the NBB. Cryopreserved brain tissues were stored in -80ºC until use.

Human cell cultures

The melanoma cell line MelJuSo was cultured in IMDM (Lonza) supplemented with 8% FCS. Rab7-GFP-transfected MelJuSo was generated as reported previously. The HEK293T cell line was grown in DMEM medium (Lonza) containing 8% FCS and 1% penicillin-streptomycin. Peripheral blood samples from patients and age- and gender-matched healthy individuals were collected in Vacutainer CPT tubes (BD). Peripheral blood from buffy coats (Sanquin) was layered on Ficoll-Paque solution (Amersham Biosciences). Isolation of peripheral blood mononuclear cells (PBMC) was performed using standard density-gradient centrifugation. We used CD14 microbeads (Miltenyi Biotec) or a Percoll density gradient to isolate monocytes from PBMC fractions, resulting in a purity of $CD14^+$ cells of >90% as determined by flow cytometry. For differentiation into moDC, isolated monocytes were cultured in the presence of GM-CSF (600 U/ml; BioSource International, Camarillo, CA) and IL-4 (400 U/ml; R&D Systems, Minneapolis, MN). After washing and seeding, moDC were matured by treatment with LPS (1 µg/ml) for 16 h. Macrophages were differentiated from primary monocytes by using Teflon conical flasks and culturing in RPMI with 5% normal human serum for five days.

Antibodies and flow cytometry

Maturation induction of moDC was checked using the following monoclonal antibody panel in a single tube containing mouse anti-human CD11c FITC, HLA-DR APC (L243), CD86 PerCP-Cy5, CD80 APC-H7, CD83 PE-Cy7 (all BD), CD40 V450, (BD Horizon) and CLIP PE (Santa Cruz Biotechnology). For the MelJuSo cell line, we used anti-HLA-DR APC-H7 (BD) or Cy3 (made by J. Neefjes) and anti-CLIP PE or Cy5 (made by J. Neefjes) antibody to stain for HLA-DR and CLIP surface expression, respectively.

Cells were blocked with 10% normal human serum for 10 min (MelJuSo cells) or 30 min (moDC) and stained with antibodies for 30 min in PBS/0.2%BSA/0.01%NaN_3 buffer. After washing, stained cells were assessed with an LSR-II flow cytometer and analyzed using FACSDiva software (both BD). For ***in situ*** assessment of CLEC16A expression, we used a rabbit anti-CLEC16A polyclonal antibody (Sigma-Aldrich). CLEC16A expression levels in primary immune cells were determined by flow cytometry using a rat CLEC16A monoclonal antibody (generated by J.J. Priatel) labeled to AlexaFluor647. To generate this antibody, F344 (SAS FISCH) rats were immunized with a 27-mer C-terminal human peptide ($CLEC16A_{954-980}$: VIVNETEADSKPSKNVARSAAVETASL) linked to KLH and standard monoclonal antibody production services performed by ImmunoPrecise Ltd (Victoria, Canada). The fidelity of hybridoma clones were initially screened by ELISA using peptide-BSA coated wells and subsequently, by probing HEK293T cells transfected with a vector alone (pEGFP, Clontech) or bearing full-length human CLEC16A DNA. Monoclonal CLEC16A antibody 7A4 was found to bind both denatured and paraformaldehyde-fixed protein by immunoblotting and flow cytometry, respectively. Conjugation of 7A4 antibody to AlexaFluor647 was performed by the antibody facility of the University of British Columbia (www.ablab.ca).

Isolation of mRNA and quantitative q-PCR

Total RNA was extracted from PBMC using the GenElute Mammalian Total RNA kit (Sigma) according to manufacturers' instructions. For isolation of mRNA from WM, a gentleMACS Dissociator (Miltenyi Biotec) was used followed by standard cell lysis and RNA precipitation as for PBMC. Samples were treated with DNAse I (Invitrogen) to remove contaminating DNA. Reverse mRNA transcription (1 µg) was performed using Superscript II (Invitrogen) to synthesize cDNA. Quantitative real-time PCR was performed for gene expression analysis with TaqMan technology (Applied Biosystems). Expression levels of genes of interest were corrected for rRNA levels of 18S for PBMC, and GAPDH and confirmed with GusB for MelJuSo cells and post-mortem brain tissue using commercially available primer/probe assays (Applied Biosystems). We selected specific primer and probe combinations from the Universal Probe Library (Roche, Supplementary Table 1).

Isolation of DNA and SNP detection

DNA was isolated from blood pallets obtained in EDTA tubes according to standard laboratory practice. MS-associated SNP in CLEC16A were assessed on an Illumina 610K array (Illumina).

Production of DNA constructs

CLEC16A cDNA (GenBank no BC112897.1) was cloned into the Bgl2-EcoR1 sites of 2HA-C1 and mGFP-C1. CLEC16A cDNA ligation was checked using classical PCR and CLEC16A mRNA overexpression in cells transfected with these constructs was validated using qPCR (Supplementary Figure 4). GFP- and mRFP-RILP, FLAG-Vps18, HA- and GFP-Vps41 and HA- and GFP-Spe39 DNA constructs were produced as previously described [17, 18]. Constructs were transformed into ***E. coli*** DH5α competent

cells, which were plated out on LB agar plates containing the appropriate antibiotics to select for single colonies at 37°C. A single colony was further grown overnight in 250-500 ml LB selection medium and plasmid DNA was isolated using a Midiprep/Maxiprep kit (Roche Applied Science).

siRNA and DNA transfections

One day before transfection, fresh culture medium without penicillin/streptomycin was added. For each transfection, siGENOME© siRNA duplexes (500 nM; Thermo Fisher Scientific) were mixed with DharmaFECT transfection reagent in IMDM in 96- or 24-well plates (flat bottomed). Following 20 min incubation at RT, cells were added to the mixture and cultured for three days. For immunofluorescence analysis, cells were grown on glass cover slides (Thermo Scientific). We tested different CLEC16A siRNA duplexes: #1, UCACAGGUCUUCUUAAUUA; #2, CGGCAUGGUCCAGCGAUU; #3, UGUCUGAGAUGUACGCUAA; and #4, CGUAAAUUCUAUCAUCGUU. Scrambled and HLA-DMβ siRNA duplexes were used as siRNA transfection controls. To exclude off-target effects, we assessed the effects of different CLEC16A siRNA combinations on expression levels of both CLEC16A and HLA-DRα. To overexpress CLEC16A, RILP or HOPS complex proteins, we transfected the MelJuSo cell line with DNA constructs using either FuGENE 6 or X-tremeGENE 9 transfection reagent (Roche Applied Science). In short, DNA was mixed with transfection reagent supplemented in serum-free IMDM and incubated for 20 min at RT. The mixture was then added drop wise to the cells one or two days after CLEC16A siRNA transfection. HEK293T cells were seeded 60-70% confluent. For these cells, a mixture of serum-free DMEM, polyethylenimine (1 mg/ml diluted in PBS) and DNA was added following 30 min incubation at RT. Cells were harvested and analyzed 24-48 h after incubation.

Lentivirus production and transduction into monocytes

We obtained lentiviral pLKO.1 constructs containing specific CLEC16A shRNA from Open Biosystems (Thermo Fisher Scientific). Constructs were used with the following shRNA: #1, CAGCTCTGTATTTGACTTCTT; #2, GCTAAGACTGAACAGGATATT; #3, CCTGAACATCACCATCCACAA. For the production of lentivirus, 293T cells were transfected with packaging (pRSV-Rev, pCMV-VSV-G and pMDLg/pRRE) and shRNA constructs using polyethylenimine (PEI, Mw 25 kDa, Polysciences). In each experiment, cotransfection with a pEGFP-C1 construct showed 70-80% GFP^+ cells. Following 2-3 days of cell culture, the supernatants were filtered and lentivirus was concentrated by ultracentrifugation for 2 h. Viral particles were taken up in medium, snap-frozen and stored at -80 °C. Thawed monocytes were transduced with lentivirus in CellGro serum-free medium (CellGenix) supplemented with polybrene (4 µg/ml) and cultured in the presence of IL-4 (800 U/ml) and GM-CSF (1000 U/ml) to differentiate them into DC. DC were split 6 days after transduction and one part was matured by addition of LPS (1ug/ml) and IFN-γ (1000U/ml) for 24 h. DC differentiation and maturation status was determined by flow cytometric analysis of CD11c, HLA-DR, CD40, CD80, CD83 as well as CD86 expression.

Co-immunoprecipitations and immunoblotting

HEK293T cells transfected with HA- and GFP-tagged CLEC16A, RILP and/or HOPS complex proteins were lysed in 0.8% NP-40 lysis buffer containing 50mM NaCl, 50mM Tris-HCl, 10mM $MgCl_2$ and protease inhibitors (EDTA-free; Roche Diagnostics) for 30 min. After spinning (15 min at 14,000g), supernatants were incubated with anti-HA or -GFP antibody coupled to Protein G-Sepharose 4 Fast Flow beads (GE Healthcare) for 1 h. Beads were washed in 8% NP-40 lysis buffer and three times in 0.08% NP-40 buffer containing 50mM Tris-HCl, 500mM NaCl and 5mM $MgCl_2$ before adding sample buffer and incubation at 95°C for 5 min. Immune-precipitated proteins were detected via SDS-PAGE followed by immunoblotting on PVDF membranes for 2 h at 300mA at 4°C. The membranes were probed with an HRP-conjugated rat anti-HA (Roche) or rabbit anti-GFP antibody [19]. Protein bands were visualized using ECL (Supersignal West Dura Extended Duration Substrate; Thermo Scientific).

Immunofluorescence

Cells were fixed with 4% PFA on either poly-L-lysine coated slides (for PBMC), cover glasses (for moDC) or µ-Slide 18-well plates for MelJuSo (IBIDI). Blocking and antibody incubation were performed in PBS with 1% BSA. PBMC were blocked by pre-incubating with 10% normal human serum at RT for 10 min. Cells were incubated with primary antibodies at RT for 1h. After washing in PBS, cells were incubated with AlexaFluor secondary antibodies (Invitrogen) at RT for 1h. After cell staining with DAPI for 10 min, cover slides were mounted in Prolong Gold Antifade Reagent (Invitrogen) or Vectashield (Vector Laboratories). We used a fluorescence or confocal laser-scanning microscope (Zeiss Axioplan 2; LSM510Meta, Leica TCS SP2) together with LAS AF (Leica) and ImageJ software to analyze cells. We used LAS AF software to determine the maximum Pearson's correlation coefficient at each area.

Immunohistochemistry

Six µm frozen white matter sections were cut and mounted on glass slides. Slides were kept overnight in a humidified atmosphere at RT. The sections were air-dried at RT for 1h and then fixed with acetone containing 0.02% H_2O_2 for 10 min. Slides were air-dried for approximately 10 min and washed with PBS/0.05%Tween. Primary antibodies were diluted in TBS/0.1%BSA and the slides were incubated overnight at 4°C. To the CLEC16A antibody dilution, 10% NHS was added. Incubation with isotype-matched primary antibodies and omission of the primary antibody were performed as negative control stainings. Secondary and tertiary agents were diluted in TBS/1%BSA/1%NHS. The sections were incubated with secondary antibodies at RT for 30 min. Incubation with tertiary agents was performed at RT for 1h. HRP was detected with 3-amino-9-ethyl-carbozole (Sigma). Cover slides were counterstained with haematoxylin and sealed with Kaiser's glycerol/gelatine (Boom). Staging of MS lesions in brain white matter tissue was performed as previously reported [20]. In short, lesions were characterized using Oil Red O to detect myelin degradation products and monoclonal antibodies against HLA-II and PLP or MOG. Lesion stages included normal appearing white matter and pre-active, active demyelinating, chronic active and chronic inactive lesions.

Electron microscopy

Either peripheral blood mononuclear cells containing ±40% of monocytes or MelJuSo cells stably transduced with either CLEC16A shRNA or scrambled shRNA constructs were fixed for 2 h in a mixture of 2% paraformaldehyde and 0.2% glutaraldehyde in 60 mM PIPES, 25 mM HEPES, 2 mM $MgCl_2$, 10 mM EGTA, pH 6.9 and processed for ultrathin cryosectioning as described [21]. For immunolabeling, the sections were incubated for 10 min with 0.15 M glycine in PBS and for 10 min with 1% BSA in PBS to block free aldehyde groups and prevent aspecific antibody binding, respectively. Sections were incubated with either rabbit anti-CLEC16A antibody (Sigma Alderich) followed by swine anti-rabbit IgG as a signal strengthening step or with rabbit anti-HLA-II antibody (made by J. Neefjes) and 10 nm protein-A conjugated colloidal gold (EMlab, University of Utrecht), all in 1% BSA in PBS. Next, the cryosections were embedded in uranylacetate and methylcellulose and examined with a Philips CM 10 electron microscope (FEI, Eindhoven, The Netherlands).

Statistical analyses

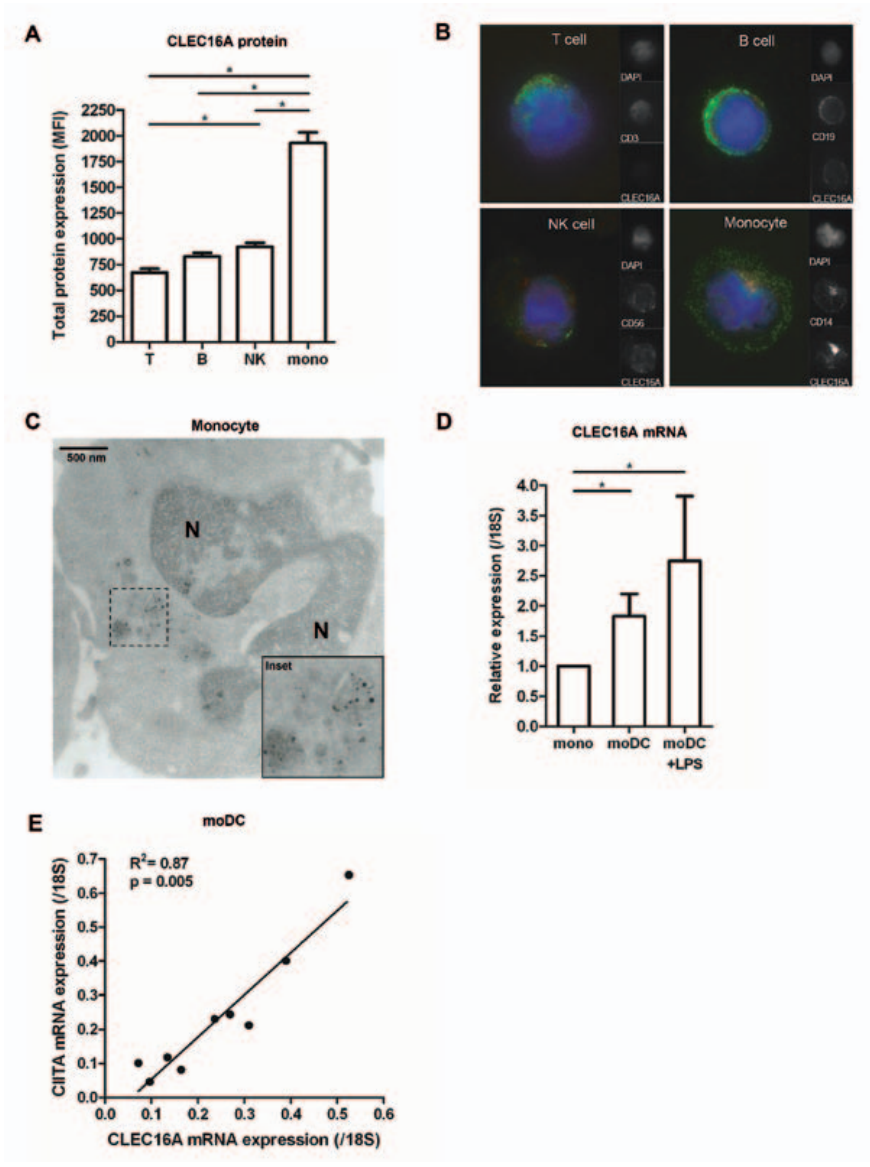
All statistical analyses were performed in SPSS 20 (IBM). Two-tailed Mann-Whitney U-tests were performed in all cases, unless stated otherwise. P values of <0.05 were considered statistically significant. Graphs were made using GraphPad, Prism v. 5.04 (GraphPad Software Inc). In the figure, p-values are denoted as * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001

Results

CLEC16A is abundant in monocytic cells and colocalizes with HLA class II and late endosomal compartments

We first explored the expression of CLEC16A in different leukocyte subsets including APC from peripheral blood of healthy donors. CLEC16A protein expression was low in T cells, moderate in NK and B cells and high in monocytes (Fig. 1A and B). This was consistent with the mRNA expression levels reported for CLEC16A in leukocyte subsets (Gene Expression Atlas; www.ebi.ac.uk/gxa). CLEC16A was predominantly localized in and around the nuclear indentation of monocytes (Fig. 1B and S1A), especially on vesicle membranes, which were highly enriched in this region (Fig. 1C). When primary monocytes were differentiated into professional APC, the level of CLEC16A expression was not altered in macrophages, but was approximately 2-fold increased in dendritic cells (DC) as compared to monocytes from the same donors. No differences in expression levels were found after maturation induction by LPS (Fig. 1D, Supplementary Figure 1B and C). In immature monocyte-derived DC (imDC), CLEC16A mRNA levels were significantly correlated with those of its neighbouring gene CIITA (R^2=0.88, p=0.003; Fig. 1E), which suggests coregulation of CLEC16A and HLA-II expression.

Figure 1. CLEC16A expression is mostly observed in APC



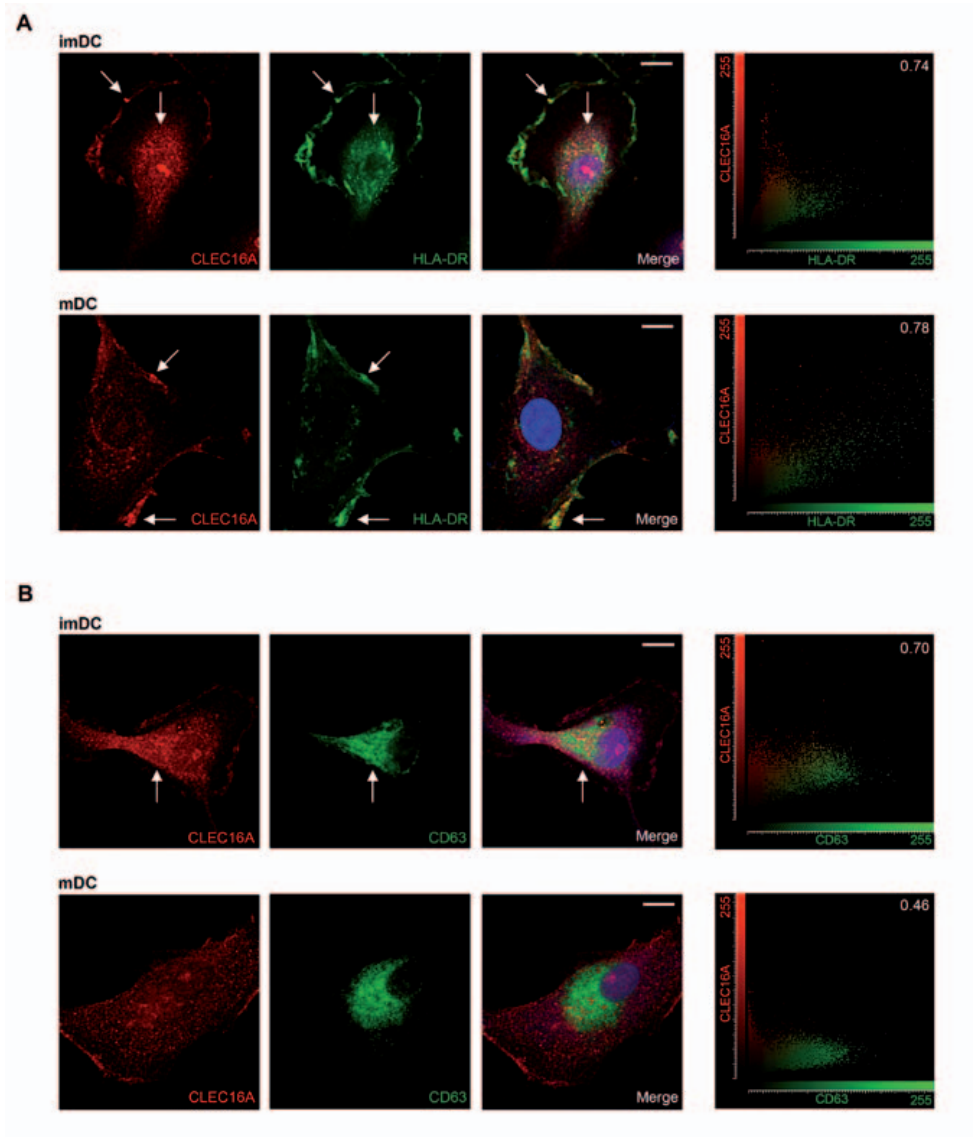
A) Flow cytometric analysis of total CLEC16A expression levels in PBMC from peripheral blood of four healthy donors. CD4 and CD8 T-cells, B-cells, NK-cells and monocytes were assessed. **B)** Immunofluoresence staining for CLEC16A in T, B and NK cells as well as monocytes of healthy donors (n=3). **C)** Vesicular localization of CLEC16A in the nuclear indentation of primary monocytes of two healthy donors. **D)** CLEC16A mRNA expression in primary monocytes as well as unstimulated and LPS-stimulated monocyte-derived macrophages (mø) and dendritic cells (DC) from the same healthy donors (n=4-5). **E)** Paired association of mRNA expression levels of CLEC16A and CIITA in immature monocyte-derived DC (n=9).

In the C-type lectin DEC-205, specific signaling motifs such as EDE triacidic clusters are present that specifically target antigens to HLA-II$^+$ late endosomal compartments, i.e. MIIC [22]. CLEC16A also contains several late endosomal targeting motifs in its C-terminus, including two EDE triads (Supplementary Figure 2), suggesting that it is directly involved in the HLA-II antigen presentation pathway. Indeed, immunofluorescence analysis of imDC showed colocalization of CLEC16A with HLA-DR (mean Pearson's correlation coefficient [PCC] = 0.75; Fig. 2A and D) as well as the late endosomal marker CD63 (mean PCC=0.67; Fig. 2B and D), predominantly in perinuclear regions. CLEC16A colocalized less frequently with early endosomal marker EEA-1 (mean PCC=0.39; Fig. 2C and D). Maturation induction of moDC by LPS caused a shift of CLEC16A and HLA-DR colocalization from perinuclear regions towards areas near or at the plasma membrane, in contrast to CD63 and EEA-1 (Fig. 2A-D). The expression of CLEC16A in primary monocytic cells and its association with HLA-II and late endosomal compartments in moDC support a function of CLEC16A in endosomal HLA-II processing by APC.

CLEC16A knockdown in APC causes abnormal maturation of HLA class II$^+$ late endosomal compartments

In ***Drosophila*** Ema mutants, endosomes fail to develop into matured late endosomal compartments [13]. To assess this for CLEC16A in the context of HLA-II in human APC, we first used the melanoma MelJuSo cell line which is a frequently used APC model. MelJuSo consistently express HLA-II and contains a large cytoplasm to optimally assess endosomal speading [23]. Different siRNA duplexes against CLEC16A were transfected into MelJuSo cells, resulting in a mean reduction of 64% in target mRNA expression for four tested CLEC16A siRNA compared to scrambled siRNA duplexes (Fig. 3A). When evaluating cytoplasmic distribution of endosomes, EEA-1$^+$ early endosomes did not show any changes (data not shown), but CD63$^+$ and Rab7$^+$ late endosomes revealed clear scattering throughout the cytoplasm after CLEC16A siRNA treatment (Fig. 3B and C; Supplementary Figure 3A and B). Subsequent transfection of CLEC16A siRNA-treated MelJuSo cells with a CLEC16A-GFP construct reversed this late endosomal phenotype (Fig. 3D, Supplementary Figure 4). Also several foci of CD63$^+$ and Rab7$^+$ late endosomes were observed (Supplementary Fig. 3A and B), suggesting that CLEC16A knockdown interferes with the biogenesis of late endosomes. To address this, we generated stable MelJuSo transductants containing lentiviral constructs encoding CLEC16A and scrambled shRNA. CLEC16A mRNA expression levels were markedly decreased in CLEC16A shRNA transductants (mean reduction of 74%), whereas CIITA and HLA-DRA mRNA levels remained unaffected compared to scrambled shRNA transductants (Fig. 3E). Electron microscopic assessment showed that CLEC16A shRNA transductants contained several multivesicular bodies (MVB) that completely lacked internal vesicles (IV) or consisted of a few remaining IV with an enlarged phenotype as compared to scrambled shRNA transductants (Fig. 3F). Three times more IV-poor than IV-rich MVB were observed in CLEC16A shRNA transductants, while the opposite was the case for scrambled shRNA transductants (Fig. 3G). Interestingly, CLEC16A shRNA transductants showed a 3-fold increase in the number of immunogold-labeled HLA-II molecules in MVB (Fig. 3F and H), without affecting their size (Supplementary Figure 3C). Together, these data imply that CLEC16A is essential for cytoplasmic localization and maturation of HLA-II$^+$ late endosomes in APC.

Figure 2. Intracellular localization of CLEC16A in primary immature and mature monocyte-derived dendritic cells



Immunofluorescence analysis of CLEC16A (red) and markers for early and late endosomes (green) in moDC by confocal microscopy. **A)** HLA-DR and **B)** CD63 were used to identify late endosomal compartments, while **C)** EEA-1 served as early endosome marker in immature and mature moDC. **D)** Quantification of colocalization of CLEC16A with HLA-DR, CD63 and EEA-1 in the perinuclear areas of immature DC and the peripheral areas near or at the plasma membrane of mature moDC.

Figure 2. Continued

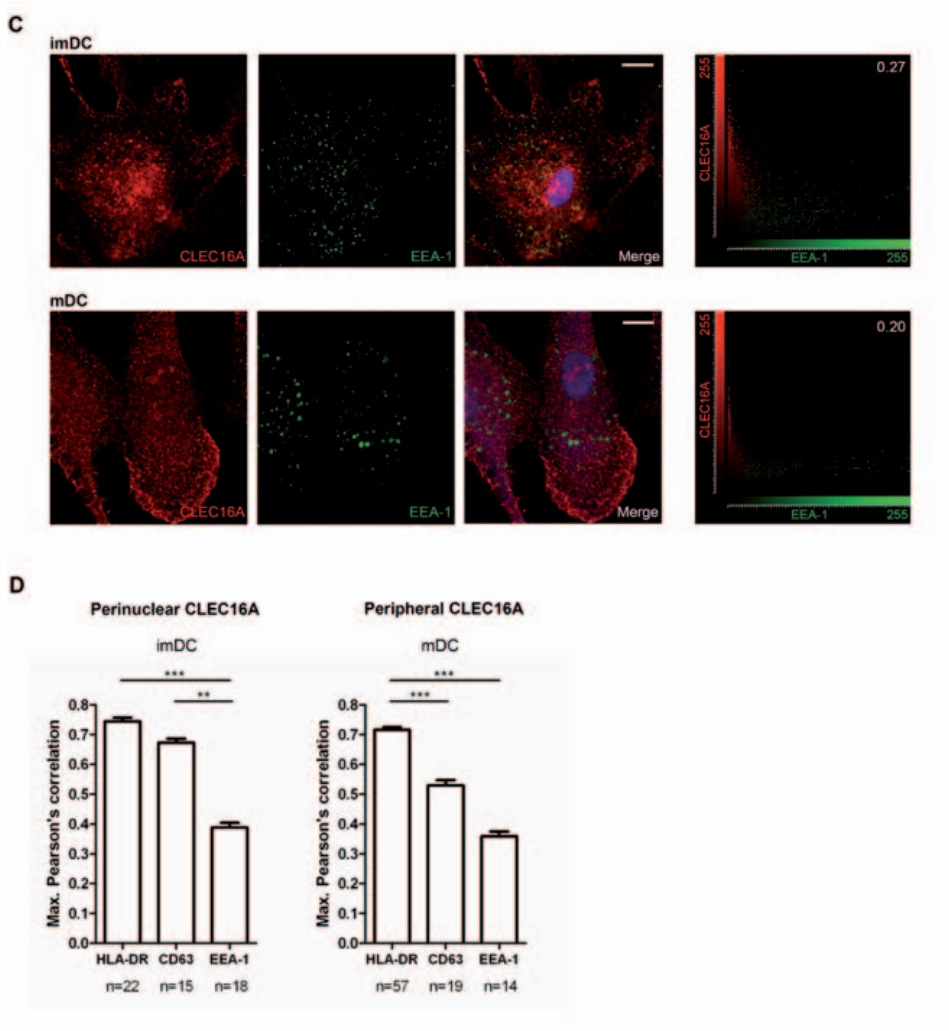
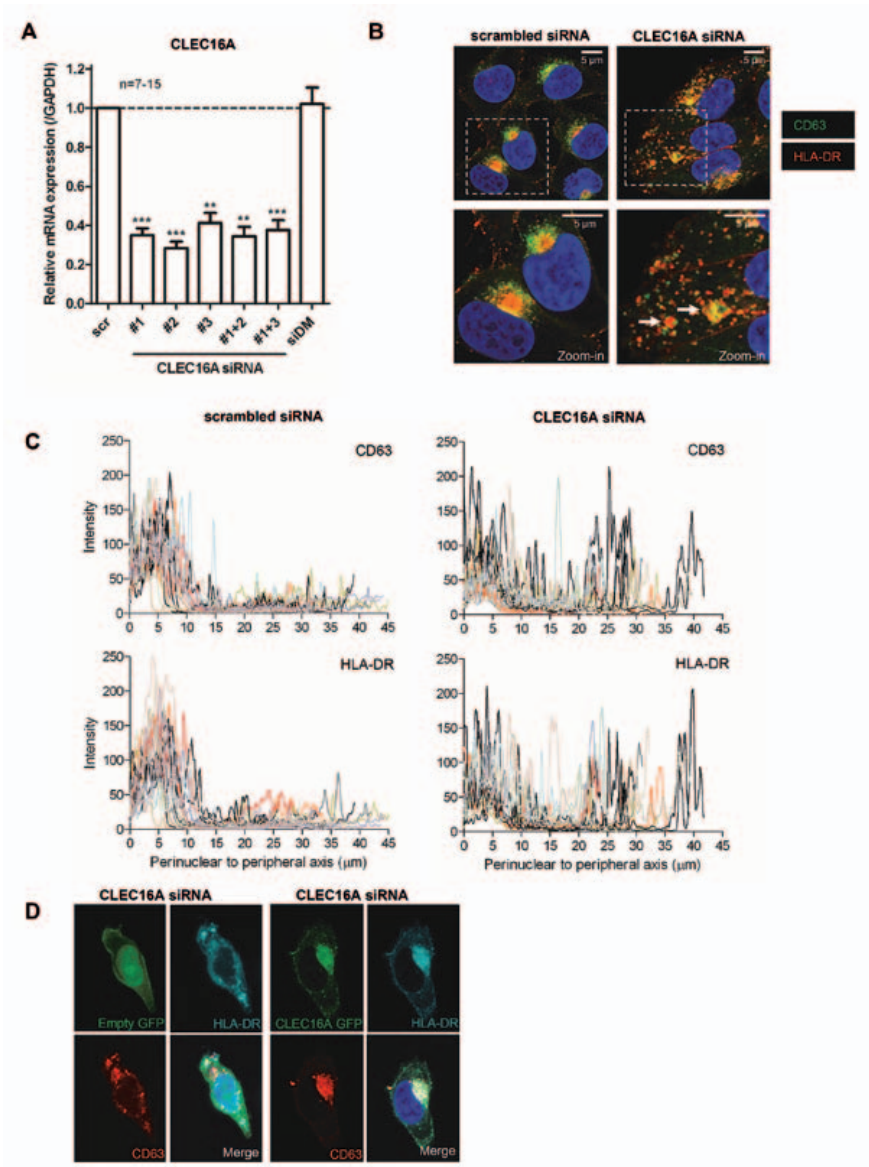
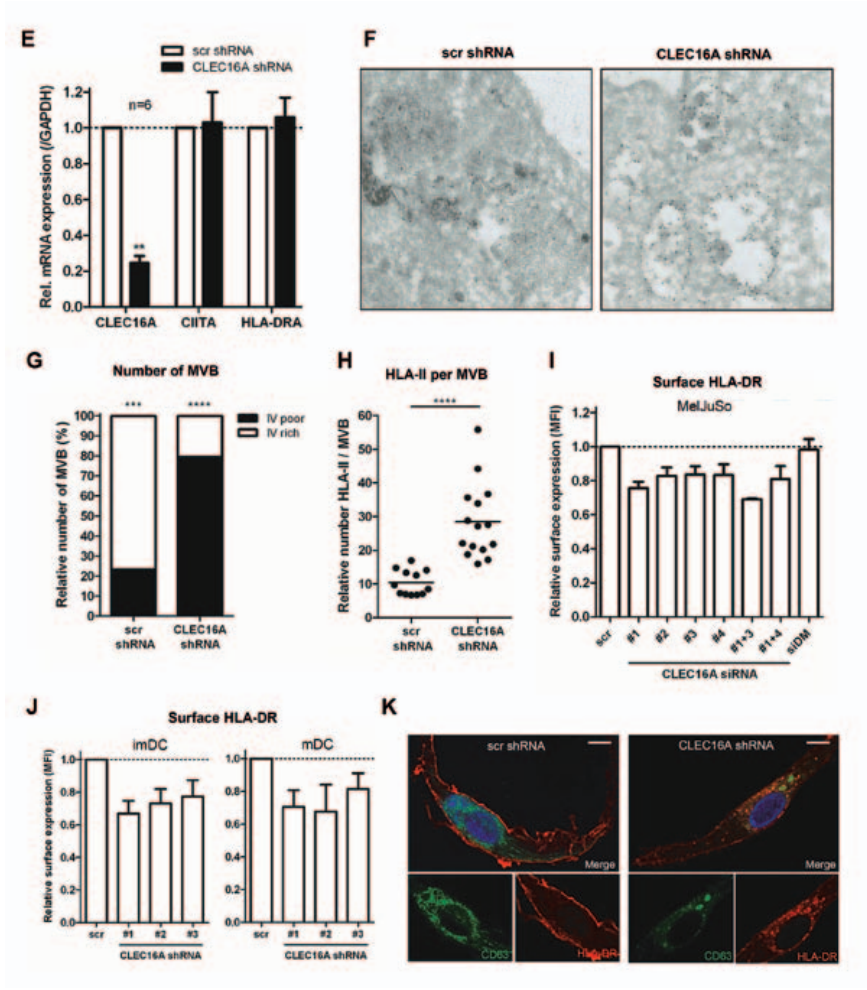


Figure 3. CLEC16A silencing affects late endosomal localization and formation in antigen-presenting cells



The melanoma MelJuSo cell line was transfected with combinations of siRNA duplexes against CLEC16A (#1, #2, #3 and #4) and analyzed after 3 days of culture. **A)** Relative CLEC16A mRNA expression levels in CLEC16A siRNA- compared to scrambled siRNA-treated cells (n=7-15). As additional negative controls, cells transfected with a pool of siRNA duplexes against HLA-DM were also analyzed. **B)** Immunofluorescence analysis and **C)** quantification of the cytoplasmic localization of late endosomes in CLEC16A siRNA- compared to scrambled siRNA-treated wild type or Rab7-GFP^{+} MelJuSo cells.

Figure 3. Continued



The stainings are representative for five separate experiments. **D)** CLEC16A siRNA-treated MelJuSo cells were transfected with empty GFP or CLEC16A-GFP constructs and assessed for cytoplasmic distribution of late endosomes. **E)** Stable MelJuSo transductants containing either scrambled or CLEC16A shRNA were analyzed with electron microscopy for MVB morphology and expression of HLA-II by labeling of anti-rabbit HLA-II antibody with gold particles. **F)** Relative expression levels of CLEC16A, CIITA and HLA-DRA mRNA in scrambled and CLEC16A shRNA MelJuSo transductants (n=6 including two different CLEC16A shRNA constructs). **G)** The number of internal vesicle-poor (IV-poor) and internal vesicle-rich (IV-rich) MVB in scrambled (n=40 and 132) and CLEC16A shRNA (n=210 and 54) transductants. **H)** The number of gold-labeled HLA-II molecules in MVB of scrambled shRNA and CLEC16A shRNA transductants. HLA-II numbers were related to the number of MVB to calculate the relative number of HLA-II/MVB in each EM image. Each EM image represented a single cell. MVB and HLA-II numbers were counted in triplicate in 12-15 different cells and were confirmed by a second independent observer. **I and J)** Relative surface expression levels of HLA-DR for scrambled siRNA- and CLEC16A siRNA-treated MelJuSo cells (I; n=4-7) as well as scrambled shRNA- and CLEC16A shRNA-treated immature (n=4-5) and mature (n=2-3) moDC, as determined by flow cytometry. **K)** Intracellular distribution of late endosomes in scrambled shRNA- and CLEC16A shRNA-treated moDC (representative for three different donors).

Silencing of CLEC16A leads to intracellular accumulation and reduced plasma membrane expression of HLA class II

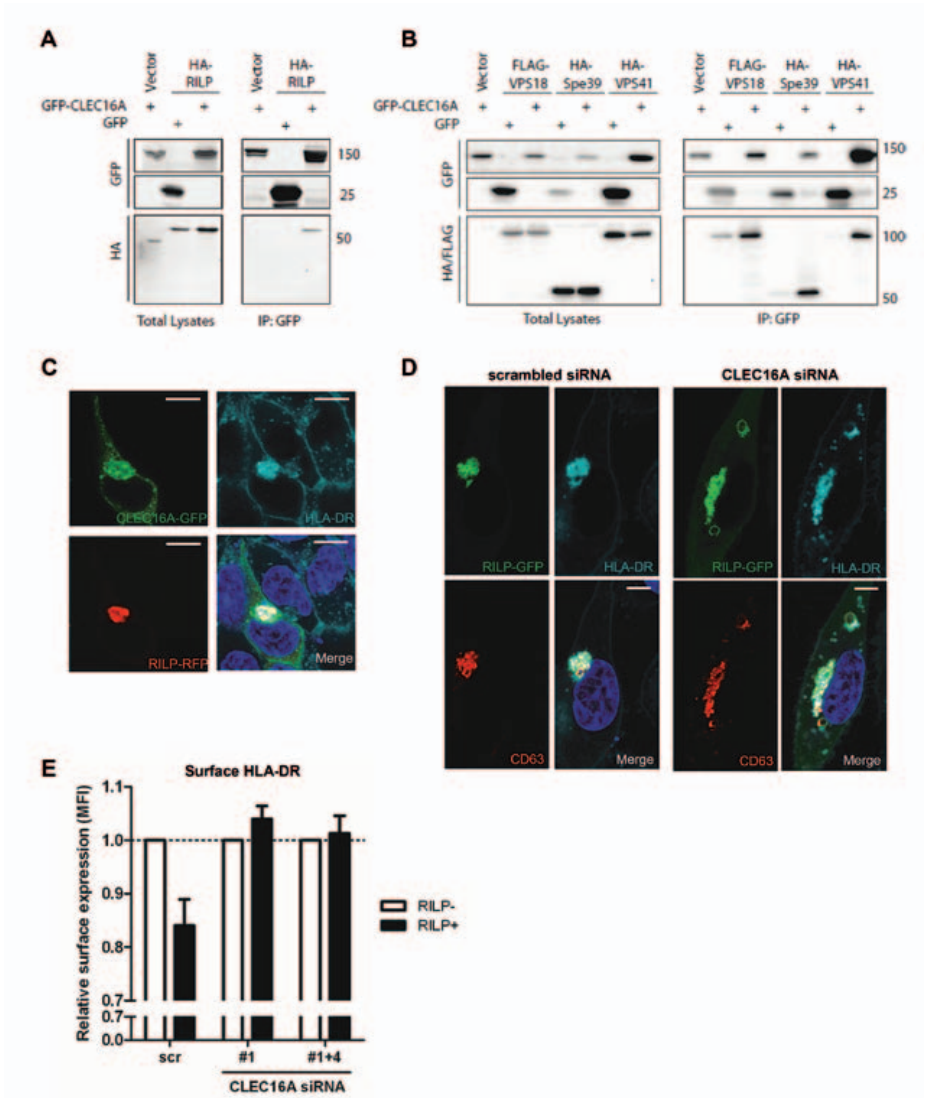
Since the function of CLEC16A was associated with late endosomal biogenesis, we next evaluated whether CLEC16A silencing also affected HLA-II biology in different APC. We assessed HLA-DR surface expression in CLEC16A siRNA-treated MelJuSo cells using flow cytometry. Cell surface expression of HLA-DR was indeed strongly reduced after CLEC16A silencing, showing a mean decrease of 21% (Fig. 3H). Simultaneous transfections of siRNA duplexes against HLA-DMβ caused a more than 2-fold upregulation of CLIP expression at the plasma membrane (Supplementary Figure 3D), indicating a high siRNA transfection efficiency. HLA-DRα mRNA levels were not reduced in CLEC16A siRNA compared to scrambled siRNA-treated cells (Supplementary Figure 3E). This verified that the effect of CLEC16A siRNA on HLA-DR surface expression was the result of cellular processes independent of HLA-DR transcription. Intracellular analysis of CLEC16A siRNA-treated cells revealed that the scattered $CD63^+$ and $Rab7^+$ late endosomes were positive for HLA-DR (Fig. 3B; Supplementary Figure 3A and B). The colocalization of HLA-DR with late endosomal foci (Supplementary Figure 3A and B) agreed with the accumulation of HLA-II in MVB within CLEC16A shRNA MelJuSo transductants, as observed by electron microscopy (Fig. 3F and H).

Next, we validated the role of CLEC16A in primary APC. Its expression was silenced using our CLEC16A shRNA lentiviral constructs for transient transductions of moDC from healthy donors (Supplementary Figure 5). Similar to MelJuSo cells, knockdown of CLEC16A caused a strong decrease in HLA-DR surface expression for immature moDC (mean reduction of 28%) and mature moDC (mean reduction of 27%, Fig. 3J). Of note, the upregulation of costimulatory molecules at the plasma membrane was impaired as well after moDC maturation (data not shown), which is in line with their processing in $HLA\text{-}II^+$ late endosomes [24, 25]. CIITA and HLA-DRA mRNA expression levels were not affected (Supplementary Figure 5). Also CLEC16A shRNA-treated moDC showed several foci and dispersed localization of $HLA\text{-}DR^+$ late endosomes compared to scrambled shRNA-treated moDC (Fig. 3K). These data show that CLEC16A affects both surface and intracellular expression of HLA-DR in different types of APC and provide evidence that this is caused by an effect on late endosomal biogenesis.

CLEC16A is directly involved in RILP-mediated distribution of late endosomes to influence HLA class II processing

In Drosophila, Ema directly associates with the HOPS (homotypic fusion and protein sorting) complex [13], a Rab7-activating complex that consists of specific Vps subunits and plays a critical role in late endosomal biogenesis in yeast [26, 27]. Another molecule that binds to Rab7, the Rab7-interacting lysosomal protein (RILP), also interacts with HOPS in the dynein motor complex to transport late endosomes along microtubuli to the perinuclear microtubule-organizing center (MTOC) [17, 18]. Since MIIC are primarily localized around the MTOC [28], we evaluated whether CLEC16A is involved in this late endosomal machinery to control HLA-II processing.

Figure 4. CLEC16A involved in RILP-mediated recruitment of HLA-II+ late endosomes to perinuclear areas



Co-immunoprecipitations of GFP from MelJuSo cells cotransfected with GFP-CLEC16A and **A)** HA-RILP or **B)** FLAG-VPS18, HA-Spe39 or HA-Vps41 constructs. GFP pull downs were analyzed for HA or FLAG expression on immunoblot. Empty GFP ('GFP') constructs were precipitated as well and served as negative controls. Results are representative for 2-3 experiments. **C)** MelJuSo transfected with CLEC16A-GFP and RILP-RFP constructs were assessed for intracellular localization together with HLA-DR. **D)** The confocal images are representative for two independent experiments. **E)** MelJuSo cells treated with scrambled siRNA or CLEC16A siRNA were transfected with a RILP-GFP construct two days after siRNA treatment. Intracellular localization (E; n=3) and cell surface expression (F; n=3) of HLA-DR was determined in RILP-GFP$^+$ and -GFP$^-$ three days after siRNA treatment.

To determine a direct role of CLEC16A in this type of machinery, we performed co-immunoprecipitations of CLEC16A in 293T cells cotransfected with CLEC16A and either RILP or Vps18, Spe39 and Vps41 as members of the HOPS complex. In pull downs for CLEC16A-GFP, we found a strong expression of RILP-HA, Spe39-HA and Vps41-FLAG on immunoblots, in contrast to pull downs for empty GFP (Fig. 4A). Control empty GFP showed similar or more expression compared to CLEC16A-GFP in total lysates of cotransfectants (Fig. 4A), which confirmed the specificity of the co-immunoprecipitates.

In CLEC16A-GFP transfected MelJuSo cells, GFP$^+$ vesicles strongly colocalized with HLA-DR in perinuclear cytoplasmic regions (Supplementary Figure 6A). Similar observations were obtained from RILP-GFP MelJuSo transfectants (Supplementary Figure 6B). Cotransfections with both CLEC16A-GFP and RILP-RFP showed a strong recruitment of CLEC16A and HLA-DR in the same perinuclear area as RILP (Fig. 4B and Supplementary Figure 6C). This likely reflects the recruitment of HLA-DR$^+$CLEC16A$^+$ late endosomes by RILP to the MTOC. To explore a functional role of CLEC16A in this machinery, we silenced CLEC16A expression and assessed the perinuclear accumulation of HLA-DR$^+$ late endosomes mediated by RILP. After transfection with RILP-GFP, perinuclear localization of HLA-DR was markedly disturbed in CLEC16A siRNA- as compared to scrambled siRNA-treated MelJuSo cells (Fig. 4C). Perinuclear foci of HLA-DR with an elongated instead of a roundish shape were observed, colocalizing with RILP and CD63. Additional foci of HLA-DR and CD63 were found in peripheral areas, of which enlarged foci were positive for RILP. HLA-DR expression at the plasma membrane showed a mean reduction of 16% in RILP-GFP$^+$ compared to GFP$^-$ cells treated with scrambled siRNA (Fig. 4E). This reduction was abolished by CLEC16A silencing (Supplementary Figure 7), since surface expression of HLA-DR was not decreased in RILP-GFP$^+$ compared to GFP$^-$ CLEC16A siRNA-treated cells (Fig. 4E). RILP overexpression did not affect CLEC16A, CIITA and HLA-DRA mRNA expression levels (Supplementary Figure 7). These data provide evidence that CLEC16A participates in a molecular complex of RILP and HOPS to mediate the trafficking of HLA-II$^+$ late endosomes to the MTOC. We argue that silencing of CLEC16A impairs late endosomal transport to perinuclear regions, thereby affecting the biogenesis of MIIC and attenuating HLA-II processing for presentation at the plasma membrane.

CLEC16A expression is increased and associates with HLA class II in multiple sclerosis patients

To determine whether this novel mechanism of CLEC16A plays a role in autoimmune disease, we assessed expression patterns and differences of CLEC16A in MS. We first analyzed CLEC16A expression in total PBMC from the peripheral blood of MS patients and healthy controls (HC; Table 1). A two-fold increase in CLEC16A mRNA expression was found for MS patients compared to HC (p=0.003; Fig. 5A). In post-mortem brain tissue of MS patients, CLEC16A mRNA expression levels were four-fold elevated in MS white matter (WM) compared to non-demented controls (NDC; p=0.028, Table 1 and Fig. 5B). Immunohistochemical stainings for CLEC16A and HLA-II in MS WM tissue showed a relatively high number of CLEC16A-positive cells in pre-active lesions, predominantly in HLA-II$^+$ perivascular infiltrates, in contrast to MS normal-appearing white matter and WM of NDC (Fig. 5C and D; n=18 and n=9, respectively). In all MS WM regions that were highly positive for CLEC16A,

high HLA-II expression was found (Fig. 5E). CLEC16A expression did not correlate with HLA-II, since HLA-II was present in some CLEC16A$^-$ areas as well (Fig. 5E). Next, we evaluated whether CLEC16A in white matter coexpressed with HLA-II in APC. Immunofluorescence analysis showed coexpression of CLEC16A with HLA-DR in different MS white matter tissues (Fig. 5F). CLEC16A was also expressed in cells positive for CD68 (Fig. 5F), indicating its presence in microglia or macrophages. The increased expression level of CLEC16A and its association with HLA-II in MS tissue supports an enhanced function of CLEC16A in APC of MS patients.

Table 1. Demographic characteristics of patients and controls in this study

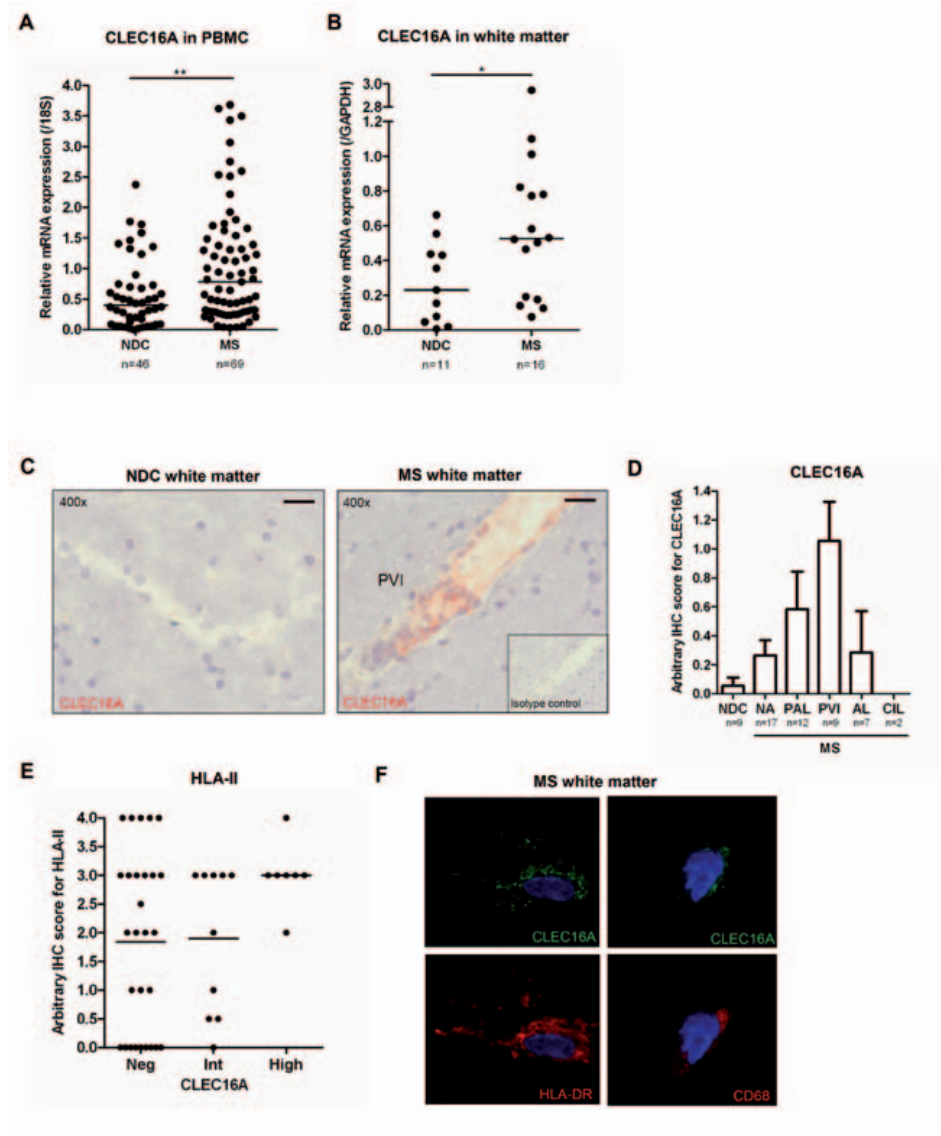
	MS	HC	NDC	MS
Age (SD)	44 (12)	37 (12)	82 (7)	65 (12)
Percentage female	82.6	71.7	45.0	20.0

Left part: PBMC samples and right part the post-mortem brain expression

Vitamin D downmodulates CLEC16A and HLA class II expression in monocyte-derived dendritic cells

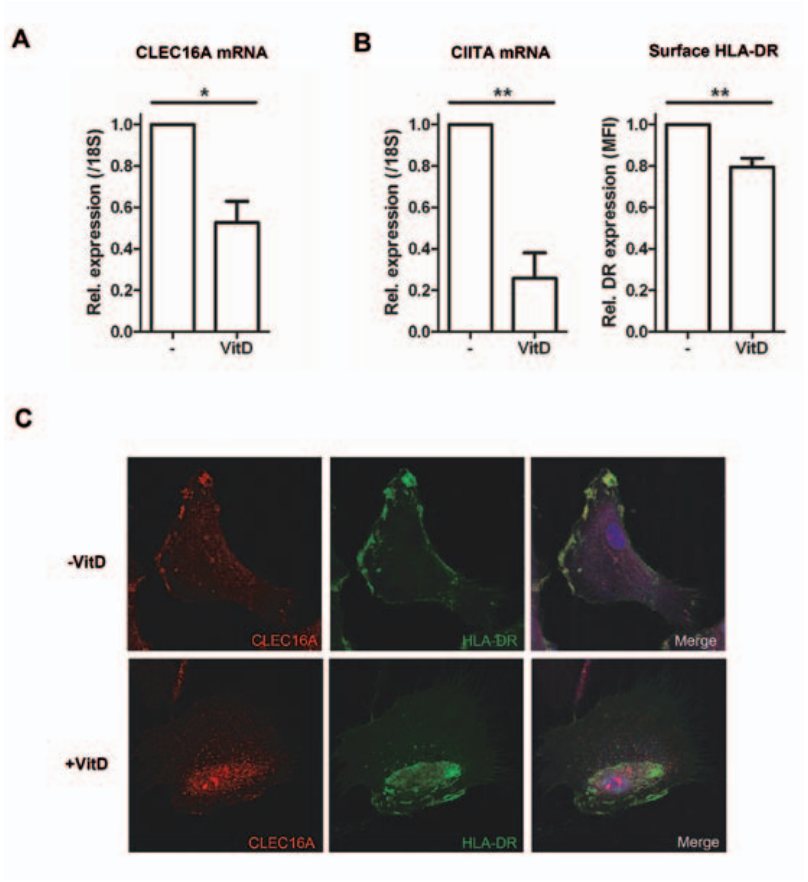
Interestingly, the strongest genetic risk factor in MS, HLA-DRB1*15, can be modulated by vitamin D [29], of which high serum levels associate with a lower risk to develop MS [30]. Also CLEC16A contains a vitamin D receptor (VDR) binding element [31]. Since vitamin D is known as an immunomodulatory agent, we explored whether vitamin D manipulated CLEC16A expression in parallel with HLA-II in primary APC. We cultured monocytes from healthy individuals with the active form of vitamin D ($1,25[OH]_2D3$) during differentiation into moDC and analyzed CLEC16A and HLA-II expression. CLEC16A mRNA expression levels were significantly reduced in vitamin D-treated as compared to untreated moDC (Fig. 6A). These differences positively correlated with levels of mRNA expression for CIITA and cell surface expression of HLA-DR in moDC, as determined by q-PCR and flow cytometry, respectively (Fig. 6B). Next, we compared the effects of vitamin D on the intracellular distribution of CLEC16A and HLA-DR in moDC. Stimulation of immature moDC with vitamin D did not influence the perinuclear localization of CLEC16A and HLA-DR (data not shown). Monocyte-derived DC cultured with vitamin D interfered with the maturation-induced relocalization of both CLEC16A and HLA-DR towards peripheral regions (Fig. 6C). These data show that one of the prime environmental factors in MS, vitamin D, reduces CLEC16A expression and its localization to the plasma membrane in line with HLA-II in moDC. This implicates CLEC16A in APC as a potential immunomodulatory target of vitamin D in MS and other autoimmune diseases.

Figure 5. CLEC16A expression is increased in primary cells of multiple sclerosis patients



A) Expression levels of CLEC16A mRNA in PBMC of peripheral blood of patients with multiple sclerosis (MS; n=69) and healthy controls (HC; n=46). **B)** CLEC16A mRNA expression levels in brain white matter tissues of MS (n=16) and non-demented controls (NDC; n=11). **C)** Representative image of CLEC16A within a perivascular infiltrate of a pre-active MS white matter lesion. **D)** Quantification of CLEC16A expression in different areas of MS and NDC white matter tissue. We used an arbitrary immunohistochemical score defined by the number of positive cells in a particular area. **F)** Immunofluorescence analysis of CLEC16A and both HLA-DR and CD68 in MS white matter using confocal microscopy.

Figure 6. Vitamin D reduces CLEC16A and HLA-DR expression in primary monocyte-derived dendritic cells



A) q-PCR to determine CLEC16A and CIITA mRNA expression in unstimulated moDC and moDC stimulated with the active form of vitamin D (1,25(OH)$_2$D3). Primary monocytes from healthy donors were continuously cultured under vitamin D conditions from the start of differentiation into DC and analyzed seven days after differentiation. **B)** Surface expression levels of HLA-DR in unstimulated versus vitamin D-stimulated moDC. **C)** Intracellular localization of CLEC16A and HLA-DR in moDC treated with and without vitamin D following maturation induction. Cultured moDC were stained on day seven. Images are representative for three different donors and >20 moDC per donor.

Discussion

The majority of new susceptibility genes recently identified in MS GWAS are immune-related and shared with many other autoimmune diseases [3, 32]. To understand the role of these genes in the complex immunopathogenesis of MS, the next pivotal step is to unravel their individual function and role in pathological mechanisms. ***In silico*** analysis of MS GWAS data implicated CD4$^+$ T cell activation and differentiation as the principal candidate mechanism in MS pathogenesis [3]. Here, we demonstrate that MS and the autoimmune disease associated risk gene CLEC16A serves as a critical regulator of HLA-II$^+$ late endosomal biogenesis to control intracellular processing and surface expression of HLA-II in APC.

In addition to the evidence that we present on CLEC16A, also other identified MS risk genes show direct involvement in the HLA-II antigen presentation pathway. For example, interferon regulatory factor 8 (IRF8) and mitogen-activated protein kinase 1 (MAPK1) function as transcription factors for CIITA [33, 34], the master regulator of HLA-II that is localized adjacent to CLEC16A. Zinc finger and BTB domain containing 46 (ZBTB46) is specifically expressed and boosts MHC-II expression in murine DC [35, 36]. Also CIITA variation in HLA-DRB1*1501$^+$ individuals has been proposed to link with MS susceptibility [37]. The function of APC, in particular monocyte-derived cells, is considered as the prime target of genetic risk variants in type I diabetes [38], of which ERBB3, CD226 and CLECL1 all are involved in antigen presentation by DC [39-41]. In line with this, CLEC16A was abundant in primary monocytes compared to other leukocyte subsets (Fig. 1A and B) and controlled HLA-II processing in monocyte-derived DC (Fig. 3J and K).

In literature, there is limited evidence for an association of CLEC16A risk genotype with expression levels. Only in the thymus, but not in peripheral blood, a significant correlation of CLEC16A risk SNP rs12708716 with relative expression levels was found,[46] which implicates a thymus-specific splicing process. The expression of CLEC16A was not different for the risk carriers of the rs7200786[A] SNP (Supplementary Figure 8). The regulation of CLEC16A and CIITA expression in moDC by vitamin D (Fig. 6), supports a joint regulatory mechanism of CLEC16A and HLA-II expression via a VDR. Indeed, in addition to VDR binding to CLEC16A, also a VDR binding site was identified specifically in the promoter of the strongest risk locus in MS, HLA-DRB1, close to the regulatory SXY module required for CIITA function [29, 42].

Knockdown of CLEC16A expression in APC had significant effects on cytoplasmic localization and maturation of HLA-II$^+$ late endosomal compartments (Fig. 3B and K). The localization of these endosomes is critical for the efficiency of HLA-II antigen presentation and is strongly linked to endosomal maturation. During their maturation, HLA-II$^+$ late endosomes are transported to perinuclear areas to undergo acidification, which activates specific enzymes that process antigens into peptides for loading onto HLA-II molecules. This maturation and transport to perinuclear areas is regulated by the dynein motor complex, which forms a bridge between the microtubuli and Rab7$^+$ late endosomes. The association of CLEC16A with two regulators of this machinery, RILP and

HOPS (Fig. 5), suggests that CLEC16A uses this mechanism to control the efficiency of HLA-II antigen presentation in MIIC (Supplementary Figure 9A). This is supported by our recent identification of RILP as a gene that affects CLIP surface expression [12].

CLECL1, which is another MS risk gene [3], functions as surface receptor to boost HLA-II expression in DC [39]. DC-SIGN and DEC205 also regulate HLA-II antigen presentation, but by targeting antigens to the MIIC [22, 43]. In contrast, the function of CLEC16A is different to that from other C-type lectins. To our knowledge, CLEC16A is the first member of the C-type lectin family that controls HLA-II antigen presentation by participating in the localization and maturation of MIIC. The role of Ema in the formation of autophagosomes points to a similar function for CLEC16A in autophagy-mediated HLA-II antigen presentation [44].

The differences of CLEC16A expression in MS (Fig. 5A and B) indicate that CLEC16A has an effect on HLA-II antigen presentation by APC. In EAE, the animal model of MS, self-antigens are recognized and internalized by APC in the cervical lymph nodes [45] and probably, when presented on MS-associated HLA-II molecules, to activate $CD4^+$ T cell subsets that contribute to CNS inflammation. Thus, the increased level of CLEC16A expression in MS points to a model in which CLEC16A promotes late endosomal maturation into MIIC in APC, thereby changing the peptide pool and enhancing the efficiency of HLA-II antigen loading and presentation. This then leads to enhanced surface expression of HLA-II/peptides complexes that triggers the activation of disease-associated $CD4^+$ T cell subsets (Supplementary Figure 9B).

In conclusion, this study uncovers a novel mechanism in which CLEC16A controls the biogenesis of $HLA\text{-}II^+$ late endosomes and underlines the relevance of CLEC16A in an autoimmune disease such as MS. Our findings couple the function of a minor risk allele, CLEC16A, to a major genetic risk factor, HLA-II, for MS and several other autoimmune diseases. The direct role of CLEC16a in the HLA-II antigen presentation pathway provides a strong rationale to identify pathogenic APC subsets with aberrant levels of CLEC16A and develop strategies to selectively manipulate this pathway in autoimmune disease.

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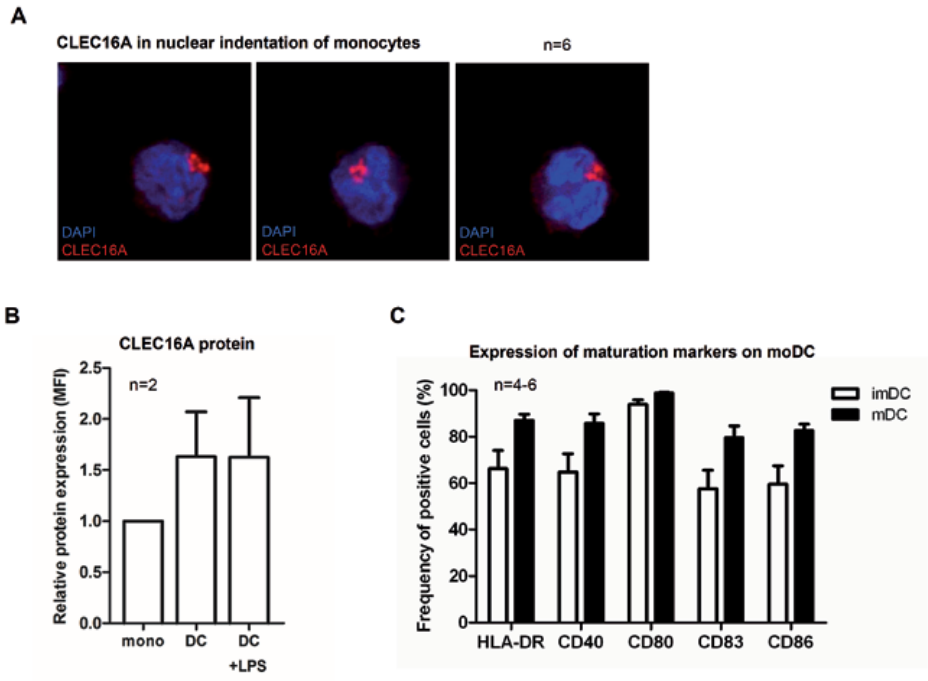
Supplementary materials

Supplementary Table 1. q-PCR primers

Gene	Forward primer	Reverse primer
CLEC16A	TGCCCCTCTACGTGTACTCA	GAGACACCGGCAGGCTAAT
CIITA	AACAGGATTCACGGATCAGC	CAGCGTGGTTAGTGTCCTCA
HLA-DRα	AGCCTCTTCTCAAGCACTGG	GGCACACACCACGTTCTCT
HLA-DMβ	GAACTCCCGGCATCTTTACA	CAACAGACAGGTGCTTTCCA

Supplementary Figure 1. CLEC16A is mainly located in nuclear indentation and increases during differentiation to DC



A) Representative immunofluorescent stainings of monocytes of six donors. **B)** CLEC16A protein expression increased during differentiation from monocyte to mo-DC. **C)** The expression of costimulatory markers is increased in mature monocyte-derived DC

Supplementary Figure 2. CLEC16A contains several tri-acidic clusters.

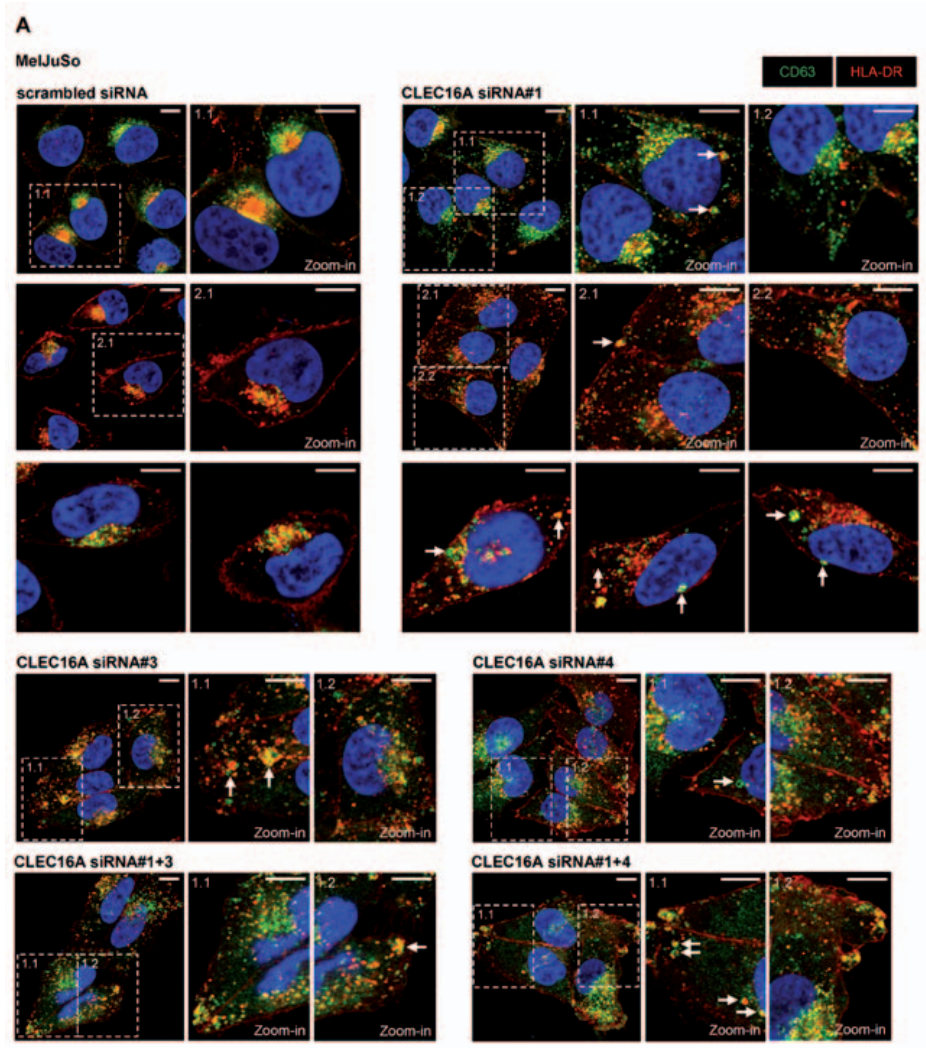
C-terminus of CLEC16A protein (NP056041.1)

```
301  kggerpkisl pvslyllsqv fliihhaplv nslaevilng dlsemyakte qdiqrssakp
361  sircfikpte tlerslemnk hkgkrrvqkr pnyknvgeee deekgpteda qedaekakgt
421  eggskgikts geseeiemvi mersklsela astsvqeqnt tdeeksaaat csestqwsrp
481  fldmvyhald spdddyhalf vlcllyamsh nkgmdpekle riqlpvpnaa ekttynhpla
541  erlirimnna aqpdgkirla tlelsclllk qqvlmsagci mkdvhlacle gareesvhlv
601  rhfykgedif ldmfedeyrs mtmkpmnvey lmmdasillp ptgtpltgid fvkrlpcgdv
661  ektrrairvf fmlrslslql rgepetqlpl treedliktd dvldlnnsdl iactvitkdg
721  gmvqrflavd iyqmslvepd vsrlgwgvvk faglldqmqv tgveddsral nitihkpass
781  phskpfpilq atfifsdhir ciiakqrlak griqarrmkm qriaalldlp iqpttevlgf
841  glgsststqh lpfrfydqgr rgssdptvqr svfasvdkvp gfavaqcinq hsspslssqs
901  ppsasgspsg sgstshcdsg gtsssstpst aqspadapms pelpkphlpd qlvivnetea
961  dskpsknvar saavetasls pslvparqpt isllcedtad tlsvesltlv ppvdphslrs
1021 ltgmpplstp aaactepvge eaacaepvgt aed
```

Predicted transmembrane domain*
Tyrosine-based motif
Dileucine motif
Tri-acidic cluster motif

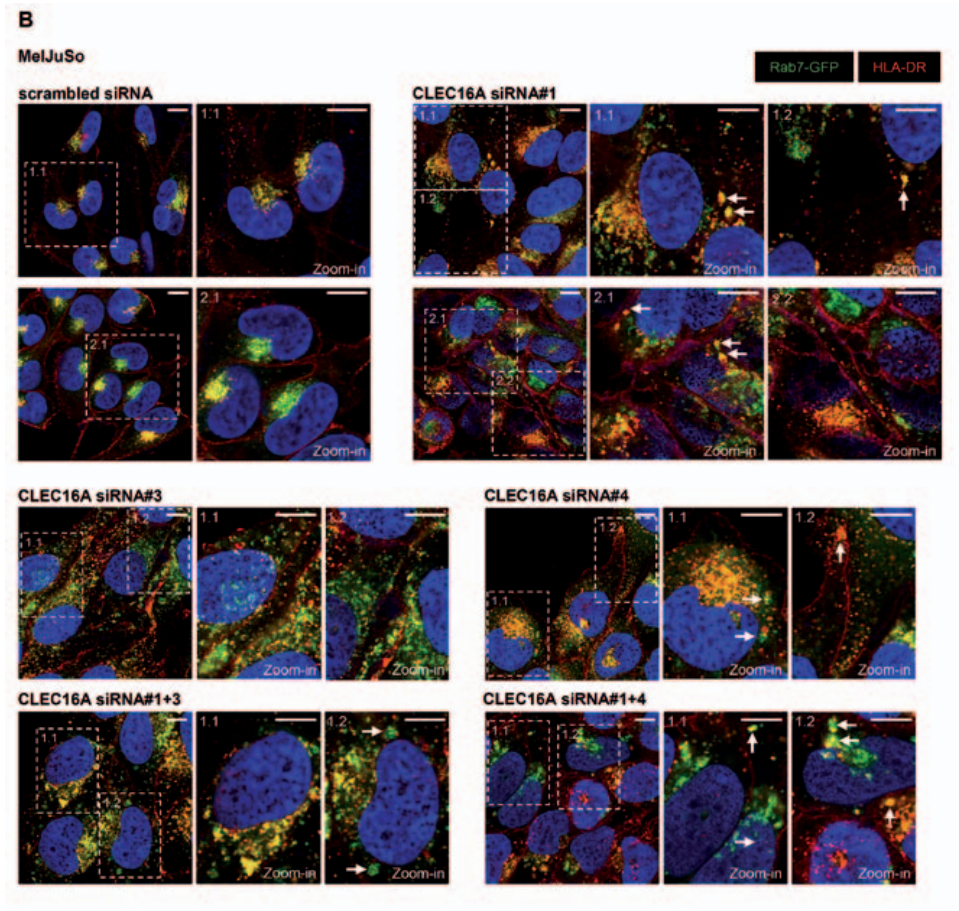
* adapted from http://www.ch.embnet.org

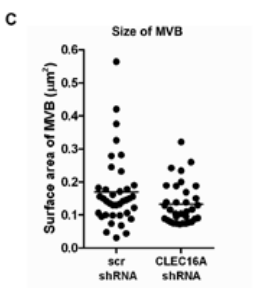
Supplementary Figure 3. Silencing with several siRNA duplexes against CLEC16A results in similar phenotype

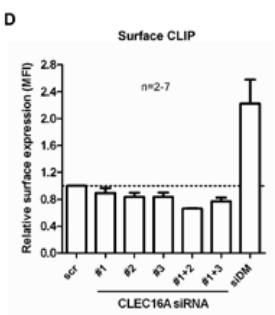


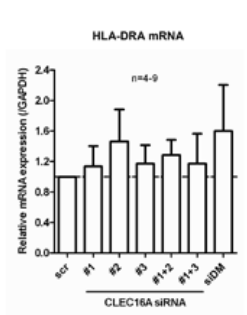
A-B) Silencing of CLEC16A with several siRNA duplexes results in a similar effects on the distribution of HLA-DR and CD63 in MelJuSo. **C)** The size of multivesicular bodies after silencing of CLEC16A with a shRNA against CLEC16A. **D)** Silencing of CLEC16A did not alter the expression of CLIP. **E)** CLEC16A siRNA did not affect the mRNA expression fo HLA-DRα

Supplementary Figure 3. Continued

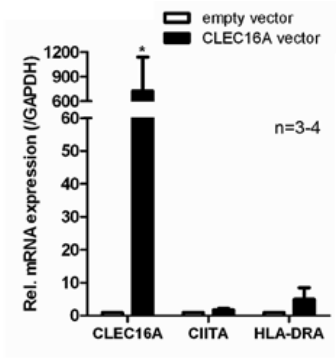




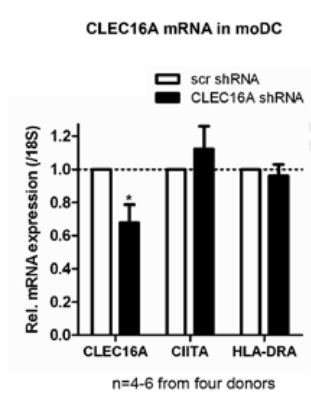




Supplementary Figure 4. CLEC16A DNA construct induces strong upregulation of CLEC16A mRNA

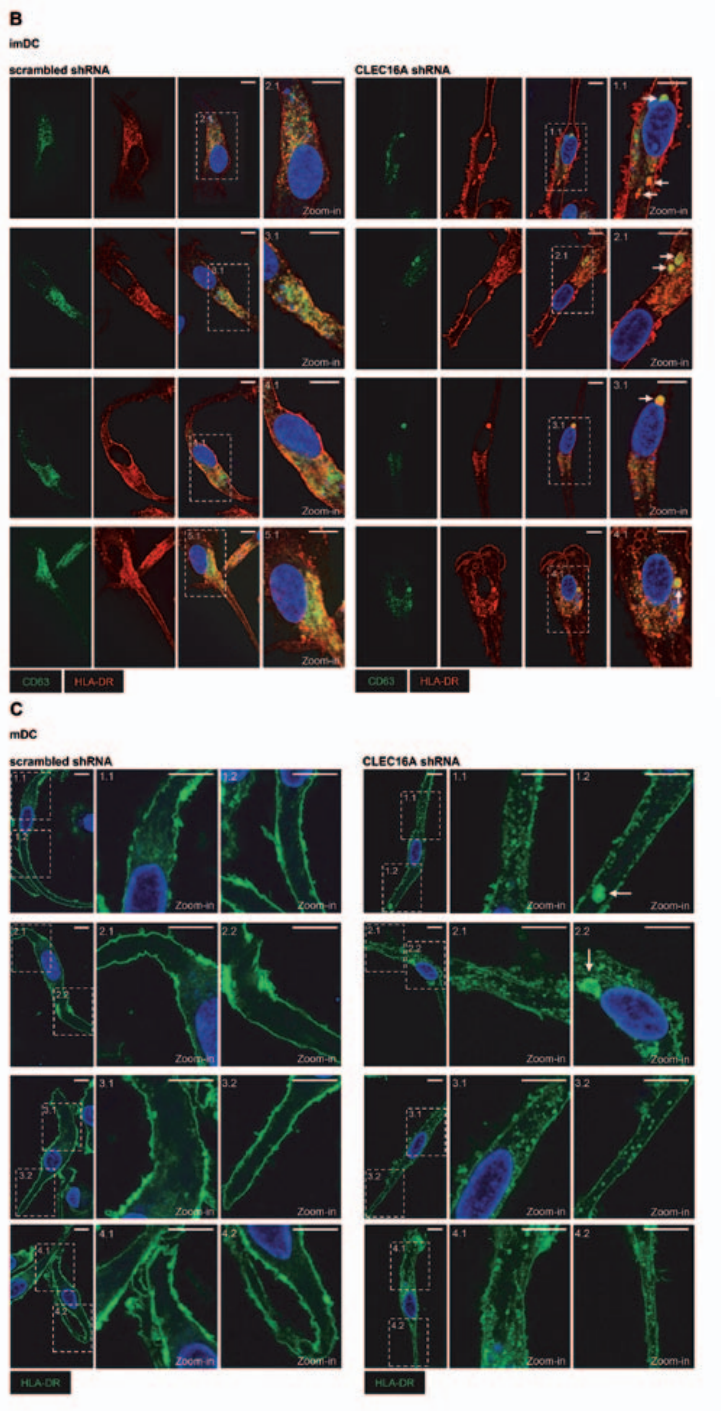


Supplementary Figure 5. shRNA against CLEC16A reduces CLEC16A, but not CIITA or HLA-DRα mRNA expression. CLEC16A shRNA affects the localization of late endosomes and HLA-DR

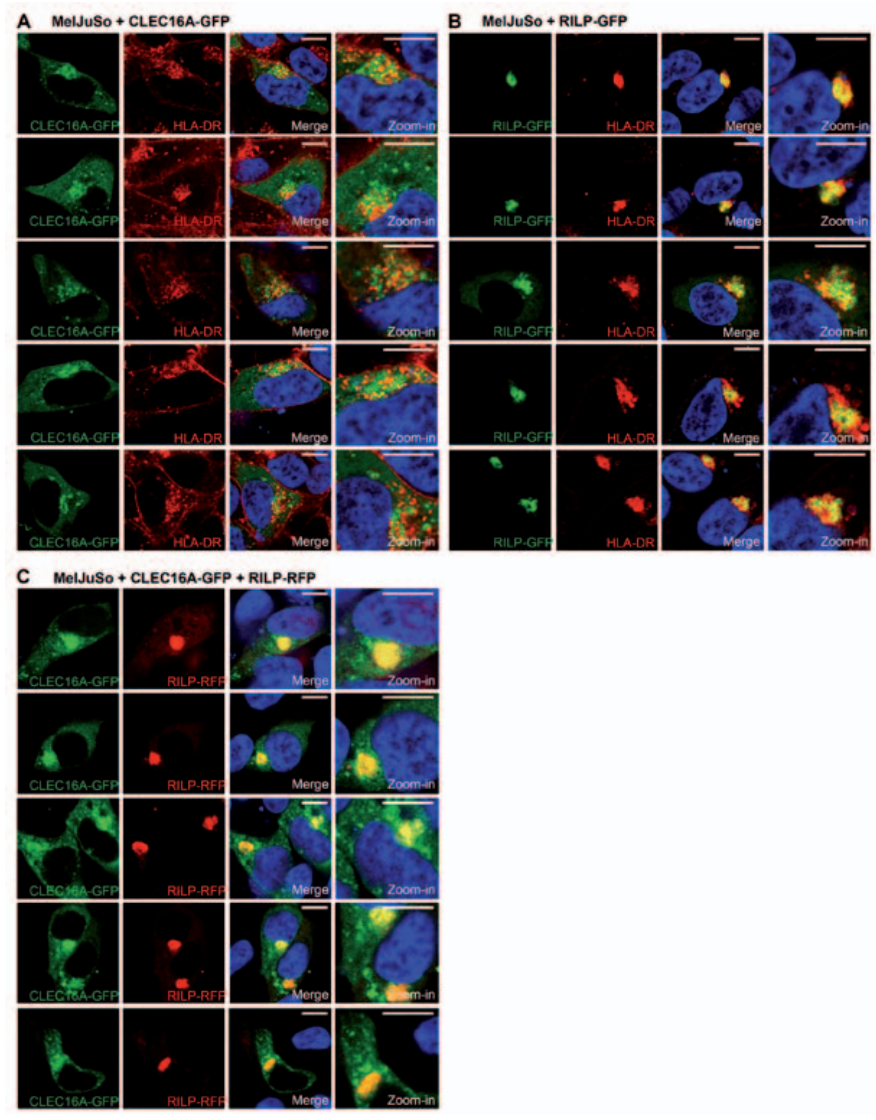


A) shRNA against CLEC16A only decreased CLEC16A mRNA and not CIITA or HLA-DRα. **B-C)** CLEC16A silencing results in enlarged foci containing HLA-DR.

Supplementary Figure 5. Continued

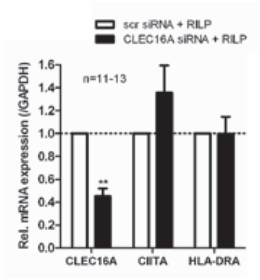


Supplementary Figure 6. CLEC16A colocalised with RILP and HLA-DR



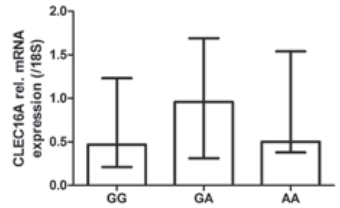
A) CLEC16A colocalised with HLA-DR. **B)** HLA-DR colocalised with RILP and **C)** RILP colocalised with CLEC16A.

Supplementary Figure 7. CLEC16A silencing did not alter the expression of CIITA or HLA-DRA

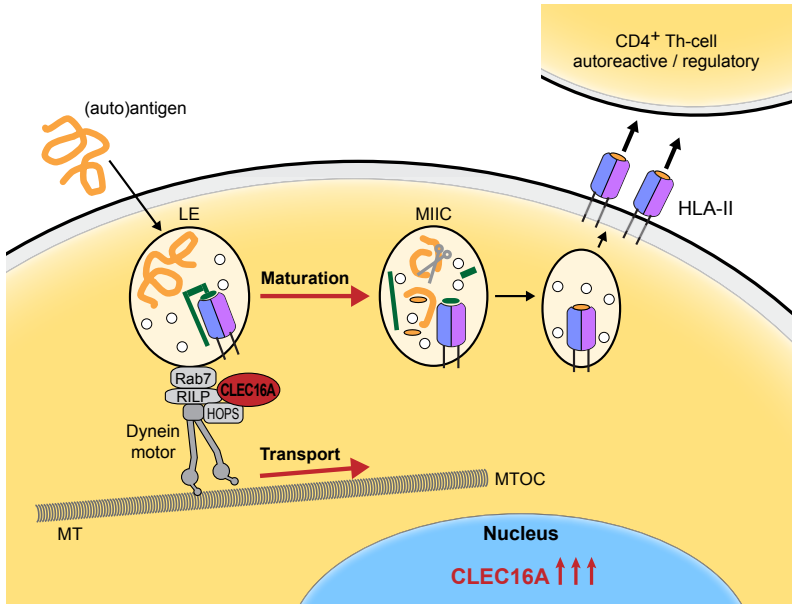


CLEC16A silencing only downregulated CLEC16A mRNA expression, but not CIITA or HLA-DRα

Supplementary Figure 8. CLEC16A mRNA expression similar between non-risk and risk carriers of CLEC16A rs7200786[A] SNP



Supplementary Figure 9. Proposed working mechanism of CLEC16A in endosomal maturation



Chapter 5

Genetic determinants of enhanced EBNA-1 IgG production in MS.

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Manuscript in preparation.

Chapter 6

Abundant kif21b is associated with accelerated progression in neurodegenerative diseases.

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E.M. Hol, B.A. Oostra, J.D. Laman and R.Q. Hintzen

Manuscript submitted

Abstract

Kif21b is one of the few multiple sclerosis (MS) risk genes with a presumed CNS function. Kif21b belongs to the kinesin family, proteins involved in intracellular transport of proteins and organelles. We hypothesised that kif21b is involved in the neurodegenerative component of MS and Alzheimer's (AD) disease. Post-mortem kinesin expression was assessed in 50 MS patients, 58 age and gender matched non-demented controls (NDC) and 50 AD patients. Kif21b mRNA expression was correlated with time from disease onset to EDSS 6.0 (sustained disability). Moreover, we assessed whether a correlation exist between kif21b expression and disease specific pathology (tangle formation for AD and grey matter demyelination in MS). Additionally, we used in situ analysis to determine which cell types expressed kif21b. Lastly, kif21b rs12122721 genotype was determined. Kif21b expression was five-fold increased in AD compared to MS and NDC aged below 62 years ($p=8*10^{-5}$), three-fold between 62-72 years (p=0.005) and not different above 72 years. No significant differences were observed between MS and NDC. Next, we assessed whether kif21b expression correlated with CNS pathology. In AD, the expression was two-fold increased in Braak stage 6 (scoring for density of neurofibrillary tangles) compared with stage 5 (p=0.003). In MS patients, kif21b correlated with the extent of grey matter demyelination (Spearman's rho=0.31, p=0.03). Next, we determined whether rapid development of neurological disability (EDSS 6.0) in MS patients correlated with kif21b. Abundant kif21b, defined as expression above the median, was associated with a two-fold accelerated development of EDSS 6.0 (median time in low kif21b group 16 years vs. high kif21b 7.5 years, log-rank test p=0.04). Given the genetic association of kif21b with MS risk, the results were stratified according to the rs12122721 [A] SNP. No association was found between kif21b expression or the time to EDSS 6 in kif21b risk SNP carriers compared to non-risk carriers. To assess whether the observed differences are specific to kif21b or that other kinesins also correlate with the time to develop EDSS, the expression of six other kinesins was assessed. No other kinesin was associated with accelerated time to develop EDSS 6.0. Immunofluorescence revealed that kif21b was expressed in astrocytes in addition to neurons. Upon astrocyte activation, kif21b increased approximately nine-fold. Abundant kif21b expression is associated with more severe pathology in MS and AD and with accelerated neurodegeneration independent of the kif21b MS risk SNP.

Introduction

Multiple sclerosis (MS) has classically been considered as an auto-immune disease of the white matter. However, the grey matter component of the pathology receives increasing attention, especially because axonal and neuronal degeneration occur already early in the disease [1]. This area of research received an impulse by novel MRI techniques to visualize grey matter lesions [2].

Recently, a large genome wide association study (GWAS) in MS patients identified 57 single nucleotide polymorphisms (SNP) associated with an increased risk to develop MS. Most of the (SNP) have important functions in immune cells, mainly in T-cell biology [3]. However, a few of these SNP may be involved in neurodegeneration. Kif21b is one of these SNP, which was also associated with MS risk in earlier studies [4, 5].

Kif21b is a relatively poorly studied member of the kinesin family, expressed most profoundly in the CNS, especially in the dendrites of neurons [6]. The kinesin superfamily contains 45 genes, known as kinesins or kifs, which are divided into 15 different families. These kifs are mainly expressed in neurons, and are localised in axons and dendrites in the CNS. Members of the kinesin family consist of a motor domain, a binding site for microtubules, and a specific cargo binding domain for the transport of different molecules and organelles. Neuronal functioning and survival largely depend on intracellular microtubule mediated transport, which is especially well developed in neurons, because long distances need to be covered along the axon [7]. The exact function and cargo transported by kif21b are currently unknown. Genetic variation in some of the kinesin family members has been linked to neurodegenerative diseases like amyotrophic lateral sclerosis (ALS), Huntington's and Alzheimer's disease [8].

Dementia is characterized by memory loss, cognitive impairments and decline in intellectual performance. The majority of patients with dementia suffer from Alzheimer's disease (AD). Neuropathological changes include the extracellular deposition of Aβ peptide in plaques, reactive gliosis [9] and intraneuronal aggregation of hyperphosphorylated Tau in tangles. Tau is a microtubule associated protein and binds to tubulin [10], along which kinesins transport their cargo. In AD, Tau is abnormally phosphorylated and therefore dissociates from microtubuli [11], leading to the formation of neurofibrillary tangles (NFT). The level of NFT strongly correlates with neuronal dysfunction and clinical progression in AD [12]. The extend of Tau pathology is reflected in the Braak stage [13].

We hypothesized that kif21b is aberrantly expressed in neurodegenerative disease and that its expression is associated with disease progression. Therefore, kif21b expression was assessed in 50 MS patients compared with 58 non-demented controls (NDC) and with 50 Alzheimer´s patients (AD). We found that cortical kif21b expression is significantly increased in AD patients compared with MS patients and NDC, independently of the MS risk genotype. Moreover, in AD and MS patients with more severe neuropathology, significantly higher expression levels of kif21b were found. This increased kif21b expression was associated with a shorter disease duration in both MS and AD patients and accelerated progression to sustained neurological disability (EDSS 6.0) in MS.

Materials and methods

Brain tissue

Brain and spinal cord tissue was obtained from the Netherlands Brain Bank (NBB), Netherlands Institute for Neuroscience, Amsterdam. The cortical tissue consisted of the medial temporal gyrus of 50 Alzheimer patients (including two AD patients: superior frontal gyrus tissue), 50 multiple sclerosis patients (including six tissues of MS patients from the superior frontal gyrus and one hippocampus) and 58 age and gender matched non-demented controls (all medial temporal gyrus). The majority of non-demented controls were free of neurological disease. However, three patients had cerebral or cerebellar micrometastases of carcinomas, one NDC had a small Schwannoma of the right vestibulocochlear nerve four NDC had old infarctions and one had mononeuropathy multiplex. None of the tumours or infarctions were located in the tissues sections studied here. White matter tissue was obtained from 18 NDC, 23 MS and three AD patients. Additionally, seven MS spinal cord samples were tested. All materials have been collected from donors from whom written informed consent for brain autopsy and the use of the material and clinical information for research purposes had been obtained by the NBB. Brain tissue was stored at -80ºC until use.

RNA isolation and real-time quantitative PCR

For isolation of total RNA 5-7 cryo-sections of 50 µm were used. RNA isolation was performed by using the GenElute Mammalian Total RNA Miniprep Kit (Sigma). RNA samples were treated with DNAse I to remove contaminating DNA (Invitrogen). Using 1.0µg RNA as a template, copy DNA (cDNA) was reverse transcribed by using Superscript II (Invitrogen). Primers and probes were selected by using the Universal ProbeLibrary Assay Design Centre (Roche). To determine target gene mRNA expression, real-time quantitative reverse transcription PCR was performed using TaqMan technology. GAPDH mRNA and GUSB (for brain tissue) or 18S (for astrocyte cultures) RNA levels were measured as a control to normalize for RNA input. Reference gene primers and probes were obtained from Applied Biosystems. An Applied Biosystems 7900 Sequence Detector was programmed for the initial step of 2 min at 50 °C and 10 min at 95 °C, followed by 40 thermal cycles of 15 s at 95 °C and 1 min at 60 °C. For calculation of mRNA expression levels, Ct values per gene were applied to standard curves, generated for each gene of interest. Sequences of the primers are listed in Supplementary Table 1a.

DNA isolation from brain tissue

5-10 mg of brain tissue was lysed using cell lysis solution (Qiagen) and Proteinase K (MP Biomedicals LLC) was added and incubated at 55°C over night. Samples were treated with RNAse A solution (Qiagen) and incubated at 37°C for 1 h. Protein precipitation solution (Qiagen) was added, and the samples were placed on ice for 5 min. 100% isopropanol was added to the supernatant and DNA was precipitated with 70% ethanol and hydrated in Tris EDTA buffer (TE-buffer) at 55°C for 5 min and stored at 4°C for SNP detection.

SNP detection in brain tissue

Risk SNP carriership of rs12122721 [A] in brain tissue was assessed in order to analyze whether this corresponds with mRNA expression levels. SNP detection was performed using Taqman assay (Applied Biosystems) as previously described [14]. NDC, MS and AD donors were all in Hardy Weinberg equilibrium (p=0.72) and the call rate was 98.9%.

Immunohistochemistry

Immunohistochemistry was performed on frozen sections of brain tissue to detect kif21b protein. Briefly, 6 µm frozen sections were cut and thawed onto gelatine-chrome alum coated glass slides. Slides were kept overnight at room temperature in humidified atmosphere and air-dried for 1h. Thereafter, slides were fixed in acetone containing 0.02% (v/v) H_2O_2. Slides were then air-dried for 10 min, washed with PBS and incubated with optimally diluted primary antibody specific for kif21b. Incubation with secondary antibody was performed, followed by tyramide signal amplification (TSA) according to the manufacturers' protocol (Invitrogen). HRP activity was revealed by incubation for 10 min at RT with 3-amino-9-ethyl-carbazole (AEC, Sigma) substrate, leading to a bright red precipitate. Slides were embedded with glycerol-gelatin (Boom). Incubation with isotype control antibodies of irrelevant specificity and omission of primary antibody were used as negative controls.

Grey matter lesion staging

MS grey matter brain lesions were staged on the basis of demyelination criteria, as described earlier [15, 16]. Grey matter tissue was stained with monoclonal antibodies against PLP, NeuN and MAP2 using immunohistochemical detection. The slices were scanned using a Nanozoomer microscope (Hamamatsu). Lesions were digitally staged according to the classification scheme for grey matter demyelinating lesions, using the Nanozoomer Digital Pathology software version 1.2. Shortly, lesions were scored as mixed white-grey matter (Type I), intracortical (Type II), subpial (Type III) and lesions stretching from the pia mater until the border of white matter (Type IV). Two independent observers (KLK, MvM), blinded to clinical information and kif21b expression, scored grey matter lesions based on PLP staining, on a standardized scoring form. Morphometric information regarding total area of the slide, area containing white matter in the tissue (based on absence of MAP2 and NeuN expression), total number, types and area of grey matter lesions was assessed. Moreover, expression of HLA-II was scored as well as meningeal infiltration. In addition, when the slide contained white matter lesions, the area and HLA-II expression of these lesions was scored.

Immunofluorescence

Double-labelling of kif21b expressing cells with NeuN, MAP-2, HLA-II or CD68 was performed using immunofluorescence as described previously [17]. Sections were first incubated with primary antibody mixture for 1h, followed by incubation of a mixture of secondary antibodies for 30 min. Sections were mounted in Prolong Gold (Molecular Probes) with 80 ng/ml DAPI (Molecular Probes).

Confocal fluorescence images were obtained on a Leica SP5 confocal system (Leica Microsystems CMS GmbH), equipped with an Argon laser (488 nm), Diode lasers (405 nm) and HeNe laser (633nm). Images were taken using a 63x NA 1.4 objective. Images were recorded using standard Las-AF software version 2.6.3 (Leica Microsystems CMS GmbH). Possible crosstalk between the different fluorochromes, which could give rise to false-positive co-localization of the signals, was completely avoided by careful selection of the imaging conditions. Emission windows for Alexa Fluor 488 dyes were between 498-578nm and for Alexa Fluor 647 between 643-775nm, and Kalman averaging was used.

All antibodies used in this study are indicated in Supplementary Table 1b.

Astrocytoma cell line cultures

U251MG human astrocytoma cells (ECACC 89081403, passage 28) were cultured in Dulbecco's modified Eagle's medium (DMEM) with GlutaMAX/ Ham's F-10 Nutrient Mix 1:1 containing 10% fetal bovine serum (FBS), 10 U/ml penicillin G and 10 mg/ml streptomycin (1% P/S) (all Invitrogen) at 37°C/5% CO_2.

Primary human astrocyte isolation and culture

For primary human adult astrocyte cultures, we obtained freshly dissected post-mortem subcortical white matter from a 79-year-old female control with a post-mortem delay of <18 hours (h) and a pH of the cerebrospinal fluid of 6.30 from the NBB. The tissue was collected in 25 ml cold Hibernate A (Invitrogen), and mechanically dissociated into small pieces. The tissue was digested with 0.2 % trypsin (Invitrogen) and 0.1% DNAse I (Invitrogen) at 37° C, while shaking for 30 min. Next, 2 ml FBS was added to the mixture and, subsequently, the cells were collected by centrifugation. The pellet was taken up in DMEM without phenol red containing 10% FBS, 2.5% Hepes, and 1% P/S (all Invitrogen), and the suspension was filtered through a 60 µm mesh screen. Then, Percoll (Amersham/GE Healthcare) was added (half of cell suspension volume), and this mixture was centrifuged to separate cells, debris and myelin at 3220 relative centrifugal force (rcf) at 4°C for 30 min. The second layer (glial cell containing fraction) was collected and washed with complete DMEM (containing 10% FBS, 1% P/S, 2.5% Hepes and 1% gentamycin, all Invitrogen). After centrifugation, the pellet was taken up in complete DMEM and cells were seeded in a 6-cm uncoated culture dish. Microglia will adhere to the dish and the astrocytes will be present in the medium. After 6 h at 37°C/5% CO_2, the medium, containing astrocytes, was taken off, centrifuged, and the microglia depleted pellet was seeded onto poly-L-lysine coated wells (PLL, Sigma-Aldrich, 15 µg/ml in PBS, 1 h at room temperature) in DMEM/Ham's F12 GlutaMAX medium containing 5% FBS and 1% P/S (all Invitrogen).

Cytokine treatment in astrocytic cultures

U251MG astrocytoma cells or primary human astrocytes were plated in 24-well plates (25.000 cells per well) in their respective culture medium and allowed to adhere overnight. Then, medium was

replaced and cells were cultured in medium with vehicle (PBS) or medium with a combination of recombinant human IFN-γ (R&D systems, 50 ng/ml) and recombinant human IL-1β (R&D systems, 50 ng/ml). After 48 h, cells were collected for mRNA isolation.

Statistical analysis

Gene expression data and presence of the SNP compared to kif21b expression levels were analyzed using Kruskal Wallis test for multiple groups. For subgroup comparisons, Dunn's Multiple Comparisons test was used. Mann Whitney U-test was performed to test differences between two groups. To determine correlations, non-parametric Spearman's test was used. For survival analysis, the time between onset of MS and reaching EDSS 6.0 [18] was calculated and kif21b expression was dichotomized with expression above and below the median. Kaplan-Meier curves were constructed and differences in time to reach EDSS 6.0 were tested with a log-rank test. Hazard regression was performed to correct survival data for confounding variables. Statistical analysis was performed using SPSS version 20 (IBM). Graphs were made in GraphPad Prism and Dunn's multiple comparisons test were also performed in GraphPad Prim version 5.04 (GraphPad Software Inc.). p-values <0.05 were considered statistically significant and are denoted in the figures as * $p<0.05$, ** $p<0.01$ and *** $p<0.001$.

Results

Characteristics of AD and MS patients and NDC

We assessed the size of the investigated tissue and the percentage of grey matter in the tissues of MS, AD and NDC and these characteristics were similar (Supplementary Figure 1). No significant differences in kif21b expression were found between tissues obtained from the medial temporal gyrus or the superior frontal gyrus (Supplementary Figure 2). The age at death, pH of the CSF, and post-mortem delay (PMD) were significantly different between NDC, MS and AD (Table 1). Linear regression showed no clear trend for kinesin expression and PMD or pH, in contrast to age at death (Supplementary Table 2). Thus, for disease specific kinesin expression, stratification according to age at death was performed based on the age quartiles of NDC.

Table 1. Clinical and demographical data

	NDC (n=58)	MS (n=50)	AD (n=50)	p-value
Age at death (SD)	71 (13)	63 (13)	66 (9)	0.004
Median post-mortem delay in hours (IQR)	7:35 (6:18-9:16)	6:57 (5:25-8:15)	5:20 (4:27-6:13)	$4*10^{-7}$
pH (IQR)	6.64 (6.45-6.94)	6.50 (6.36-6.71)	6.47 (6.34-6.67)	0.02
Percentage female	72	74	76	0.92
Kif21b rs12122721 genotype n (%) GG AG AA Undetermined	 27 (46.6) 26 (44.8%) 3 (5.2%) 2 (3.4%)	 31 (62.0) 15 (30.0) 4 (8.0%) 0 (0)	 21 (42.0%) 26 (52.0%) 3 (6.0%) 0 (0)	 0.24

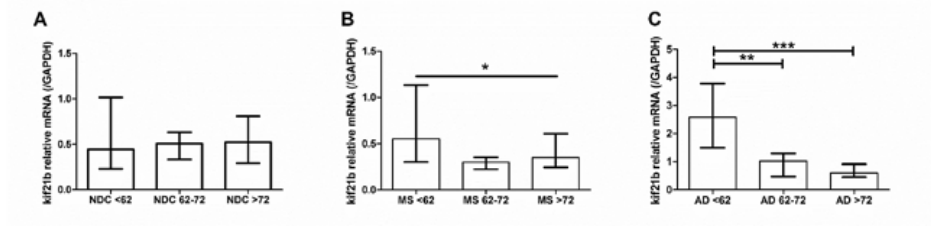
IQR: interquartile range

Kif21b is increased in AD patients compared with MS and NDC

During physiological aging in NDC, no changes in kif21b expression were observed with increasing age (p=0.9, Fig. 1A). However, a significant decrease of kif21b over age was observed in MS (p=0.03, Fig.1B) and AD patients ($p=5*10^{-4}$, Fig. 1C). Comparing MS, AD and NDC in the different age groups, we found that kif21b is significantly increased in AD compared with MS patients and NDC. Interestingly, in the age category below 62 years of age, an approximately five-fold increase in kif21b was observed in AD patients compared with MS. Kif21b in AD compared to NDC was approximately six-fold increased ($p=9*10^{-5}$), whereas no significant differences were found between MS and NDC. In the age group from 62-72 years, a three-fold increase in AD compared with MS and two-fold between AD and NDC (p=0.005) was observed. In the elderly patients (>72 years), no differences between MS, AD and NDC were found (p=0.23, Fig. 2A). Next, we assessed whether the alterations in kif21b expression might

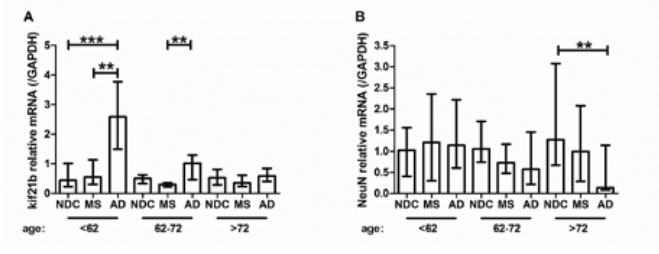
reflect differences in neuron density. Therefore, we used NeuN (RBFOX3) as marker for neuron density and found a significant reduction in NeuN in the AD patients compared with NDC above 72 years of age (p=0.006, Fig. 2B), whereas no differences between these groups in kif21b expression were found (Fig. 2A). Additionally, we determined the expression of other CNS specific markers, GFAP (astrocytes) and MBP (oligodendrocytes). In the youngest AD, we observed a significant increase of GFAP (p=0.02) compared with MS and in MBP (p=0.003) compared with NDC and MS (Supplementary Figure 3). No significant differences between males and females were observed or between primary progressive and secondary progressive MS (Supplementary Figure 4 and 5 respectively). Additionally, we validated kif21b expression levels corrected for PMD as there were slight, though significant differences in PMD between the different patients groups. Similar results were found for kif21b expression corrected for PMD as with the uncorrected kif21b expression, indicating that it is unlikely that the PMD has influenced the kif21b expression (Supplementary Figure 6).

Figure 1. Kif21b expression in the grey matter does not change during physiological ageing, but is significantly increased in young Alzheimer patients.



Kif21b expression was stratified according to the age at death into three categories based on age at death of the NDC (<25th percentile represents <62 yrs, between 25-75th percentile equals 62-72 yrs or >75th percentile is >72 yrs). Kif21b was compared between the three age categories in **A)** NDC, **B)** MS and **C)** AD.

Figure 2. Cortical kif21b is significantly increased in younger Alzheimer patients compared with MS and NDC.



A) Kif21b expression is significantly increased in AD patients younger than 62 years at death compared with MS and NDC younger than 62 yrs and in AD patients between 62-72 yrs compared with MS patients in the same age category. **B)** No significant differences were found in the expression of NeuN, a neuron specific marker, in the youngest two age categories. However, NeuN expression significantly decreased in elderly AD patients compared with NDC.

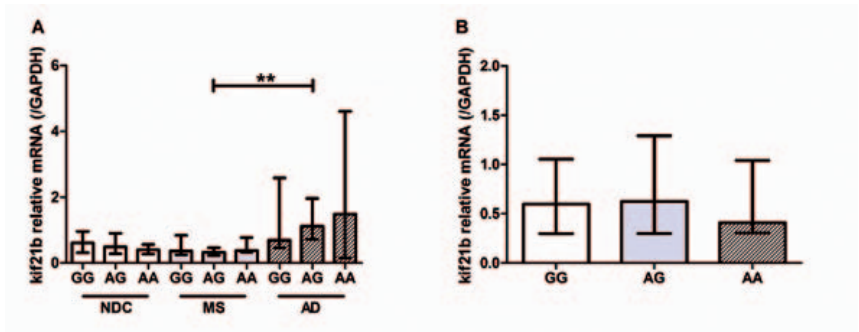
Kif21b in spinal cord correlates with cortical kif21b expression of MS patients

The medial temporal gyrus is preferentially affected in AD, while this area is affected in approximately 34% of clinically isolated syndrome patients (CIS, the first attack of neurological deterioration in MS) [1]. To investigate whether kif21b levels in a commonly affected area in MS correlates with expression in the temporal gyrus, we assessed kif21b expression in seven matched brain and spinal cord tissues. We found a clear trend between expression in brain and spinal cord in MS patients (p=0.06, Supplementary Figure 7).

No correlation between increased kif21b expression and kif21b risk SNP

We hypothesised that kif21b expression is influenced by the kif21b risk SNP and therefore kif21b expression was stratified according to rs12122721 [A] risk allele carriership in the three patients groups. In none of the three groups, carriership of the risk allele correlated with kif21b expression (all p>0.54, Fig. 3A). Even after pooling all donors for the three groups, no difference in kif21b expression between the different genotypes was found (p=0.74, Fig. 3B).

Figure 3. No differences between MS risk genotypes and kif21b expression levels.



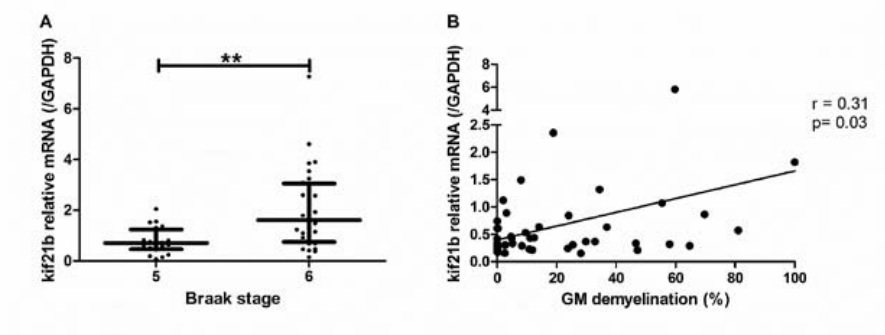
A) Kif21b expression was compared within NDC, MS and AD and stratified according to kif21b rs12122721 [A] risk genotype and within genotypes between the different diseases. No obvious differences between the risk genotypes or diseases were observed. **B)** The three groups of donors were pooled and kif21b expression was stratified according to the rs12122721 genotypes. No significant differences were observed between the three genotypes.

More severe pathology is associated with increased kif21b expression in AD and MS

Next, we hypothesised that kif21b expression correlates with the severity of neuropathology in Alzheimer and MS. AD pathology was staged according to the Braak criteria [13] by experienced neuropathologists from the Netherlands Brain Bank. Kif21b mRNA expression was stratified according to these criteria. Kif21b expression is significantly higher in Braak stage 6 compared with Braak stage 5 (p=0.003, Fig. 4A). Additionally, we assessed whether the level of kif21b expression correlated with the extent of grey matter demyelination in MS patients. A significant correlation between kif21b and

the percentage of cortical demyelination was found (Spearman's rho=0.31, p=0.03, fig. 4B), indicating that kif21b is increased with more severe neuropathology.

Figure 4. Kif21b expression correlates with both AD and MS pathology.

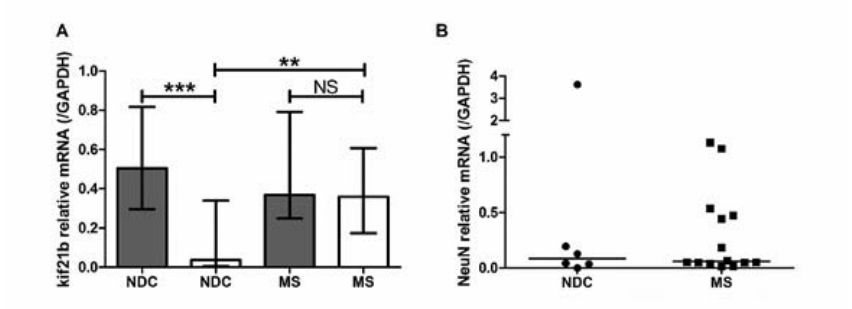


A) Kif21b expression was stratified for AD patients according to the Braak criteria. AD patients with more severe AD pathology (Braak stage 6) had significantly higher kif21b expression levels. **B)** For MS patients, the total area of grey matter demyelination was quantified and percentage of cortical demyelination was calculated. With increasing percentage of GM demyelination, enhanced kif21b expression was observed.

Kif21b is approximately ten-fold more abundant in MS white matter compared to NDC

In view of the significant correlation between the amount of grey matter demyelination and kif21b expression, we hypothesised that kif21b is increased in the white matter (WM) of MS patients, a common area of demyelination in MS. Kif21b mRNA expression was assessed in the white matter of 11 NDC and 16 MS patients (Supplementary Table 3) and we found that kif21b was approximately ten-fold increased in MS compared with NDC (median and IQR of kif21b expression relative to GAPDH in NDC 0.037, 0.006-0.34 vs. MS 0.36, 0.17-0.34, p=0.03, Fig. 5A). Comparing the levels of expression between the white and grey matter in NDC revealed that kif21b is highly expressed in the grey matter and hardly in the WM as expected with a kinesin highly enriched in dendrites of neurons. Interestingly, in MS patients the levels of expression in the WM and GM are comparable (Fig. 5A). Next, we assessed whether these differences in kif21b expression in the WM of MS patients and NDC could be explained by differences in neuron density. No differences in NeuN expression levels in the WM of NDC and MS patients were found (p=0.65, Fig. 5B), but GFAP and MBP were increased in MS patients (Supplementary Figure 8).

Figure 5. In MS white matter, kif21b is approximately ten-fold increased compared with NDC white matter.



A) Kif21b expression in the white matter of MS and NDC was determined. MS patients had approximately ten-fold increased kif21b expression compared with NDC (white bars). As a reference, the kif21b cortical expression levels are shown (grey bars). In MS patients, kif21b white matter expression equalled kif21b grey matter expression, whereas in NDC kif21b expression in the white matter compared with the grey matter was significantly lower. **B)** No significant difference in the expression of NeuN in the white matter of MS patients compared with NDC was observed. Thus the number of neurons is equal and this could not explain the differences in kif21b expression.

Kif21b protein is increased in AD patients and expressed in neurons and astrocytes

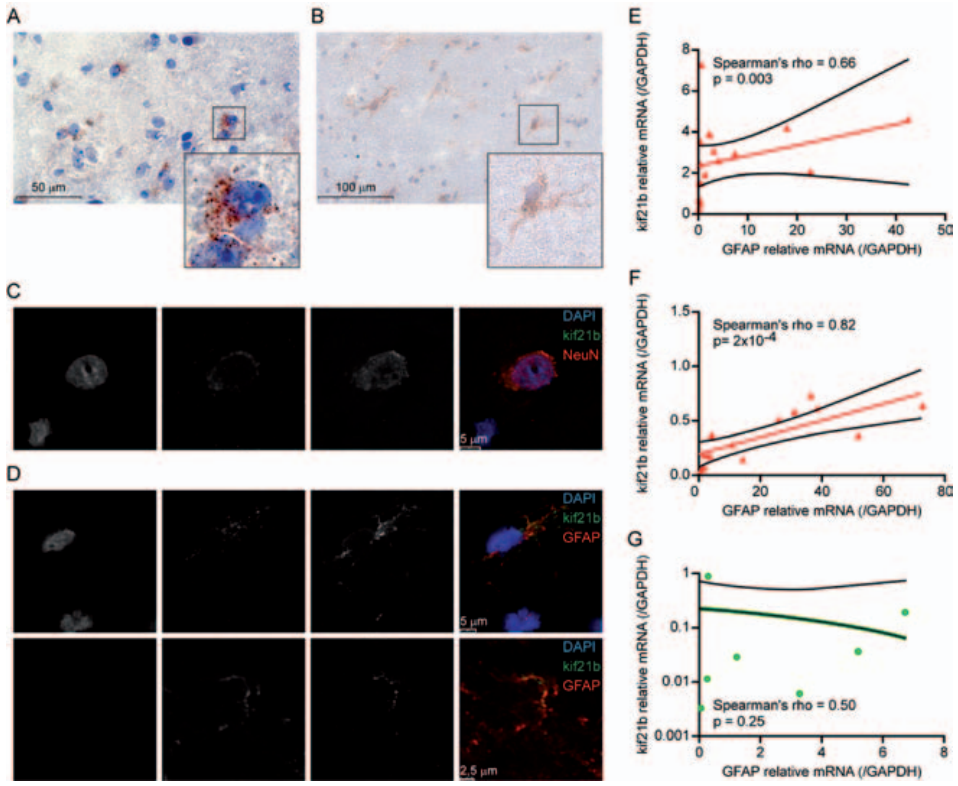
Using immunohistochemistry, we next assessed whether kif21b protein is also increased in the cortex of AD patients compared with NDC and MS (Supplementary Table 3) to validate our mRNA findings in grey matter tissues. Firstly, we assessed which cell types expressed kif21b. Both glia cells and neurons expressed kif21b (Fig. 6A). Next, we semi-quantitatively scored kif21b expression in age-, gender- and genotype matched NDC, MS and AD patients. Kif21b expression was higher in AD patients compared with MS and NDC, both in neurons and in glia cells (Table 2), confirming the mRNA data (Fig. 2). Next, we assessed whether the kif21b protein was also expressed in the white matter. In one NDC out of 16, we could not detect any kif21b protein in the white matter, whereas in 11 out of 14 MS patients kif21b protein was highly expressed in the WM (Fig. 6B). In the WM of three AD patients, we also observed kif21b protein expression. The WM expression of kif21b is highly variable within individual tissues, some areas had very high kif21b expression, whereas other regions are completely kif21b negative (Supplementary Table 4) and this hampers reliable quantification. This variation possibly indicates that kif21b is expressed in activated cells. Lastly, we assessed which cell types expressed kif21b. No kif21b expression was found in microglia cells (Supplementary Figure 9A and B) or in SMI32 positive axons (Supplementary Figure 9C and D). High kif21b expression was found as expected in neurons (Fig. 6C) and interestingly also in astrocytes (Fig. 6D). Only in young AD patients, GFAP mRNA correlated with kif21b expression in the grey matter (Fig. 6E, Supplementary Figure 10). In frontotemporal dementia, especially young patients have reactive astrocytosis [19], comparable with our findings. Additionally, also in the white matter of MS patients (Fig. 6F), but not NDC (Fig. 6G), a positive correlation between GFAP and kif21b mRNA expression was found, again pointing to activation of astrocytes.

Table 2. Quantification of kif21b expression in situ

Patient id	Age at death	Gender	Presenting symptom	Time to EDSS 6.0 (years)	Neuropathology assessment		Kif21b [1]		Kif21b genotype
					Braak stage	Amyloid	Neurons	Glia cells	
NDC2	49	F	NA	NA	0	ND	+	-	GA
NDC4	53	F			0	0	+/-	-	GA
NDC6	56	F			0	0	+/-	-	GG
NDC8	62	F			1	0	+/-	-	GA
NDC10	70	F			0	B	+	+	GA
NDC13	50	M			1	0	-	-	AA
NDC29	77	M			1	B	+/- / +	-	GG
NDC30	78	M			1	A	+/-	+/-	AA
NDC36	82	M			1	A	+/-	-	GG
MS4	50	F	Myelitis	7	ND	ND	-	-	GG
MS6	56	F	ND	4 *	ND	ND	+	+	GG
MS8	63	F	Optic neuritis	17	0	0	+	-	GA
MS10	70	F	Cerebrum	30	1	0	-	-	GG
MS23	56	M	Myelitis	2	ND	ND	-	-	AA
MS25	57	M	Myelitis	6	ND	ND	+/-	-	GG
MS40	74	M	Myelitis	8	3	0	-	-	AA
AD2	57	F	NA	NA	6	C	+	+/-	GA
AD4	57	F			6	C	+	-	GG
AD6	59	F			5	C	+/++	+/++	GA
AD8	63	F			5	C	+/-	-	GG
AD10	70	F			5	C	+	-	GA
AD13	42	M			6	C	+/-	+/-	AA
AD15	54	M			6	C	+	+/-	AA
AD20	58	M			4	C	++	++	GG
AD42	76	M			5	C	+	+/-	GA

[1] scored as: - no positive cells, +/- 1-2 positive cells per field, + maximum of ~30% of the cells positive, ++ ~60% of the cells positive, +++ ~80% positive cells and +++ (virtually) all cells positive
* time to EDSS 9.0
NA not applicable, ND not determined or documented
Neuropathology assessment based on Braak criteria [13].

Figure 6. Kif21b is expressed in astrocytes and costains with GFAP in AD grey matter and MS white matter.



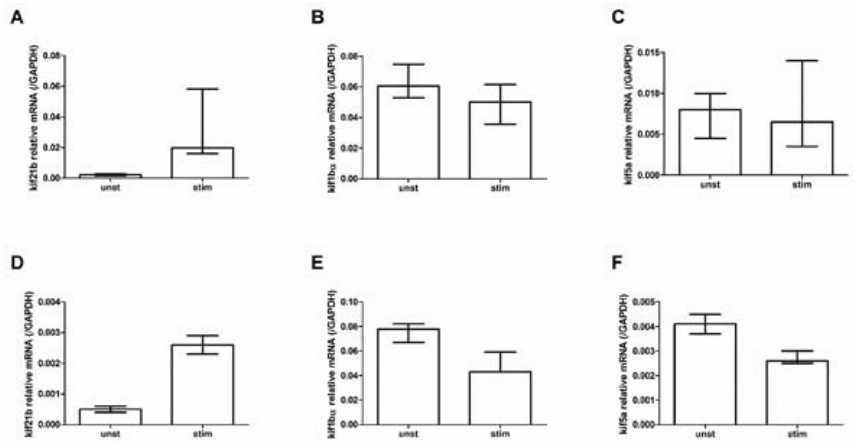
Representative immunohistochemical staining of kif21b expression in **A)** the grey matter (out of 25 investigated tissues, Table 2) and **B)** in the white matter (out of 26 samples, Supplementary Table 4). Based on morphology, different cell types express kif21b. Immunofluorescence staining in six tissues revealed that **C)** kif21b is expressed in the somata of neurons and **D)** kif21b is expressed in the cell body as well as the processes of astrocytes. Kif21b expression correlates with GFAP expression **E)** in the young AD patients in the grey matter, **F)** in MS white matter, **G)** no correlation was found between GFAP and kif21b in white matter of NDC.

Kif21b is upregulated during astrocyte activation

Most knowledge on kifs focused on neurons and virtually nothing is known regarding kinesins in astrocytes. Therefore, we decided to determine kif21b in astrocytes further. We asked whether astrocytes express a basal level of kif21b or that the observed kif21b expression in both WM and GM astrocytes reflects reactive astrocytosis. The astrocytoma cell lines U251 was stimulated with IL-1β and IFN-γ for 48 h and kif21b expression was compared between unstimulated and stimulated conditions. The activation status was determined by assessing IL-6 production (Supplementary Figure 11A and B). Upon astrocyte activation, kif21b increased approximately nine-fold (Fig. 7A). To assess whether this is specific for kif21b or that other kinesins are also induced and therefore reflecting

cell activation in general, kif1bα and kif5a expression levels were also determined. The expression of kif1bα did not change during astrocyte activation (Fig. 7B), whereas kif5a slightly decreased (Fig. 7C). Lastly, we determined whether this mechanism is also observed in primary astrocytes isolated directly post-mortem, to corroborate the U251 study. Similar, but more pronounced changes in kinesin expression upon activation were observed (Fig. 7D-F).

Figure 7. Expression of kif21b, but not other kinesins increases upon astrocyte activation.



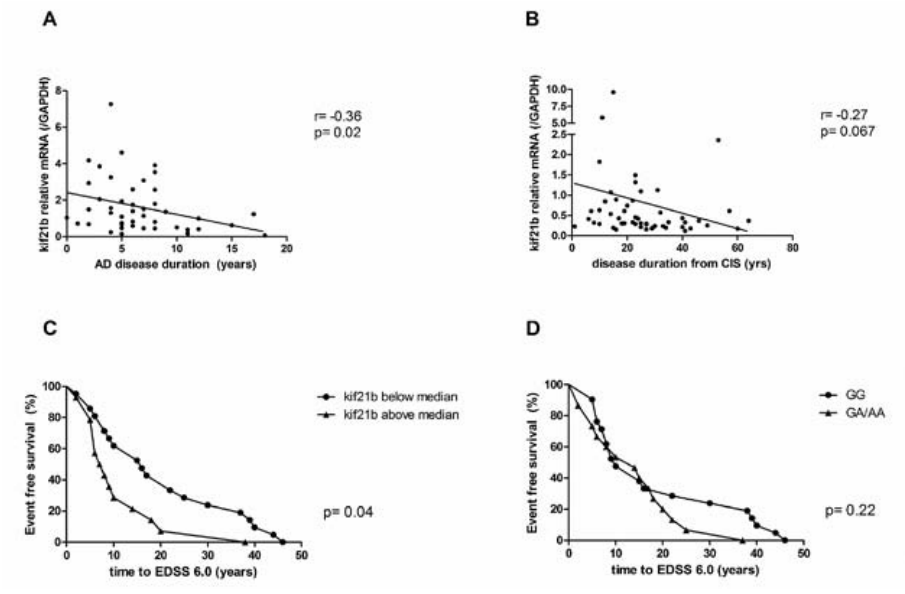
The astrocytoma cell line U251 was stimulated with IL-1β and IFN-γ for 48h in three independent experiments in duplo. **A)** Kif21b expression increased approximately nine-fold upon stimulation, whereas **B)** kif1bα did not change **C)** and kif5a slightly decreased. Next, primary isolated astrocytes of a NDC were also stimulated in one experiment in triplo and similar changes in kinesin expression were found **(D-F)**

High levels of kif21b expression are associated with a more rapid and severe disease course in MS and AD

Given the correlation between kif21b and neuropathology, we hypothesised that kif21b expression contributes to more rapid progression in neurodegeneration. Therefore, we assessed whether a correlation between disease duration and kif21b expression existed. In AD, high kif21b expression was associated with a significantly shorter disease duration (Spearman's rho -0.36, p=0.02, Fig. 8A) and a similar trend was observed in MS (Spearman's rho -0.27, p=0.067, Fig. 8B). Next, we assessed whether kif21b levels in MS also correlated with a shorter time to reach EDSS 6.0, a measure for sustained disability. Kif21b was dichotomised as expression above or below the median and a Kaplan Meier survival curve was constructed. Kif21b expression above the median is associated with an approximately two-fold more rapid progression to EDSS 6.0 compared to expression below the median (Log Rank test, p=0.04, Fig. 8C). MS patients with abundant kif21b expression had a three-fold increased risk to have an accelerated disease course (hazard ratio (HR) for abundant kif21b in the development of sustained disability corrected for age at onset 3.0, 95% CI 1.4-6.4, p=0.003), independent of gender. Given the significant correlation between kif21b and the percentage

of GM demyelination, we assessed whether the time to develop EDSS 6.0 is independent of GM demyelination. Abundant kif21b expression adjusted for the percentage of GM demyelination revealed that a rapid progression to EDSS 6.0 is independent of the percentage of GM demyelination. Moreover, GM demyelination is not associated with the time to develop EDSS 6.0 (Supplementary Table 5). No significant difference in time to reach EDSS 6.0 between kif21b risk SNP carriers and non-risk SNP carriers (p=0.22, Fig. 8D) was observed, indicating that the accelerated neurodegeneration is independent of the MS risk SNP (HR 1.3, 95% CI 0.61-2.79, p=0.07). Additionally, adding the kif21b risk SNP to the Hazard regression model for the effect of abundant kif21b expression on the time to develop EDSS 6.0 did not alter the results, indicating that kif21b expression is the explanatory factor (Supplementary Table 5). Given the association between astrocyte activation and kif21b expression, we assessed whether high expression of GFAP correlated with an accelerated time to EDSS 6.0 and found no association (HR 0.97, 0.45-2.07, p=0.94). Thus, abundant kif21b expression is an independent predictor for accelerated progression to develop EDSS 6.0 (Supplementary Table 5).

Figure 8. Abundant kif21b expression is associated with a shorter disease duration and accelerated progression to sustained neurological disability.



Kif21b expression was correlated with the disease duration of **A)** Alzheimer patients and **B)** multiple sclerosis patients. In both diseases, shorter disease duration was associated with abundant kif21b expression. **C)** Kif21b expression in MS patients was dichotomised for the expression above the median and below the median and survival analysis was performed in MS patients. MS patients with kif21b expression above the median had a significantly shorter time to develop EDSS 6.0, the neurological score for sustained disability. **D)** The time to develop EDSS 6.0 was stratified according to kif21b MS risk SNP carriership. The rs12122721 [A] SNP is not associated with accelerated time to develop EDSS 6.0

Other kinesins are not associated with accelerated neurodegeneration

Lastly, we assessed whether abundant kinesin expression in general is associated with a more rapid neurodegeneration in MS. Therefore, we tested six other kinesins of different kinesin families. Two of these kinesins have previously been associated with MS (kif1b and kif5a), although subsequent validation of kif1b failed [20], whereas the other kinesins are unrelated to MS. Members of the kinesin-1 superfamily (kif5a, b, c and the kinesin light chain, KLC) are associated with AD**.** For kif5a specifically, we observed a trend that abundant expression is associated with a shorter time to develop EDSS 6.0 (HR 2.0, 95% CI 0.97-4.2, p=0.06). None of the other kinesins showed a trend towards accelerated neurodegeneration (Supplementary Table 6), indicating that specific kinesins are involved in a more rapid disease course.

Discussion

Genetic variations in the kif21b locus confer a modestly increased risk to develop MS [3]. However, the underlying mechanisms are currently completely unknown. We here show that abundant levels of kif21b are associated with accelerated progression to EDSS 6.0 and a shorter disease duration in MS. Similarly in AD, high levels of kif21b expression are found in patients who have had a short disease duration. Moreover, increased levels of kif21b are associated with more severe neuropathology in both AD and MS. The observed differences in expression and progression were independent of the MS risk SNP. Similar to our findings, Harding et al found no association between the kif21b SNP and the time to develop EDSS 6.0 [21]. We and others have functionally investigated the intronic rs12122721 [A] SNP, whereas recently the rs7522462 [G] SNP was reported in the largest MS GWAS. Rs7522462 is located in an open reading frame nearby the kif21b gene. These SNP are in linkage disequilibrium with the each other (R^2 >0.73, based on http://www.broadinstitute.org/mpg/snap/) and therefore it is unlikely that this has affected the results.

Genetic variations in both kif21a and kif21b are linked to several human diseases. Microduplications in the kif21b locus were identified in two patients with neurodevelopmental disorders. These two patients had delayed motor and cognitive development [22]. Kif21a is a family member of kif21b and has similarities in amino acid composition, but its expression pattern is different. Kif21a is expressed throughout neurons and kif21b is mainly expressed in dendrites. Kif21a is linked to congenital fibrosis of the extraocular muscles type 1 (CFEOM1), a disease characterised by absence of motor neurons of the midbrain and in the superior division of the oculomotor nerve [23]. Moreover, CFEOM1 with a Marcus Gunn jaw-winking phenomenon is associated with another kif21a mutation [24]. Variation in the kif21b locus is also strongly associated with ankylosing spondylitis (M. Bechterew) [25] and moderately with ulcerative colitis [26, 27]. This suggests that kif21b has an additional function in the immune system, which is beyond the scope of the current report.

Also other kinesin family members are genetically associated with human diseases. For example, a variant in kif5a locus has been linked with the risk to develop rheumatoid arthritis [28] and is a candidate SNP in MS [29]. Moreover, kif5a mutations are associated with the development of hereditary spastic paraplegia (SPG10), a neurodegenerative disease. At least one mutation in kif5a decreases both the anterograde and retrograde transport flux of neurofilaments [30]. Interestingly, we observed a trend that abundant kif5a is also associated with a more rapid development of sustained disability in MS (Supplementary Table 6). Additionally, a SNP in kif1b has been implied as risk SNP for MS [31], but a subsequent study failed to replicate this finding [20].

Kif21b, a plus end-directed motor kinesin, is produced in the cell body of the neuron, after which it is transported to the dendrites, where it is mainly expressed. Kif21b might be involved in delivering currently unknown cargoes to the distal regions of dendrites [6]. The physiological function of kif21b and the mechanisms by which abundant kif21b and possibly kif5a in neurons contribute to accelerated neurodegeneration remain to be determined. Whether abundant kif21b is the cause or

consequence of the accelerated neurodegeneration needs to be investigated. Possible mechanisms in a causative model are that abundant kif21b expression leads to increased transport and thereby to increased energy consumption. The energy production might fail and this may result in tissue damage. In MS brain, numbers of mitochondria were higher, which might support this hypothesis [32]. Kif21b expression may also be increased as a response to tissue damage. Recently, it was shown in experimental autoimmune encephalomyelitis (EAE), an animal model of MS, that during Theiler's murine encephalomyelitis virus (TMEV) infection, kif21b expression is significantly increased already 14 days post infection (dpi). However, this increase was transient, as kif21b normalised 42 dpi and remained normal until 196 dpi [33].

Increasingly, it has been recognised that in addition to white matter lesions, also grey matter pathology in MS occurs already in early in the disease [2]. One of the major pathological changes besides demyelination in MS is axonal damage, however relatively little is known about neuronal damage [34]. It is intriguing to speculate that kinesins might play a pivotal role in these processes as they are important for axonal transport and thereby for neuron integrity, function and survival. Currently, as far as we are aware of, hardly anything is known about the expression and distribution of kinesins in MS or AD. In AD, extracellular depositions of insoluble Aβ peptide occur in the plaques. Moreover, in neuronal cell bodies hyperphosphorylated Tau molecules can aggregate. Interestingly, Tau is a microtubule-binding protein, which is able to inhibit phosphorylation of kinesin light chains, leading to the dissociation of the transported cargo from kif5 [35, 36]. Although there is evidence that the heavy and light chains of kif5 are important for AD [37, 38], other kinesin family members might also play a role in APP transport. It is therefore interesting that kif21b expression is increased in AD and that abundance correlates with a shorter disease duration in both MS and AD, and with the time to develop sustained disability in MS.

Additionally, we showed that kif21b is also expressed in activated astrocytes Abundant levels of kif21b expression have been linked to a poorer prognosis of several forms of cancers. Increased kif21b expression was observed in gliomas, brain tumours with a very poor prognosis, especially the glioblastoma multiforme with a survival rate of less than a year [39]. Increased kif21b levels were also observed in three well-characterised astrocytoma cell lines, KINGS-1, no. 11 and Becher compared with benign gliomas or healthy brain tissue [40]. This is comparable to our findings that kif21b expression increases during astrocyte activation. High levels of kif21b expression in neurodegenerative diseases are associated with a more rapid disease course as we have shown here, again pointing to an important role of kif21b in astrocytes. The mechanistic consequences of abundant kif21b expression and the upregulation of kif21b upon activation in astrocytes remain speculative as the function of kinesins in general and kif21b specifically in astrocytes is currently unknown. It might be that kif21b is upregulated to support the trophic function of astrocytes [41]. The increased kif21b might lead to more vesicle transport, mediating enhanced release of gliotransmitters and dysfunction of the trisynapse, which may impair cognitive functioning. Kif21b might thus be important for the neuron-astrocyte interaction [42, 43]. In a subset of familial ALS patients, mutations in the antioxidant enzyme

Cu/Zn superoxide dismutase 1 (SOD1) have been found [44]. In a model with cultured mouse motor neurons with SOD1 mutations, no or delayed neurodegeneration was observed. However, when wildtype motorneurons were co-cultured with astrocytes bearing the SOD1 mutation, the survival of the neurons was significantly decreased [45]. This is due to currently unknown soluble factors produced by the mutant astrocytes [46], which might be transported by kif21b.

It is interesting that more severe AD pathology is associated with enhanced kif21b expression. Reactive astrocytes are found in regions with amyloid beta deposits in AD pathology and these astrocytes have accumulated neuronal amyloid beta 42 [47]. Astrocytes can be activated when co-cultured with microglia cells in the presence of amyloid beta [48]. Additionally, astrocytosis is mainly observed in younger frontotemporal dementia patients, and this is supportive for our findings regarding the correlation between kif21b and GFAP only in the young AD patients [19]. Astrogliosis is observed in MS white matter lesions, whereas GFAP expression in grey matter lesions is not increased [49,50]. This supports the correlation between GFAP and kif21b in WM of MS patients, but not in cortical demyelination (Fig. 6F and Supplementary Figure 10). The role of astrocytes in the neurodegenerative component of multiple sclerosis is also increasingly being recognised [51].

Further research on the cargo transported by kif21b and the exact function of this kinesin will be important to gain more insight into the exact mechanisms whereby kif21b contributes to neurodegeneration. Understanding the function of kif21b in neurons and astrocytes, may shed new light on possible mechanisms to therapeutically target neurodegeneration and malignant brain tumours.

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Supplementary materials

Supplementary Table 1. Primers and antibodies used in this study

Supplementary Table 1a. Primers

Gene	Forward primer	Reverse primer
Kif1a	cttggcgacatcactgacat	gctggacagggctgagag
Kif1bα	GCATTAAAGGCTGTTTTACTACCG	GTAAGGCACTGGGGCACA
Kif1bβ	GAAGCTGGAGCTCCTACATGA	ACGAGTCGCTCAGGGATTT
Kif1c	gttcattcgggagcaacact	gctccagagtgaccaccact
Kif5a	acacgagaagagcaccaagc	gactgctcatgtcgctcgta
Kif5b	CTGAAGACGCAAATGTTGGA	TGCATATTGTCTTGATCCCTTC
Kif21b	GGCTGGACCTGAGTTCAAAG	GGTCAAGGATCTCCTCGTTG
NeuN	GGGGAACCCCTACACCAA	GAATTCAGGCCCGTAGACTG
GFAP	AGAGGGACAATCTGGCACA	CAGCCTCAGGTTGGTTTCAT
MBP	AGCCCTCTGCCCTCTCAT	GGAGCCGTAGTGAGCAGTTC
IL-6	gatgagtacaaaagtcctgatcca	ctgcagccactggttctgt
GAPDH	Commercial kit Applied Biosystems	
GUSB	Commercial kit Applied Biosystems	

Supplementary Table 1b. Antibodies

Marker	Clone	Isotype	Dilution	Supplier
Kif21b		rIgG	1/25	Atlas
MAP2	HSM5	mIgG1	1/400	Pierce
PLP	plpc1	mIgG1	1/2000-1/4000	Bioconnect
MOG	Z12	mIgG2a	1/200	Generous gift from S. Amor
HLA-II	CR3/43	mIgG1	1/1600-1/20000	Dako
GFAP	ASTRO6	mIG1	1/50*-1/200	Thermo Scientific
CD68	EBM11	mIgG1	1/200	Dako
NeuN	MAB377	mIgG1	1/400*-1/6000	Chemicon
SMI-32	SMI-32	mIgG1	1/100*-1/400	Sternberger monoclonals
Secondary antibodies				
RAM-Ig-HRP	NA	rIg	1/100	Dako
RAM-Ig-bio	NA	rIg	1/400	Dako
SAV-AF488	NA	NA	1/100	Molecular Probes
GAM-Ig-AF594	NA	GIg	1/300	Molecular Probes
Isotype controls				
mIgG1	MAB002	-	Depends on primary antibody concentration	R&D Systems
mIgG2a	MAB003	-	1/358	R&D Systems
Rabbit Ig	NA	-	1/125	R&D Systems

* Antibody dilution used for immunofluorescence; NA, not applicable

Supplementary Table 2. Age at death is a significant predictor for the levels of kif21b expression in AD patients, but not in MS or NDC.

	pH CSF			PMD			Age at death		
disease	Correlation coefficient (95% CI)	R2	p-value	Correlation coefficient (95% CI)	R2	p-value	Correlation coefficient (95% CI)	R2	p-value
NDC	0.012	$2*10^{-4}$	0.93	0.090	0.008	0.52	-0.067	0.005	0.63
MS	-0.26	0.07	0.09	0.096	0.009	0.51	-0.24	0.06	0.10
AD	-0.27	0.07	0.06	-0.088	0.008	0.55	**-0.55**	**0.31**	**$3*10^{-5}$**

Correlation coefficients between kif21b expression and pH of the CSF, post-mortem delay and age at death were assessed by linear regression. Significant correlations are indicated in bold.

Supplementary Table 3. Demographic characteristics of white matter donors included in this study.

	NDC (n=18)	MS (n=23)	AD (n=3)
Age at death (SD)	80 (6)	59 (14)	81 (4)
Female/ male (n)	8/10	17/6	2/1
Post-mortem delay in hours (IQR)	7.0 (5.58-8.20)	7.15 (5.40-8.32)	4.15 (4.0-8.35)
pH CSF	6.42 (6.20-6.83)	6.54 (6.33-6.76)	6.66 (6.04-8.28)
Age at onset (SD)	NA	35 (8)	74 (8)

IQR, inter-quartile range

Supplementary Table 4. Kif21b protein expression in the white matter is highly variable.

	HLA-II	kif21b		MOG	kif21b		ORO	kif21b
NDC1	0	0/3		4	0/3		0	0/3
NDC2	1/2	2		4	0.5/2		0	0.5/2
	0	0.5/1						
NDC3	0	0		4	0		0	0
NDC4	2	2		4	0/2		0	0/2
	0.5	0.5/1						
	0	0/1						
NDC5	2	2		4	0.5/2		0	0.5/2
	3	0.5						
NDC6	2	0.5/2		4	0.5/2		0	0/2
	0.5/1	0.5/1		2	1			
NDC7	0	0/2		4	0/2		0	0/2
NDC8	1	0.5		4	0		0	0/2
	2/3	0		2	0			
	2/3	2						
NDC9	0.5	0/2		2	1/2		0	0/2
				4	0/2			
NDC10	0/0.5	0/2		4	0.5/1		0	0/2
				2	1/2			
				1	2			
NDC11	0.5	1/2		0	0		0	0/2
	0.5	0						
NDC12	0.5	0		4	0/2		0	0/2
	0.5	1/2						
MS1	2/3	3		4	2/3		0	2/3
	1	2/3						
MS2	2	0		0	0		3	2
	3	2		4	1/2		3	0
	3	0						
MS3	2	1/2		2	0.5/1		0.5	2
	1	2		4	1/2			
	1	1/2						
MS4	3/4	0/2		4	0/2		0	0/2
	0	0		1	0		2	0
MS5	3	1/3		4	1/3		0	1/3
				1	3		0	0
MS6	1/2	0		1	0			
				4	0			
				3	0			
MS7	0/0.5	0		4	0		0	0
MS8	1/2	0		4	0		0	0
MS9	2	0/3		2/3	1/2		0	0/3
				4	2/3			
				2/3	0			
MS10	0.5	0/2		3/4	0/2		0	0/2
	2	2						
MS11				3	2/3		0	1/3
				4	1/3			
AD1	0	0/3		4	0/3		0/0.5	0/3
AD2	0	0.5/3		3	0.5/3		0	0.5/3
AD3	1/2	0/3		1	0		0	0/3
				4	0.5/2			
				4	3			

Staining was scored as: 0= no positive cells, 0.5= 1-2 positive cells per field, 1= maximum of ~30% of the cells positive, 2= ~60% of the cells positive, 3= ~80% positive cells and 4= (virtually) all cells positive

Supplementary Table 5. Abundant kif21b expression is an independent predictor for the time to develop EDSS 6.0 in MS

Variable	Hazard ratio (95% CI)	p-value	Additional variables in HR model	Adjusted HR (95% CI)	Adjusted p-value
Abundant kif21b expression	2.2 (1.01-4.6)	0.047	Abundant kif21b expression	3.0 (1.4-6.7)	0.006
			Age at onset	1.05 (1.02-1.08)	0.004
			Abundant kif21b expression	3.7 (1.6-8.7)	0.003
			Age at onset	1.06 (1.02-1.1)	0.001
			GM demyelination	1.01 (1.0-1.03)	0.08
			Abundant kif21b expression	2.99 (1.29-6.94)	0.01
			Age at onset	1.06 (1.02-1.10)	0.005
			GFAP expression above median	0.65 (0.27-1.55)	0.33
			Abundant kif21b expression	3.0 (1.3-6.7)	0.009
			Age at onset	1.05 (1.01-1.08)	0.006
			MS risk SNP rs12122721 [A]	1.13 (0.51-2.5)	0.76
			Abundant kif21b expression	3.4 (1.5-7.8)	0.004
			Age at onset	1.05 (1.02-1.08)	0.004
			Gender	1.8 (0.76-4.3)	0.18
GM demyelination	1.01 (1.0-1.03)	0.18	GM demyelination	1.01 (1.0-1.03)	0.1
			Age at onset	1.04 (1.01-1.08)	0.02
GFAP expression above median	0.97 (0.45-2.07)	0.94	GFAP expression above median	0.69 (0.30-1.58	0.38
			Age at onset	1.04 (1.00-1.08)	0.027

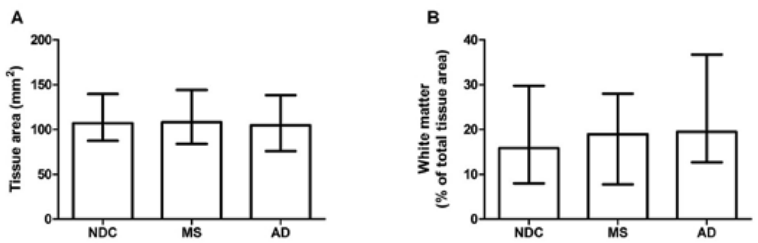
Abundant kif21b expression (defined as above or below the median expression) was adjusted for several variables in a Hazard regression model. Unadjusted and adjusted hazard ratios for time to develop EDSS 6.0 are indicated. Abbreviations: GM, grey matter; HR, hazard ratio; SNP, single nucleotide polymorphism

Supplementary Table 6. Abundant expression of several other kinesins is not associated with accelerated development of sustained disability

Kinesin	HR* (95% CI)	p-value*	Previous neurological disease associations [1]	References
Kif1a	0.77 (0.37-1.6)	0.48		
Kif1bα	1.38 (0.63-3.02)	0.43	1) MS GWAS, not validated 2) Charcot-Marie-Tooth disease (human mutations, mouse model)	1-3
Kif1bβ	0.97 (0.48-1.95)	0.97	1) MS GWAS, not validated 2) Charcot-Marie-Tooth disease, both human mutations and mouse model	
Kif1c	0.94 (0.48-1.97)	0.97		
Kif5a	2.02 (0.97-4.2)	0.06	1) MS candidate gene study 2) Kinesin-1 superfamily and kinesin-1 light chains implicated in transport of APP in AD 3) Hereditary spastic paraplegia (SPG10)	4-7
Kif5b	1.41 (0.65-3.06)	0.38	Kinesin-1 superfamily and kinesin-1 light chains implicated in transport of APP in AD	5, 7

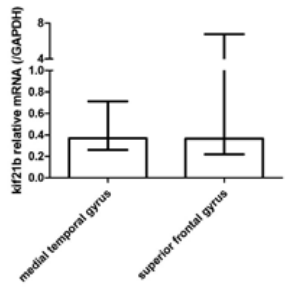
Abundant expression of several other kinesins and the time to develop EDSS 6.0 was assessed in a Hazard regression model in MS patients.
* Adjusted for age at onset
[1] Note, mutations in single patients or families are not taken into consideration in this table.

Supplementary Figure 1. No significant differences in the size of the tissue or the percentage of white matter between MS, AD and NDC



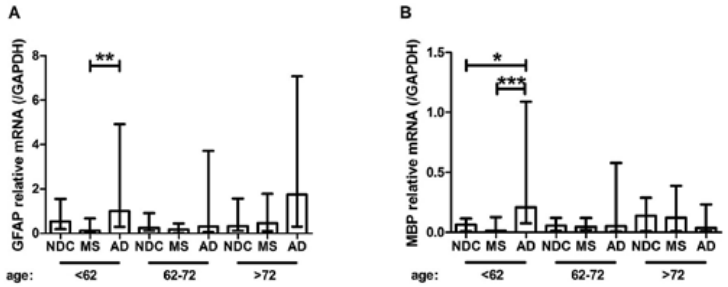
Morphometric parameters of the tissues investigated in this study were obtained using the NDC.view software (Hamamatsu). **A)** No significant difference was found in the total area of the tissue between 57 NDC, 49 MS and 50 AD (p=0.84). **B)** The percentage of white matter in the investigated tissues was calculated as percentage of the total tissue area. No significant differences were found between MS, AD and NDC (p=0.37). Statistics were calculated using a Kruskal-Wallis test.

Supplementary Figure 2. No significant difference in kif21b expression in MS patients between the medial temporal gyrus and the superior frontal gyrus



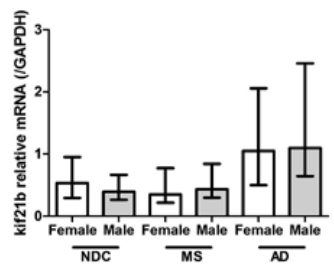
Kif21b expression was assessed in the medial temporal gyrus (n=44) and in the superior frontal gyrus (n=6). No significant regional differences were found (Mann Whitney U-test p=0.87).

Supplementary Figure 3. GFAP and MBP mRNA are significantly increased in the younger AD patients



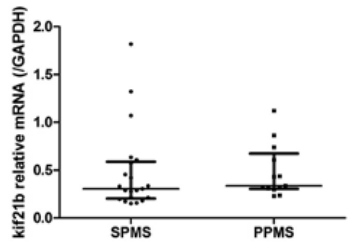
In the different age categories in NDC, AD and MS patients, the expression of **A)** GFAP and **B)** MBP was compared using a Kruskal-Wallis test with Dunn's multiple comparison ad hoc analysis. For both GFAP and MBP, the variation in AD was large.

Supplementary Figure 4. No differences in kif21b expression between males and females in the three donor groups



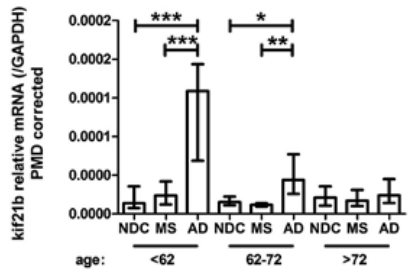
Since MS has a strong female predominance, we assessed whether kif21b expression was different between males and females. No significant gender differences were found in NDC, MS or AD (Mann Whitney U-test per disease, all p<0.26).

Supplementary Figure 5. No significant difference between distinct MS disease forms



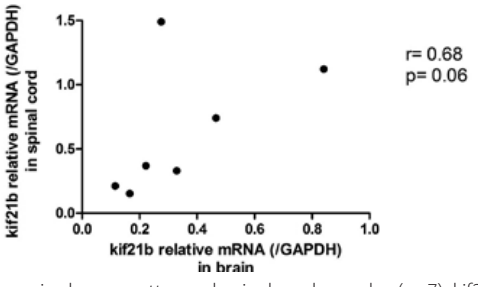
As we observed that abundant kif21b expression was associated with accelerated neurodegeneration, we assessed whether differences between primary progressive MS (PPMS, n=13) and secondary progressive MS (SPMS, n=21) exist. No significant difference in kif21b expression between SPMS and PPMS was found (Mann Whitney-U test, p=0.24).

Supplementary Figure 6. Correcting kif21b expression for post-mortem gave similar results as uncorrected kif21b expression



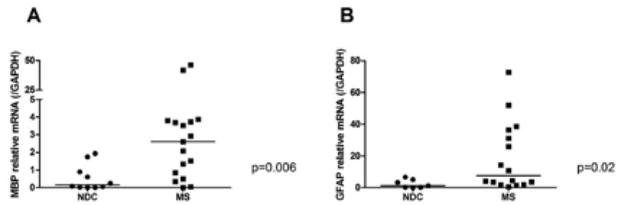
Since in AD patients, post-mortem delay (PMD) was a significant predictor for kif21b expression (Supplementary Table 2), we assessed whether correcting kif21b expression for PMD would alter the results. A similar pattern as in the main text Figure 2 was observed for the differences in kif21b expression between AD, MS and NDC in the different age categories. Statistics were calculated using a Kruskal-Wallis test with Dunn's Multiple Comparison Test.

Supplementary Figure 7. Kif21b expression levels are correlated in paired MS GM and spinal cord samples



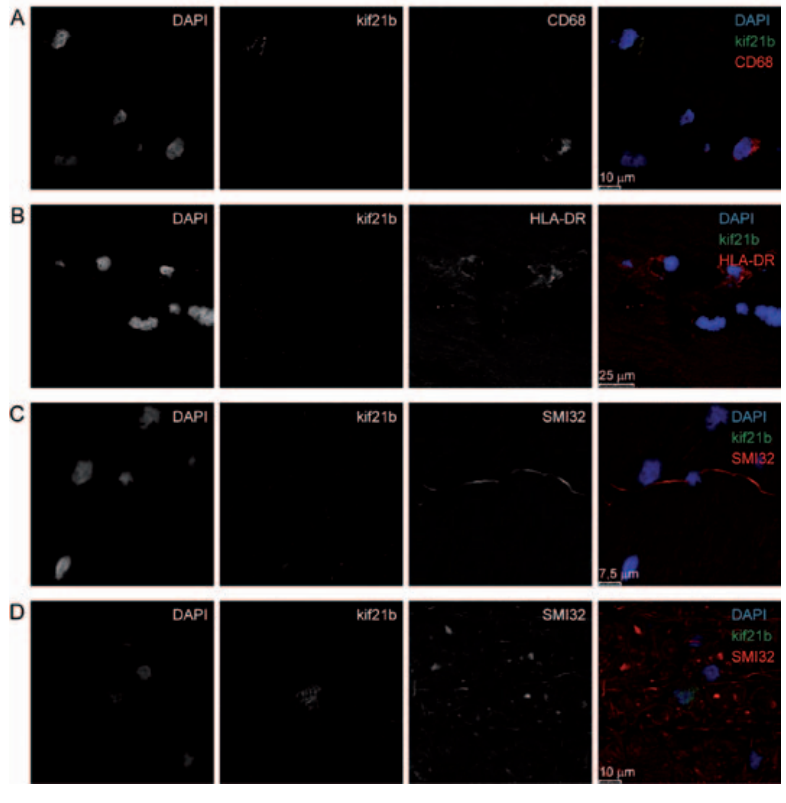
In paired grey matter and spinal cord samples (n=7), kif21b expression was determined. Kif21b expression in grey matter tissue and spinal cord samples gave similar results (p=0.06). Additionally, no differences between medial temporal and the superior frontal gyrus (Supplementary Figure 1) were found, indicating that the kif21b expression levels are probably not influenced by regional differences.

Supplementary Figure 8. In MS white matter, MBP and GFAP expression are significantly increased



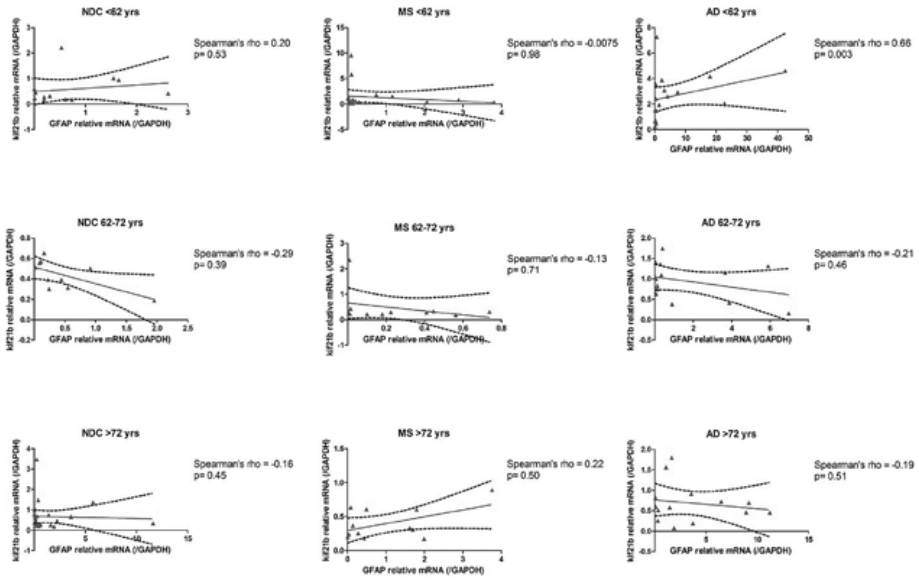
In the white matter of MS patients and NDC, the expression of **A)** MBP and **B)** GFAP was assessed. No significant difference between MS and NDC was found for NeuN (Fig. 6B, main paper). As expected, MBP was significantly increased, probably at least partially due to remyelination, and GFAP was slightly increased in MS patients, possibly due to astrogliosis. p-values were calculated using a Mann Whitney U-test.

Supplementary Figure 9. Kif21b is not expressed in microglia cells or in SMI32 positive axons.



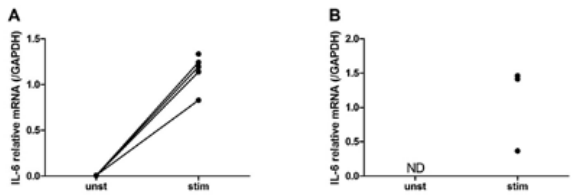
No kif21b expression was found in the white matter of in **A)** CD68 positive microglia/ macrophages or **B)** HLA class II positive cells. Additionally, no kif21b expression was observed in SMI32 positive axons **(C-D)**.

Supplementary Figure 10. In NDC and MS patients and in elderly AD patients no correlation between cortical GFAP and kif21b expression exist.



Correlation between kif21b and GFAP mRNA expression in the cortex of NDC (left column), MS (middle column) and AD (right column) stratified according to age categories < 62 years of age (upper row), 62-72 years (middle row) and above 72 years (lower row). Please note that the axes are different in the different figures. The graph for AD patients <62 years of age is also displayed in the main paper (Fig. 4E).

Supplementary Figure 11. IL-6 increased upon astrocyte activation.



The U251 astrocytoma cell line **(A)** and primary astrocytes **(B)** were activated with IL-1β and IFN-γ for 48 h. IL-6 mRNA increased, indicating that the astrocytes are activated.
ND= not detected

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Chapter 7

General discussion

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Guided choice of selected pathways for further studies

Currently, 57 polymorphisms in or around genes are known to modestly increase the risk to develop MS in addition to the HLA as major genetic risk factor. However, how these SNP mechanistically contribute to this increased risk for the development of MS is very poorly understood. Well-designed functional, mainly immunological, studies are needed to increase our understanding of the pathogenic mechanisms caused by these SNP and how these SNP contribute to the development of MS.

This chapter is divided into three parts. Part one discusses the outcome of functional investigations regarding the mechanistic consequences of MS-associated SNP and aberrancies in the underlying pathways described in this thesis. Additionally, alterations in these pathways in other autoimmune diseases will be discussed. The second part provides a critical appraisal regarding the outcomes and interpretation of GWAS results. Moreover, the current stage of genome research in MS as well as the caveats in MS genetics will be introduced. This part also includes other published functional immunogenetic studies in MS. In the last part, future perspectives in the field of genetics and clinical immunology of MS will be presented.

Part one

Reflection on functional immunogenetic studies

During the last six years, several large MS GWAS have been performed. The next critical step in MS research is to assess ***how*** the MS-associated SNP ***functionally*** contribute to the increased risk to develop MS. The main conclusions from functional immunogenetic studies described in this thesis are summarised in Box 1.

Box 1. Main conclusions from the studies described in this thesis

1. In MS patients, IL-7Rα expression is increased on CD8 effector memory T-cells. This functionally leads to enhanced phosphorylation of STAT5 and subsequently aberrant expression of the cytotoxic molecule granzyme A (**chapter 2.1**)
2. The IL-7Rα is also aberrantly expressed on CD8 regulatory T-cells of MS patients (**chapter 2.2**)
3. The highest levels of soluble IL-7Rα are found in risk allele carriers of the IL-7Rα MS risk SNP. However, the levels of sIL-7Rα expression are lower in MS patients. Also IL-7 levels are decreased in MS (**chapter 3**)
4. The C-type lectin CLEC16A is a critical regulator of endosomal function and maturation, regulating the transport of endosomes. Via these functions, HLA class II surface expression is controlled (**chapter 4**)
5. SNP in the loci of NF-κB1, BATF and TMEM39A are associated with enhanced humoral immune responses against the latency associated EBV peptide EBNA-1. Additionally, HLA-DQA1/DRB1 allele carriers have increased levels of EBNA-1 IgG (**chapter 5**)
6. Abundant expression of the kinesin kif21b is associated with more severe neuropathology. Additionally, high levels of kif21b are associated with accelerated progression to sustained neurological deficits. In addition to neurons, kif21b is also highly expressed in astrocytes. Astrocyte activation increases kif21b expression (**chapter 6**)

Physiological roles of the IL-7 and IL-7Rα pathway

One of the first SNP identified to be associated with an increased risk to develop MS is the IL-7Rα. IL-7 and IL-7Rα are interesting candidate molecules to contribute to autoimmunity. In man, engagement of the IL-7Rα is important for the rearrangements of the kappa chain of the immunoglobulin in the large pre-B-cell stage. During maturation of B-cells, the IL-7Rα is downregulated and not expressed anymore. Moreover, signalling via the IL-7Rα is important for developing T-cells in the thymus. In the double negative stage, the IL-7Rα is expressed and signalling via this pathway is important for the rearrangement of the T-cell receptor [1]. In the periphery, IL-7 and IL-7Rα are important for homeostatic proliferation of T-cells and for the maintenance of the pool of T-cells. After binding of IL-7 to the IL-7Rα, JAK1 and JAK3 are recruited, leading to the phosphorylation of STAT5. Subsequently, survival signals are induced via the modulation of BCL2 and BIM [2,3].

The IL-7Rα pathway in autoimmunity

Functional alterations in the IL-7 and IL-7Rα pathway have not been assessed thoroughly in MS and therefore we investigated this pathway in detail. In **chapter 2.1**, we determined the expression of the IL-7Rα on functional T-cell subsets of MS patients and healthy controls (HC). We found an increased frequency as well as increased levels of expression of the IL-7Rα on CD8 effector memory T-cells of MS patients. Also on other CD8 T-cell subsets and on CD4 effector T-cells, the levels of the IL-7Rα are increased. Moreover, IL-7 stimulation leads to increased phosphorylation of STAT5 and subsequently to enhanced expression of the cytotoxic protein granzyme A. It is intriguing to speculate that these cells might be autoreactive T-cells. Myelin-specific CD4 T-cells can be expanded and detected better after IL-7 stimulation [4,5]. Interestingly, in MS white matter lesions, IL-7 protein expression by astrocytes was observed [6]. Moreover, in these lesions, more restricted clonally expanded CD8 T-cells compared with CD4 T-cells have been observed [7]. The increased expression of the IL-7Rα on CD8 T-cells might contribute to clonal expansion and survival of these cytotoxic T-cells. Notably, the increased IL-7Rα expression does not correlate with the rs6897932 MS risk SNP. Alternative splicing of the IL-7Rα caused by this SNP only affects the expression of the soluble and not the membrane-bound IL-7Rα receptor [8].

In other autoimmune diseases, aberrancies in the IL-7Rα pathway have been implicated as well. For example, in rheumatoid arthritis (RA) increased levels of IL-7Rα have been observed on T-cells, macrophages and B-cells in the inflamed joint [9]. IL-7Rα expression on monocytes and macrophages was also observed in another study with RA patients [10]. Whether myeloid cells in MS also express the IL-7Rα is currently unknown. In lupus patients and in anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis, the expansion of memory CD8 T-cells correlated with a poorer prognosis. Interestingly, microarray analysis of sorted CD8 T-cells revealed that several genes in the IL-7Rα pathway were associated with worse prognosis in these patients [11].

IL-7Rα on regulatory T-cells in MS

The balance between regulatory T-cells (Treg) and potentially autoreactive T-cells is important. In the normal immune repertoire of healthy individuals, some myelin reactive T-cells can be found [12]. These cells are presumably kept under control by Treg. Different subsets of Treg have been described and especially Treg in the CD4 compartment are studied thoroughly. The characterisation of Treg depends on high expression of CD25 (IL-2RA) and low expression of CD127 (IL-7Rα). Treg express the master transcription factor FoxP3 intracellularly [13]. FoxP3 is a suppressor of the promoter of the IL-7Rα [14]. The simplest subdivision of regulatory T-cells is naturally occurring Treg (output of the thymus) and induced Treg (in the periphery). Another subdivision is naive and memory Treg. In addition to cells with regulatory capacities of the CD4 lineage, also CD8 regulatory T-cells [15], IL-10 producing B-cells (also termed regulatory B-cells, Breg) [16] and regulatory NK-cells [17] have been described, the latter two will not be discussed further here.

Extensive investigation of CD4 Treg has been performed for MS. The main observation of several studies is that no numerical differences in CD4 Treg between MS patients and healthy controls occur. Functionally, the suppressive capacity of at least CD4 Treg of MS patients is impaired compared with HC [18]. Within the CD8 regulatory T-cell compartment in MS (CD8 Treg characterization solely based on the expression of CD25 and FoxP3), no numerical differences between patients and controls were found [19]. Accordingly, we also did not observe numerical differences in CD8+CD25hiFoxP3+ Treg in 32 MS compared with 37 HC (unpublished data). Interestingly, in MS patients having a relapse, the number of CD8 Treg is significantly decreased compared with MS patients in remission or with HC [20]. When we assessed the CD8 regulatory T-cells and stratified the CD8+CD25hiFoxP3+ cells according to the expression of CD127, we found a significantly increased proportion of CD8 Treg, still expressing CD127 in MS patients (**chapter 2.2**). This might indicate that FoxP3 is not fully functional or active. It would be interesting to assess whether these CD8+CD25highCD127+FoxP3+ T-cells have an altered suppressive capacity compared with the CD127- counterparts in MS patients.

In contrast to impaired functional suppressive capacity of Treg in MS patients, it was shown that effector T-cells of MS patients are resistant to the suppressive capacity of CD4 Treg. Interestingly, also Treg obtained from healthy donors could not inhibit the proliferation of effector T-cells of MS patients [21]. Resistance of effector T-cells to Treg induced suppression has also been shown in other diseases, for example in juvenile idiopathic arthritis [22].

Soluble IL-7Rα in autoimmune diseases

To fully understand the IL-7Rα pathway in MS, it is also important to determine the expression of the soluble form of the receptor as well as its ligand. A transfection study regarding the rs6897932 MS risk SNP showed that this SNP is associated with increased splicing of exon 6, which encodes the transmembrane domain of the IL-7Rα [8]. In **chapter 3**, we determined the expression of the sIL-7Rα and IL-7. Quite unexpectedly, the levels of expression of the IL-7Rα were decreased in MS patients compared to healthy controls fully matched for the rs6897932 SNP. This is discrepant with a previous,

smaller study, which found no alterations in sIL-7Rα levels between HC and MS [23]. Nevertheless, in concordance with the previous study, we found a strong correlation between sIL-7Rα and the rs6897932 risk allele carriership. The sIL-7Rα is produced due to alternative splicing [24] caused by the non-synonymous SNP rs6897932 [8].

In RA patients, increased sIL-7Rα levels were found compared to healthy controls. Of note, the MS-associated IL-7Rα risk SNP is not associated with increased RA risk. Fibroblast-like synovial cells produce the sIL-7Rα upon exposure to the pro-inflammatory cytokines IL-1β and/or TNF-α. Interestingly, high sIL-7Rα also correlated with poorer responses to disease-modifying treatments [25]. Whether sIL-7Rα is increased in MS patients suffering from a relapse compared with patients in remission is currently unknown.

IL-7 in autoimmunity

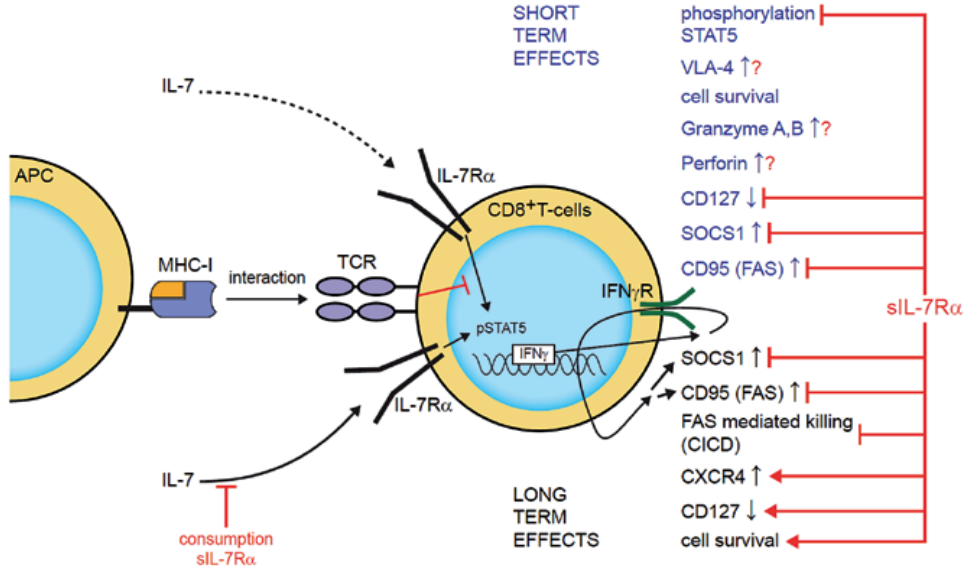
In **chapter 3**, we also investigated systemic IL-7 levels in serum of MS patients and healthy controls. We found significantly lower IL-7 concentrations in MS patients compared with healthy controls. Contrary to our study, Haas and colleagues found increased levels of IL-7 in MS patients (11.2 pg/ml) compared to HC (5.1 pg/ml) [26]. In another study by Lundstrom et al, IL-7 plasma levels were 8 pg/ml in MS and 4 pg/ml in HC, whether or not this was statistically significant different was not assessed unfortunately [27]. The apparent discrepancy between these studies can be explained by differences in methodology (collection of blood in different types of tubes) and the included patients (in remission in our study vs. mixed remission and relapse patients or with the disease duration respectively). Also in RA, discrepancies between studies regarding IL-7 levels in patients and controls have been observed (reviewed in Churchman et al. [28]). Importantly, the main difference in IL-7 levels in RA studies is found in healthy controls and not in patients. Also in our study compared with the study by Haas et al., the difference in results was mainly due to the IL-7 levels in the healthy controls. The median and interquartile range of IL-7 we observed in the HC was 11.3 pg/ml, IQR 8.9-14.0 pg/ml and this is in the generally accepted range of 13 ± 5 pg/ml found in the majority of studies [28]. Haas et al. found very low levels of IL-7 in HC, thereby overestimating the difference between patients and controls. Given the very narrow concentration range of IL-7 in blood, a critical view on reported concentrations in both patients and controls is necessary. Enhanced IL-7 serum levels in MS patients predict the response to IFN-β treatment, especially in patients with low IL-17F [29]. In this study, extremely high concentrations of IL-7 in HC (90.2 ± 28.8 pg/ml) and similar levels for MS patients were found.

In RA patients resistant to anti-TNF-α treatment, IL-7 levels remained stable during treatment [30]. Early RA patients treated with the first line treatment Methotrexate, only IL-7, IL-18 and TNF-α decreased during longitudinal follow up. The only cytokine associated with clinical changes (number of swollen joints, tender joints and erythrocyte sedimentation rate) was IL-7 [31]. Mechanistically, TNF-α and IL-1β induce the expression of IL-7 in synovial cells [32] and IL-7 induces TNF-α expression in macrophages [33], thus providing an amplification loop in the inflamed joint. At sites of RA pathology, IL-7 expression is enhanced in synovial tissue [30], comparable with IL-7 expression on astrocytes in MS lesions [6].

Fine tuning of the IL-7Rα pathway is important to prevent autoimmunity

The balance between IL-7, sIL-7Rα and cell-associated IL-7Rα is important. IL-7 levels are under physiological conditions limited to prevent autoimmunity [34]. A recent study showed that IL-7 downregulates membrane-bound IL-7Rα via two independent mechanisms. Binding of IL-7 to the IL-7Rα leads to internalization of the receptor and reduces IL-7Rα mRNA expression in CD8 T-cells [35]. This is in line with our findings that IL-7 is reduced in MS patients and that the expression of the IL-7Rα on the cell surface of T-cells is increased (**chapter 2.1 and 3**). Additionally, sIL-7Rα regulates the availability of IL-7 by inhibiting the consumption of IL-7 [27]. Decreased sIL-7Rα levels in MS would thus indeed lead to decreased IL-7 levels as we have observed (**chapter 3**). sIL-7Rα alters cellular responses, both short and long term [30]. In naive CD8 T-cells, membrane-expressed IL-7Rα is regulated at least partly by T-cell receptor (TCR) signalling. Under continuous IL-7Rα signalling, IFN-γ is produced, inducing FASL in an autocrine fashion. This leads to the activation of caspase-3 and cell death. Interestingly, during intermittent IL-7Rα signalling, engagement of the TCR inhibits the expression of IFN-γ and thereby promotes cell survival [36]. The complex interactions between IL-7, sIL-7Rα and membrane-bound IL-7Rα are summarised in Figure 1. Whether these mechanisms also regulate CD4 T-cell responses is currently unknown.

Figure 1. IL-7 signalling has multiple functional effects on CD8 T-cells



Continuous IL-7 signalling (solid line) has different short and long-term effects on CD8 T-cells. Additionally, the effects of intermittent IL-7 signalling (dashed lines) can be inhibited by TCR signalling via inhibition of IFN-γ induced changes in gene expression. The inhibitory effects of the soluble IL-7Rα on IL-7 actions are summarised.

From the studies in **chapter 2.1 and 3**, it is apparent that the balance in the IL-7Rα/ IL-7 pathway in MS patients has shifted towards enhanced cellular responses. Also in other autoimmune diseases like RA, this phenomenon has been observed. Several studies showed that different interventions in the IL-7Rα pathway reduce EAE scores. Culturing naive T-cells under Th1 or Th17 polarizing conditions with addition of IL-7 greatly enhanced Th1 responses, but not Th17 cells. Inhibition of IL-7 and blockade of the IL-7Rα in EAE resulted in less severe EAE by reducing the number of naive and effector memory CD4 and CD8 T-cells. This blockade partly prevented T-cell migration into the CNS, reducing EAE scores [29]. Another EAE study using IL-7Rα knockout mice showed that these mice are resistant to EAE, but paradoxically the deletion of the IL-7Rα reduced frequencies of Th1 and Th17 cells [37]. In conclusion, it is apparent that inhibition IL-7Rα/IL-7 pathway reduces EAE, but the underlying immunological mechanisms of the IL-7Rα/IL-7 pathway in EAE immune pathogenesis are incompletely understood due to these conflicting results. Also in an arthritis mouse model, IL-7 controlled homeostatic proliferation of CD4 T-cells. IL-6-induced phosphorylation of STAT3 was responsible for the production of IL-7 [38]. In a mouse model using LCMV virus infection, IL-7 augmented the anti-viral immune response, by inhibiting the SOCS3 pathway, increasing T-cell cytokine production and effector function as well as increasing the naive T-cell pool. These effects were dependent on IL-6 [39]. In a vaccine-induced anti-tumour mouse model, IL-7 augmented Th17 responses via IL-6. IL-7 repressed the negative regulator of T-cells, Cbl-b and lowered the TGF-β expression [40]. Interestingly, Cbl-b is one of the MS risk genes.

Given the aberrancies in the IL-7Rα pathway and the biological role of this pathway in T-cell biology, it is a promising candidate for therapeutic interventions. Possible interventions are IL-7Rα blockade using monoclonal antibodies or inhibiting the downstream signalling of the IL-7Rα with small molecule JAK inhibitors. Blocking antibodies might have serious safety issues since the IL-7Rα is widely expressed in both developing B- and T-cells and on the majority of CD4 and CD8 naive and memory T-cells. Blocking the IL-7Rα might result in (cyto)toxicity and possibly severe side effects, like opportunistic infections. Inhibiting the JAK downstream adaptor proteins might be more promising, although selectivity of these drugs is currently a problem. Nevertheless, Tofacitinib, a non-selective JAK inhibitor, showed comparable results to anti-TNFα treatment in RA [41, 42] and is now FDA approved. Second generation selective JAK 1/2 and JAK 1/3 inhibitors are being developed, and currently tested in clinical trials for several autoimmune diseases. This category of drugs is a promising new group of drugs to be tested in MS.

Antigen presentation in MS

In **chapter 4**, we have investigated the function of the C-type lectin CLEC16A. Several polymorphisms in the CLEC16A locus have been associated with MS and other autoimmune diseases like type I diabetes mellitus [43], autoimmune thyroid disease [44], primary biliary cirrhosis [45], other non-autoimmune diseases like selective IgA deficiency [46] and Alzheimer's disease (sub-genome wide significance) [47]. C-type lectin receptors (CLR) are members of the pattern recognition receptors (PRR), which are important innate signalling receptors. CLR are characterised by a carbohydrate recognition domain,

although this domain does not necessarily bind carbohydrates [48]. CLR on dendritic cells (DC) are able to bind fucose or mannose-terminated glycan carbohydrates among other ligands. Binding of these pathogen structures leads to the internalization of the CLR, processing of these structures into peptides and subsequently to antigen-presentation to T-cells via HLA [49]. These functions of CLR make them attractive candidate molecules to be involved in autoimmunity. Currently, very little is known regarding the function of CLEC16A, especially in humans. Only two studies analysed the function of Ema, the ***Drosophila*** orthologue of CLEC16A. These studies implied that Ema is involved in endosomal maturation [50] and autophagy [51]. In humans, only the correlation between the ratio of the short and long isoform of CLEC16A in peripheral blood and thymic tissue in relation to the MS risk SNP has been investigated. Only in thymic tissue, one of the MS-associated CLEC16A SNP correlated with ratio between the expression of the long and short isoform [52]. Whether these two isoforms have different biological functions is currently unknown.

Some of CLEC16A SNP described in different studies correlated weakly with the expression of SOCS1 and DEXI [53]. Additionally, in type I DM the SNP in intron 19 physically interacted with the promoter of DEXI, a gene with a currently unknown function [54]. Interestingly, a SNP in SOCS1 is associated with MS [55] and the inhibitory function of SOCS1 in cytokine signalling may be important in MS.

In **chapter 4**, we have shown that CLEC16A has an important role in human endosomal transport by a direct interaction with the cellular transport molecule Rab7-interacting lysosomal protein (RILP) and the associated homotypic fusion and protein sorting (HOPS) complex [56]. CLEC16A regulates late endosomal transport and the functioning of MHC-II loading compartments (MIIC) and thereby the expression of HLA-II on the plasma membrane.

Although the importance of HLA class II in the genetics of MS has already been established in the seventies of the previous century, relatively little is known regarding antigen presentation in MS. Antigen presenting cells (APC) containing myelin debris were found in lymph nodes of several MS patients and only in a minority of HC [57, 58]. Additionally, also neuronal antigens are found in both MS and HC in these lymphoid tissues [59]. Currently, it is unknown which peptides are specifically bound in the peptide-binding groove of HLA molecules in MS patients and if these peptides differ from HLA-matched HC. Additionally, to our knowledge no studies exist on altered expression of CLIP or the invariant chain [60] in MS compared with HC. This lack of knowledge regarding antigen presentation in MS is quite unexpected, because of the long-standing HLA-II association with MS and because several of the polymorphisms associated with MS are either directly or indirectly involved in antigen presentation. For example, MAPK1, IRF8, NF-κB1 [60], IL12A (encoding IL12p35), IL12B (IL12p40) [61], Vit. D regulatory elements [62], PKC [63] and CLECL1 [64] are all associated with the regulation and induction of antigen presentation. Additionally, MS-associated risk genes were analysed using pathway analysis software. This revealed the strongest association for antigen presentation in MS risk. In addition to antigen presentation, neural and axonal guidance genes were also significantly associated with MS susceptibility [65].

Besides SNP associated with HLA class II antigen presentation, also SNP important for HLA class I presentation have been associated with MS in a candidate gene study. A SNP in the endoplasmatic recticulum aminopeptidase 1 (ERAP1) gene, which is associated with ankylosing spondylitis (M. Bechterew), was also significantly associated with MS [66].

In conclusion, it will be important to further investigate antigen presentation in MS and determine the functional consequences of non-HLA SNP on the function and expression of HLA.

The role of MS genetics in an altered humoral immune responses against EBV

Enhanced immunoglobulin responses against EBV peptides are observed in MS patients. The most consistent finding is increased IgG levels against the latency associated EBNA-1 protein in MS patients, whereas responses against the (re-)activation associated protein EA-D and the viral capsular antigen VCA are more heterogeneous [67]. The genetics of MS underscore the importance of immune activation. Therefore, we hypothesised that MS genetics predispose to the altered immune response against EBNA-1. One of the genes involved in the enhanced IgG response against EBNA-1 is HLA-DQA1/DRB1. Carriership of the strongest genetic risk factor for MS is associated with enhanced IgG levels against EBNA-1, but not with IgG levels against the varicella zoster virus (VZV). Interestingly, also SNP in NF-κB1 and BATF, both transcription factors expressed in B-cells, and TMEM39A, a gene with unknown function, were associated with increased EBNA-1 IgG levels (**chapter 5**). Currently, relatively little is known regarding the expression or function of BATF in MS. BATF physically interacts with the early growth response gene (EGR-2) which is important for the inhibition of IL-17A production, but not IFN-γ in CD4 T-cells. EGR-2 knockout mice have severe EAE and increased numbers of Th17 cells. Human EGR-2 and BATF expression in CD4 T-cells is induced upon CD3 stimulation. In a small group of MS patients, EGR-2 but not BATF expression was decreased in CD4 T-cells [68]. Lastly, NF-κB1 was associated with increased EBNA-1 IgG levels in MS. Several MS associated genes are involved in the NF-κB pathway, for example Cbl-b which inactivates NF-κB [69] and MERTK which is an inhibitor of NF-κB [70]. Additionally, the C-type lectin CLEC16A might be involved as several C-type lectins are upstream of the NF-κB pathway [71]. Interestingly, MALT1, another MS risk SNP, together with several other proteins like BCL-10 and CARMA-1, is upstream of the canonical NF-κB pathway [72]. The paracaspase activity of MALT1 is capable of activating T-cells [73-75]. MALT1 knockout mice have strong lymphocytic infiltration into the CNS, but fail to develop EAE. Functionally, MALT1 deletion affects Th17 cells without affecting the signature transcription factors of Th17 cells. These cells fail to cleave RelB, the suppressor of the canonical NF-κB pathway. Alteration in the NF-κB pathway due to deletion of MALT1 did not affect Th1 cells [76]. Also BATF is important for the development of Th17 cells [77]. From the GWAS hits in MS, it is apparent that multiple SNP associated with or within the NF-κB pathway are important in the pathogenesis of MS. It will be important to further study these SNP functionally to assess if they contribute to possible alterations of this pathway in MS.

CD8 regulatory T-cells may be important in the control of EBNA-1 IgG responses

MS genetics explains only a minor fraction of the variance (approximately 10%) of EBNA-1 IgG levels. Therefore, other mechanisms must contribute. In a mouse model of lupus, CD8 Treg inhibit follicular Th-cells (Tfh). Inhibition of Tfh-cells lowered the germinal centre (GC) dependent IgG production [78-80]. Additionally, it was shown that BATF is involved in CD8 T-cell exhaustion during chronic HIV-1 infection [81, 82]. Whether this also applies to other chronic viral infections is currently unknown. Finally, we showed that the number of IL-7Rα negative CD8 regulatory T-cells in MS patients is significantly decreased (**chapter 2.2**). Therefore, we hypothesised that the percentage of CD8 Treg might correlate with EBNA-1 IgG levels. We found in HC, but not in MS patients, a significant inverse correlation between EBNA-1 IgG and CD8 Treg (**chapter 5**). This might indicate that CD8 regulatory T-cells are important to control abbarant B-cell responses against EBNA-1. Notabably, the number of healthy controls negative for EBV and EBV exposed HC, without EBNA-1 IgG levels is currently very low. Hence, this finding needs to be validated in a larger independent cohort of HC.

The role of EBV in MS pathogenesis

A recent meta-analysis found that EBNA-1 IgG seropositivity is associated with an OR of 4.5 for the risk to develop MS [67]. The majority of studies point to the importance of EBV in MS etiology. However, some EBV seronegative patients have been described in the literature [83]. This could reflect that EBV is not a prerequisite for the development of MS, or alternatively ascertainment bias by experimental design of the study, or misdiagnoses. In our study is it highly unlikely that ascertainment bias has occurred as we screened for antibodies against three important EBV epitopes in patients with a long disease duration. Also in pediatric MS, EBV seronegative patients have been identified [84]. Unexpectedly, only in MS patients above 25 years of age, EBNA-1 IgG levels are increased compared with HC, whereas below 25 years no significant differences were described [85]. Mechanistically, this implies that not only variations in the host genome are contributing to the increased EBNA-1 IgG levels observed in MS. The increase of EBNA-1 IgG above 25 years of age in MS patients might be explained by a second hit with another micro-organism leading to immune activation, or infection with a second EBV strain. Co-infection with multiple EBV strains has been described in healthy persons. Co-infections are commonly occurring and are variable over time [86]. Currently, only a few rather small studies have assessed strain differences or co-infection in MS. Co-infection with EBV type 1 and 2 was more prevalent in MS cases than in healthy controls [87]. Using sequencing technologies for two EBV genes, EBNA-1 and BRRF2, extensive polymorphisms in the virus genome were observed in both MS and HC. The frequency of the polymorphisms in these genes was marginally different between cases and controls, indicating that the exact association between MS and EBV is rather complex with regard to strain differences between patients and controls [88].

It is currently unclear how EBV mechanistically contributes to MS pathogenesis, but several models have been proposed. Firstly, it was hypothesized that EBV alters antigenic exposure. EBV is capable to induce αB-crystallin expression, which can be presented by B-cells in an HLA-DR dependent manner.

αB-crystallin is considered to be a candidate auto-antigen in MS [89]. Another candidate antigen induced via transactivation by EBV is the endogenous retrovirus HERV-K18, which can act as a superantigen [90]. Secondly, it might be possible that an EBV peptide is similar to a peptide expressed in the human brain leading to molecular mimicry, although no formal evidence for pathogenic molecular mimicry in MS exist. Thirdly, it is possible that EBV infects auto-reactive B-cells. These B-cells start to divide and become clonally expanded B-cells. Clonally expanded B-cells in the CSF of MS patients have been described in several studies [91, 92]. Clonally related B-cells are present in different CNS compartments, namely in the CSF, brain parenchyma (both NAWM and inflammatory lesions) and meninges [93]. Whether these B-cells are autoreactive remains to be determined. In a provocative study, nearly all MS patients had EBV positive B-cells within the CNS. Additionally, activated CD8 T-cells were found in areas with EBV-infected B-cells [94]. However, subsequent studies failed to replicate these findings [95, 96]. Lastly, EBV infection might alter gene expression in MS patients via epigenetic mechanisms. It would be of interest to determine which genes are epigenetically modified during EBV infection and if these genes significantly overlap with MS risk SNP.

The genetic component of neurodegeneration in MS

In parallel with the neuro-inflammatory component of MS, a second important hallmark of MS is neurodegeneration. Although the majority of SNP in MS is associated with immune related genes, some of the polymorphisms have a presumed function in the CNS, like GALC, MANBA, MMEL1, ZNF746 and kif21b.

In **chapter 6**, we assessed the role of the intracellular transporter molecule kif21b in MS. Kif21b belongs to the family of kinesins. Kif21b is a plus-end kinesin, important for transport from the neuron soma into the dendrites, where it is highly enriched. Currently, the cargo of kif21b is unknown [97]. Given the importance of kif21b in neuronal transport, it is a likely candidate to be involved in the neurodegenerative component of MS.

We found that kif21b is significantly increased in areas with more severe neuropathology, both in MS and Alzheimer's patients. Additionally, patients having enhanced kif21b expression levels had a shorter disease duration. Lastly, MS patients having abundant levels of kif21b had a shorter time to reach EDSS 6, a measure for sustained disability in MS. The effects were independent of the kif21b MS risk SNP. Kif21b protein expression was found in neurons and quite unexpectedly, kif21b expression was found in astrocytes, the most abundant cell type in the CNS accounting for approximately half of the human brain cells. Virtually nothing is known regarding the expression or function of kinesins in astrocytes. Overexpression of Tau in primary rat astrocytes decreased kinesin levels and increased tubulin synthesis. Kinesin-dependent traffic was disrupted and this eventually led to fragmentation of the Golgi apparatus and astrocytic death [98]. Additionally, kinesins and dynein transported endosomes in astroglial cells [99]. Interestingly, astrocyte activation upon LPS stimulation is dependent on CLEC16A [100], which is involved in endosomal transport (**chapter 4**) and maturation [50]. Moreover, CLEC16A is an important mediator in the regulation of HLA-II membrane expression (**chapter 4**).

During the last decade, a variety of new functions of astrocytes has been described, in addition to their functions in brain homeostasis [101], the regulation of the BBB, and scar formation in areas with tissue damage. Astrocytes might also serve as antigen presenting cells within the CNS. Within MS lesions, but not in the NAWM, astrocytes express HLA class II molecules [102]. Whether astrocytes express co-stimulatory molecules is a matter of debate. Co-stimulatory molecules on astrocytes seems to be absent in vitro [103], but in chronic active MS lesions astrocytes express CD80 and CD86 [104]. Additionally, astrocytes are capable to produce T-cell priming cytokines, like IL-12 and IL-23 [105] and produce chemokines and adhesion molecules to attract T-cells to the CNS [106, 107]. Via the constitutive expression of FASL on the endfeet of astrocytes, they can induce T-cell apoptosis [108]. Astrocytes are able to produce IL-7 [6] and upregulate HLA-I during viral infections [109], factors important for the increased expression of the IL-7Rα on CD8 effector memory T-cells (**chapter 2.1**). Lastly, the importance of astrocytes in auto-immunity was shown in neuromyelitis optica (M. Devic), where pathogenic auto-antibodies against the water channel aquaporin-4 expressed on astrocytes induce demyelination [110]. The role of astrocytes in grey matter MS pathology is currently less certain than in the white matter pathology. Besides the role of astrocytes in immunity, their importance in neuroscience is also increasingly been recognised. Astrocytes are important in the regulation of memory, sleep and learning via their contribution to the synapse [111, 112]. Whether kif21b is involved in one or more of these processes would be interesting to investigate further.

Classically, MS is considered to be a white matter disease, but evidence is accumulating about the importance of other types of pathology than solely myelin destruction. Grey matter (GM) pathology and the development and extent of GM lesions became more evident. From early pathology studies, using post-mortem grey matter tissue, it was concluded that contrary to the inflammatory white matter lesions, grey matter lesions hardly contain infiltrating leukocytes. However, it was recently shown in a large study using biopsy tissues from patients with early active neurological disease, suspected for a malignancy, but eventually diagnosed with MS, that early grey matter demyelinating lesions contain numerous T-cells and macrophages and to a lesser extent also B-cells [113]. Apparently, the nature of the grey matter lesions changes over time with regard to the inflammatory component, while white matter lesions are less prone to change their appearance.

The concept that neuroinflammation precedes neurodegeneration is challenged by new MRI techniques. It is now clear that neurodegeneration occurs already in the earliest disease stage (CIS). This notion came from studies investigating the rate of atrophy in CIS patients. Already in CIS and recently diagnosed RRMS patients, significantly more atrophy was observed compared with age-matched controls [114]. Both grey lesions and atrophy as well as white matter lesions are developing rather simultaneously and largely independent of each other. Neurodegeneration is thus not per se a consequence of neuroinflammation [115].

Currently, MRI assessment for clinical purposes is mainly used to study the number and location of lesions within the white matter. It has been techniqually challenging to visualise cortical lesions with MRI given their small size, the poor contrast with the surrounding normal grey matter, and due

to partial volume effects of the CSF [116]. Recently, using new algorithms like double inversion recovery (DIR), cortical lesions can be detected rather well [117]. A few studies found a correlation between the total volume and number of grey matter lesions and the development of sustained neurological disability and the severity of cognitive impairment [118, 119]. However, MRI is still unable to detect the full extent of grey matter lesions [120, 121], probably underestimating the effect of GM lesions on clinical outcome.

There is an urgent need to increase our understanding of the biology and the underlying mechanisms of the neurodegenerative process in MS, because currently no treatments are available slowing down or stopping the accumulation of progressive neurological damage. Given the relatively low numbers of polymorphisms in genes with a presumed CNS function, it is very suitable to investigate the mechanisms underlying the development of sustained disability thoroughly. Better understanding of this process may assist the development of new drugs, slowing down this devastating process.

Part two

Critical appraisal of genetic research in MS

This part aims to give a critical appraisal of genome research in MS. Additionally, a summary of the current stage and the expectations of genomic research of the near future will be given. A general summary of the current stage of genomic research is given in Box 2, the different items listed here will be further discussed below.

Box 2. Key points in genomic research in MS

1. MS risk is associated with 57 SNP, of which 54 are located in or nearby genes. Only 4 polymorphisms are non-synonymous SNP
2. Allocation of genes is not completely unbiased
3. The current SNP have a high prevalence in the general population, limiting the clinical applicability
4. The reported SNP are proxies for other SNP, which might be the true causal variant
5. In the discovery phase of genomic research, the effect estimate of a SNP is often inflated
6. Functional immunogenetic studies are only performed for a limited number of SNP. The majority of immunogenetic studies found functional alterations
7. Currently, not all genetic variance of MS risk is explained (missing heritability)
8. Gene-gene and gene-environmental interactions are currently largely unidentified in MS

What have we learned from GWAS in MS?

Until now, 57 genome-wide significant SNP are associated with a very modestly increased risk to develop MS. Fifty-four of these SNP are implicated to be in or nearby a gene. The number of SNP associated with MS will increase in the near future using combined datasets of the IMSGC and the WTCCC.

Currently, only four SNP are presumed to alter the amino acid sequence based on the substitution of a nucleotide, a so-called missense SNP. These four genes are the IL-7Rα, MPV17L2, TNFRSF1A and DKKL1 (based on reports of the NCBI SNP database). Only functional alterations for the IL-7Rα [23] (and **chapter 3**) and TNFRSF1A [122], associated with the MS risk SNP have currently been shown.

One of the main conclusions from the largest GWAS in MS performed until now was that the MS associated SNP are mainly located in immune related genes. Only a few genes have a presumed function in the CNS. Critics of GWAS argue that some of the GWAS genes were already implicated in the (immuno)pathogenesis of MS. Therefore, this unbiased GWAS might not have added too much new insight in MS pathogenesis. However, numerous new genes not implicated or studied in MS before have now been discovered. In-depth analysis of some of these genes has revealed aberrancies in the underlying pathways in MS, such as in CD6, IL-7Rα, TNFRSF1A and CLEC16A (see paragraph on functional immunogenetics for more details). It is expected that the number of new immunogenetical studies will increase in the near future.

In the landmark paper on the genetics of MS, a SNP corresponds with a certain gene based on several criteria. The SNP must be within the actual gene or the nearest gene was selected, unless there were ´strong biological reasons´ to select another gene. These reasons were not further specified. It is thus questionable whether some of the reported genes are results of a fully unbiased genetic screen. A more critical appraisal of this unbiased screen revealed that some of the reported immune SNP are not within an immune gene itself, but actually lying within another, mainly non-immune related gene (Table 1, based on NCBI SNP database and Affymetrix product sheet for the 610K SNP array). Recently, in a follow-up paper regarding some of the SNP with sub-genome wide significance, two of the original reported SNP in NF-κB1 and TNFRSF6B were now attributed to MANBA and ZBTB46 respectively [123], supporting this notion.

The SNP attributed to NF-κB1 and TMEM39A might be in MANBA and TIMMDC1 (Table 1). The function of both genes is currently unknown and therefore it is hard to speculate how these genes could be involved in the enhanced IgG response against EBNA-1 as we observed in MS patients (**chapter 5**). Whether these genes also have a function in the immune system is currently unknown, but MANBA is highly enriched in leucocytes, suggesting that MANBA has an immune function as well. TIMMDC1 is expressed in a variety of human tissues as well as in leukocytes (based on www.biogps.org).

Another important aspect that should be mentioned is that the majority of SNP are not located within the gene itself. These SNP are thus not likely involved in altered functioning of the protein.

Table 1. Critical appraisal of reported MS risk genes

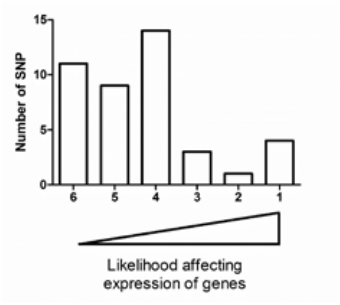
Reported gene	Reported to be immune gene	Actual gene	Function	Actual gene immune gene	Reference
VCAM1	Yes	SLC30A7	Cellular efflux of zinc	No	124
TMEM39A	No	TIMMDC1	Mitochondrial membrane	No	PF
NF-κB1	Yes	MANBA	Glycosyl hydrolase 2 family member; important for lysosomal storage	No	125
MYB	No	AHI1	Abelson helper integration site 1; important in cortical and cerebellar development	No	126
ZNF746	No	ZNF767	Zinc finger family member	No	PF
MYC	No	PVT1	Oncogene; might regulate miRNA expression	No	127
CYP27B1	Yes	AGAP1	Involved in membrane trafficking and cytoskeleton dynamics and direct regulator of the adaptor-related protein complex 3 on endosomes	No	128
CD40	No	NCOA5	Nuclear receptor co-activator	No	PF
TNFRSF6B	No	ZBTB46	Zinc finger and BTB containing 46; transcription factor for classical DC	Yes	129-131

PF: presumed function based on nomenclature of the gene

Twenty-five of the 57 reported SNP are actually intergenic SNP. It will be important to determine the actual location of these SNP and thereby how these SNP functionally contribute to MS risk, via either transcriptional or regulatory effects on associated or other regional genes (see paragraph on application of systems biology for more details).

A possible consequence of intronic SNP is altered splicing, which might alter proteins or their function. Recently, the ENCODE (Encyclopaedia of DNA Elements) project reported their data. One of the major findings was that 80% of the genome has biochemical functions. Regarding GWAS, SNP are enriched within non-coding functional elements. They confirmed that the majority of SNP are indeed outside the coding region of genes [132]. Combining GWAS data, high-throughput experimental data on regulatory regions in the genome and improved computational models for DNA binding sites resulted in powerful tools to assess functional consequences of SNP or a SNP in linkage disequilibrium [133]. Applying these tools to MS GWAS showed that only the minority of SNP is currently associated with (presumed) altered regulatory function of genes (Fig. 2). It should be noted that 15 MS-associated SNP are currently not available in the RegulomeDB database [134]. For the SNP in CD40, MYB, CXCR5 and CYP27B1 experimental evidence sugests that they affect gene expression. Additionally, it is likely that the SNP in MYC is affecting gene expression.

Figure 2. The majority of MS-associated genes is not likely to affect gene expression by disruption of DNA binding sites



Using RegulomeDB [134], the likelihood of MS-associated SNP altering the expression of genes was assessed. Experimental data regarding altered gene is available for four MS-associated SNP, one SNP is likely to affect gene expression and the majority of SNP have minimal binding evidence. SNP with class 1 evidence alters gene expression assessed with eQTL analysis. Class 2 evidence is likely to affect gene expression based on transcription factor binding sites and DNase peaks. SNP with class 3 to 6 have minimal or no data supporting a role in the regulation of gene expression. Detailed information regarding the classification of SNP into one of these categories can be found on the RegulomeDB website (http://regulome.stanford.edu/).

From the MS-associated SNP, 25 are relatively common with prevalence in the general population around or above 70%. Nineteen SNP are relatively rare with frequencies below 30%. The rarest variants are SOX8, PTGER4 and SP140, all non-immune genes. It is generally assumed that especially these rarer variants might have a major role in the pathogenesis of a disease, as negative selection mechanisms during evolution have prevented these SNP from becoming commonly distributed across the population. Conversely, common variants had a positive effect on evolution during the past centuries (for example, in the protection against infections), but are now negatively affecting human health due to changes in the environment.

It should also be noted that the currently reported SNP may not be the causal SNP. The reported SNP might be a proxy for the real causal variant. These might be in linkage disequilibrium with each other, which reflects a statistical association between two different variants in close physical proximity to each other on a chromosome. Deep replication (also termed fine mapping) or sequencing of the region of interest is necessary to identify the real causal variant, especially for SNP in large LD blocks or in regions containing multiple genes. It should be noted that it will be challenging to disentangle the real causal variant in very large LD blocks [135]. The importance of deep replication has been shown by the Immunochip consortium. From a GWAS in celiac disease, the THEMIS/PTPRK locus was found to be associated with celiac disease. From an immunological point of view, THEMIS was the most interesting gene in this region, because THEMIS is important for thymic T-cell selection. Using deep replication with the Immunochip, it was shown that not THEMIS, but PTPRK is the causal gene in this locus [136]. The function of this gene is currently unknown. Importantly, this locus is also associated with MS.

Lastly, multiple SNP might be involved as well. Testing haplotypes or multiple SNP in a region may reveal the real causal SNP for the association. One example where the originally reported SNP is not the causal SNP is the IL-2RA (CD25). The rs2104286 and rs12722489 SNP in the IL-2RA were previously associated with an increased risk to develop MS with OR of 1.19 and 1.25 respectively [137]. However, in a study assessing 26 SNP in this locus in a large number of trio families and affected sib-pairs, it turned out that the combination of rs2256774 and rs3118470 with a relative risk of 3.5 was more powerful to discriminate between MS patients and controls than the two previously associated IL-2RA SNP [138]. Currently, very few studies have performed deep replication or sequencing the region of interest to establish the real causal variant. This is important to be able to study the functional effects of a SNP further. The fact that the real causal variant is not captured yet or that these SNP are not present on the current commercial SNP arrays may contribute to the issue of missing heritability (discussed later) [139]. We did not observe functional consequences of the SNP in CLEC16A (**chapter 4**) and kif21b (**chapter 6**), and this might indicate that these SNP are not the causal SNP.

Importantly, the OR of the first GWAS providing evidence for a certain SNP to be associated with a certain trait is often inflated, probably due to population diversity between studies or biases. Several types of bias might account for this, for example publication, lag-time and sampling bias[140]. Therefore, a meta-analysis on all available GWAS data on MS is necessary to assess whether some of the very modestly MS-associated variants are true associations or false positive results.

Functional immunogenetics

A large number of SNP associated with MS risk are in or nearby receptors and molecules with an important function in the immune system. Elucidating how these SNP are affecting the expression and functioning of immune cells is a critically important step in improving our understanding of MS immunopathogenesis. Currently, only a very limited number of studies has been performed to unravel how these SNP are affecting immune functioning and therefore only a limited number of SNP with functional consequences are known. Below, eight SNP associated with altered function or expression in MS will be summarised briefly.

CD6

CD6 is an adhesion molecule and the CD6 rs17824933 risk allele is associated with impaired proliferation of long-term activated T helper cells. In both CD4 and CD8 T-cells, the CD6 risk allele leads to consistent downregulation of the expression of exon 5, which is the ALCAM binding domain. ALCAM is the ligand for CD6 and this explains the decreased proliferative response [141].

CD25, CD127 and CD58

The CD25 (IL-2RA) rs2104286 risk allele is associated with increased expression of CD25 by naive CD4 T-cells and decreased expression on memory Th-cells in healthy controls [142]. IL-2 has a dual role in the immune system, firstly it is a very potent proliferative stimulus after activation of naive T-cells and secondly it is important for the induction and maintenance of regulatory T-cells in the memory compartment. Interestingly, functionally active Treg express very low levels of the IL-7Rα. IL-2 and IL-7 belong to the same family and these two cytokines have both similar and opposing functions (reviewed in Katzman et al [143]). The IL-7Rα SNP (rs6897932) is associated with increased levels of the soluble form of the receptor [23]. Surface expression of the IL-7Rα was also increased on CD8 effector T-cells, independent of the genotype [144], as well as on CD8 regulatory T-cells of MS patients [145]. A polymorphism in CD58 (rs2300747) is implicated to alter the expression of CD58 mRNA in peripheral blood mononuclear cells (PBMC), affecting the functionality of CD4 regulatory T-cells [146]. Whether this is also applicable to CD8 Treg is currently unknown.

IRF8 and TYK2

Another study assessed whether the SNP in IRF8 (rs17445836) influenced the gene expression in PBMC. No association between the SNP and IRF8 mRNA expression was found, although it should be noted that only one probe for IRF8 mRNA was present on the microarray chip used. Interestingly, in this study sixteen genes were associated with the IRF8 SNP and eight of these genes are type I interferon responding genes [147]. Also a non-synonymous and functional variant of TYK2 (rs34536443) has been identified. This amino acid substitution is associated with decreased phosphorylation of TYK2 upon IFN-β signalling in T-cells. The protective allele of the SNP is associated with polarization of CD4 T-cells towards a Th2 phenotype [148]. It should be noted that this functional SNP is not the same SNP as was reported by the IMGSC and WTCCC, and which SNP is the causal one is currently unknown.

TNFRSF1A

Recently, it was shown that the SNP in TNF-receptor 1 (TNFRSF1A, rs1800693) is associated with a novel soluble form of the receptor, capable of binding and thereby blocking TNF with high affinity. This soluble TNF-R1 is caused by alternative splicing at the boundary of exon6/ intron 6, causing a frame shift. This results in a lack of the cysteine-rich domain, the transmembrane domain and the intracellular region essential for proper cellular localisation. Because of the deletion of the death domain, this variant is not able to signal via NF-κB (another MS risk gene) and induce apoptosis [122]. Although the functional consequence of TNFRSF1A SNP is interesting, caution is necessary regarding the interpretation of this finding in relation to the effects observed in the anti-TNF-α clinical trial in MS. Anti-TNF-α treatment in MS was given in an open label phase I safety study and no clinical neurological changes were found. In addition, the number of gadolinium enhancing lesions transiently increased after every infusion in the two included patients [149]. Additionally, also in a larger study with 168

patients, receiving either placebo or Lenercept (a recombinant TNF receptor p55 immunoglobulin fusion protein), clinical worsening was observed [150]. The interpretation that the SNP in TNFRSF1A is mirroring the outcome of anti-TNF-α clinical studies is preliminary, as Gregory and colleagues did not provide evidence that the SNP is associated with worse clinical or MRI outcomes. Additionally, 168 cases developing CNS demyelination upon anti-TNF-α treatment have been observed (until July, 2009) [151]. The TNFRSF1A SNP is not MS specific and the frequency of occurrence is around 40% in the general population [152]. Hence, if this SNP is associated with, or causing the adverse events of anti-TNF-α treatment, one would expect that for example in RA or psoriasis patients who are often treated with anti-TNF-α, this side effect would be observed much more frequently. Whether or not this SNP is predictive for adverse demyelinating events remains to be assessed.

CLEC16A

Lastly, for CLEC16A some expression studies have been performed. The ratio between the short and long isoform of CLEC16A was significantly different in thymic tissue, but not in peripheral blood of the CLEC16A risk allele carriers [52]. Also correlations between the expression of CLEC16A, DEXI and SOCS1, both neighbouring genes of CLEC16A on chromosome 16, were observed in thymic tissue. The presence of at least one CLEC16A risk allele was associated with lower SOCS1 and DEXI expression [53].

In conclusion, the majority of functional immunogenetic studies identified alterations in function or expression of the underlying gene. Therefore, it will be important to assess this for the remaining SNP as well to increase the knowledge on the immunopathogenesis of MS.

The EAE model is not likely suitable to study functional immunogenetics

Whether experimental studies using EAE will contribute to our knowledge about functional immunogenetics is questionable. Assessing the role of MS-associated SNP in the immunopathology and disease development will be the most informative. The consistency and reproducibility of EAE findings, as exemplified by the studies on the IL-7Rα in EAE, are limited. Additionally, mimicking the exact genetic change by a human SNP in the mouse genome will be very tough. Deleting a gene using knockout or conditional knockout mice will not be very informative about subtle changes due to SNP. Therefore, functional immunogenetics studies using elaborate and in-depth analysis of human samples will be the most promising way to understand the contribution of SNP in MS immunopathology.

SNP modestly increasing the risk to develop MS have large functional consequences

Although OR of the SNP are modest, the functional biological effects of these polymorphisms are (relatively) large. Some of the SNP have even larger biological effects compared with HLA-II association in MS, although the OR is much smaller. No correlation was found between the strength

of the biological effect and the OR (Fig. 3A), indicating that SNP with a relatively high OR do not exert their effects through stronger biological effects than SNP with a lower OR. Additionally, no association between the risk allele frequency and the biological difference was observed (Fig. 3B), indicating that SNP with a high risk allele frequency in the population can have strong effects on immune function. This suggests that the effects of SNP in MS are not related to selection mechanisms during evolution.

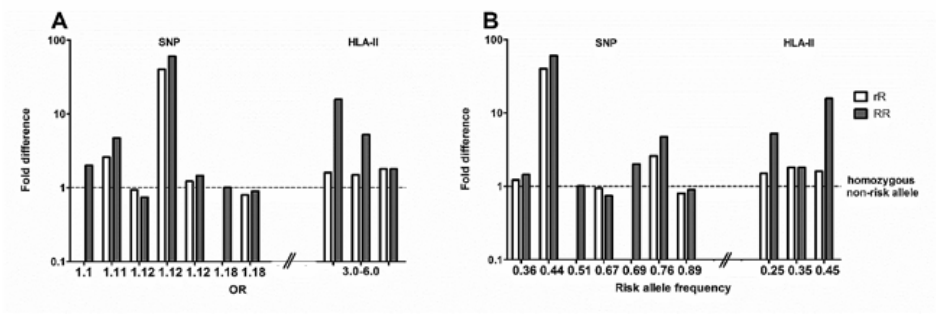


Figure 3. SNP with modest OR have strong biological effects
A) The bars represent the following SNP: TYK2, IL-7Rα, CD6, TNFRSF1A, IL-2RA, CLEC16A, CD58 and as reference the HLA-II alleles with the highest risk alleles (resp. bars are HLA-DQB1, HLA-DRB5 and HLA-DRB1). Fold differences of functional outcomes (including expression differences stratified according to the respective SNP) compared to homozygous non-risk carriers of the SNP are shown and plotted against the OR of the SNP. **B)** Functional effects of the SNP plotted against the risk allele frequency. rR denotes heterozygous carriers, RR homozygous risk carriers. Please note that TYK2 homozygous non-risk carriership is very rare and the fold change is calculated using heterozygous carriers as a reference group. For CLEC16A, heterozygous and homozygous non-risk allele carriers are pooled. Therefore, for these two genes no bar is shown for heterozygous carriers.

SNP currently not useful for diagnostic, prognostic or therapeutic purposes in MS

Currently, improvement in clinical prediction is urgently needed for better patient counseling (personalised medicine). This is important for both the conversion from CIS to MS as well as the prediction of the disease course (relatively mild versus rapid neurological deterioration). Especially because it is now widely accepted that early treatment of patients result in a more favourable disease course [153]. Predictors for the response on therapy are currently lacking. Therefore, the applicability of SNP as stable biomarkers was assessed.

The discriminative power of SNP is too low to differentiate HC and MS or CIS patients developing MS from those not developing new neurological complaints [154]. This is mainly because most of the MS-associated SNP are common in the general population and thus relatively often present in both patients and controls, although slightly more prevalent in MS. No clear differences in the weighted genetic risk score between CIS and MS patients occur and therefore the discriminatory power for CIS patients who did not develop MS and those who did is low [154]. Additionally, also in multiplex families, a MS genetic burden score (weighted log-additive model of OR of SNP) was calculated. MS cases

obtained from multiplex families have a higher genetic burden score compared with sporadic MS cases. Nevertheless, this approach does not allow to discriminate MS cases from unaffected persons [155]. Using ROC analysis, 53 MS-associated SNP currently have an AUC of 0.69. In silico analysis revelaed that the addition of 20 new SNP with an allele frequency of 0.3 and an OR of 1.1 would only yield a modest increase in the predictive power leading to an AUC of 0.70. An additional 50 SNP with OR of 1.4 and allele frequencies of 0.3 would increase the prediction to a clinically relevant AUC of 0.85 [156], while these OR are quite high compared with current GWAS results. Lastly, ten MS risk SNP did not predict the time to develop sustained disability, measured as the time to EDSS 4, 6 or 8 [157].

Some studies assessed whether the response upon IFN-β treatment can be predicted based on genotypes. Some SNP have been associated with responders vs. non-responders, however independent replication of SNP between different studies failed. This might reflect that it is impossible to predict the response to disease modifying treatments in MS, or that the real SNP associated with treatment response have not been identified [158]. In other medical disciplines, studies assessing the response on Warfarin and Coumarin (anti-coagulant treatment) showed that SNP testing is useful to assess which patients need dose adaptation [159]. Also in patients with acute myeloid leukaemia, the development of toxicity of chemotherapy can be predicted using SNP testing [160]. Lastly, also in type II diabetes, the response upon Metformin is associated with a SNP in ATM, the ataxia telangiectasia mutated gene, which is associated with the AMP-activated protein kinase activity upon treatment [161]. With the increasing number of SNP associated with MS, it might become possible to predict treatment responses.

Lastly, some studies determined whether SNP can be used to discriminate between clinically relevant subtypes or phenotypes of patients. One study found that 242 SNP are statistically associated with clinical subtypes of MS (defined based on parameters as relapsing-remitting or secondary progressive MS, age at onset, multiple sclerosis severity score (MSSS) and MRI parameters, such as brain parenchymal volume and T2 lesion load). Among these SNP were 65 SNP in the MHC region on chromosome 6. Gene ontology revealed that the top hits were associated with CNS development, calcium-mediated signalling and antigen presentation [65]. However, the largest GWAS did not find evidence for genetic associations and severity of MS, but age at onset was significantly associated with HLA-DRB1*1501 [152]. Some studies have also found that oligoclonal bands are more often associated with HLA-DRB1*1501 carriership than non-carriership [162, 163].

Currently, SNP do not contribute to clinical applications in MS, regarding the diagnostic process, prognosis of the patient or prediction of the response upon treatment, although the latter might be promising based on application in other medical disciplines. At the moment, the most promosing application of GWAS is the discovery of important and altered biological pathways. The identified SNP needs to be further assessed to determine abberancies associated with MS to increase the understanding of the immunopathogenesis of MS.

Missing heritability

Missing heritability refers to the phenomenon that genetic studies currently capture only a relatively minor proportion of the estimated genetic contribution to a disease, which is based on twin and family studies. In the large scale MS GWAS with approxiametely 10.000 patients, identifying 57 SNP, it was estimated that 0.1 of the phenotypic variance is currently captured, whereas the total variance caused by genetics lies between 0.3-0.8 [164].

So, does missing heritability play a role in MS? Both supporting and opposing arguments exist. The assumption under the missing heritability theory is that the estimation of the genetic contribution to a disease is accurately measured. This is currently estimated using the λ approach, calculated as the lifetime risk in a sibling of a patient divided by the lifetime risk of the general population. The estimates used to calculate the lifetime risk are difficult to obtain reliable and this measurement is often positively biased. Also in MS, different studies over a time period from 1988 until 2009 showed a decreasing λ [165], indicating that the variance estimated to be caused by genetics may be inflated.

Additionally, the underlying hypothesis used in GWAS is the common disease-common variant hypothesis. Using this model, one might argue that missing heritability exists in MS. Alternatively, the rare variants hypothesis assumes that common complex diseases results from accumulation of low frequency, but dominant and independently acting variants [166]. Missing heritability might be overestimated, because current GWAS are only capturing common variants (allele frequencies >5%) and not rare genetic variations (defined as minor allele frequency <0.5%). These variants are probably arising from founder effects and may therefore be population specific. Rare variants might also partially explain the heterogeneity of MS. In colorectal cancer, this hypothesis is supported by good evidence [167].

Conversely, several explanations for missing heritability in MS have been proposed. First, in the current GWAS only common variants (e.g. with a minor allele frequency of approximately 5%) are captured. The rarer variants (minor allele frequency between 1 and 5%) with predicted higher odds ratios compared with common variants are not found yet. With increasing sample sizes in GWAS, an increasing number of SNP associated with the disease has been found. The low frequency variants (minor allele frequency <0.01) are enriched for non-synonymous SNP and are therefore more likely to contribute to a disease [168]. Additionally, with the current array technologies the majority of SNP captured are intronic or intergenic SNP. The non-synonymous SNP changing an amino acid in a protein sequence and thereby possibly altering the function of a protein are not fully captured yet [139].

Second, the current GWAS have a limited power to detect gene-gene interactions and gene-environment interactions. Accounting for shared environmental factors between relatives must be performed and the current statistical methods employed in GWAS are not able to (fully) correct for these effects. Moreover, it is important to have information on exposure to certain environmental factors, but it is difficult to obtain reliable information on life time exposure of these factors. For example, ultraviolet B exposure used as a proxy for systemic levels of vitamin D prior to the diagnosis of MS, is prone to recall bias and this might affect the results. Additionally, changes over time of

environmental factors are difficult to measure. As the current GWAS are mainly case-control studies, it will be hard to gain any information on environmental factors. Gene-environmental interactions will be more reliable determined when datasets and samples, including pre-symptomatic or pre-diagnostic biological samples from large scale longitudinal prospective cohort study, like the Nurses Health Study [169], the nested case control study for US Army personnel [170] or the EPIC study [171] are combined with (epi)genetic data.

Third, in the current GWAS only SNP are assessed. Other structural variants, like insertion-deletions, copy number variations, inversions and block substitutions, which are expected to account for 20% of the genetic variance between individuals are not assessed. These variants are not necessarily in LD with SNP [172]. In schizophrenia, it was shown that large deletions at two chromosomes give rise to very large OR (above 17) [173] and in autism deletions account for approximately 1% of the cases [174]. The mechanisms by which these structural variants contribute to the development of these psychiatric diseases are currently unkown.

Lastly, family studies are powerful to detect rare or low frequency variants. However, an important contributor to the missing heritability is the parents-of-origin effect [139]. This effect is the phenomenon that a small number of human alleles are silenced and only the maternal or paternal allele is expressed. Parent-of-origin effects are difficult to detect and account for properly. If not adjusted in a meaningful manner, it might mask the contribution of rare variants [175].

Gene-environment interactions

Both genetic variants and environmental factors are contributing to the development of MS. The interaction between genes and the environment is hard to assess properly from a methodological point of view and these interactions are currently poorly studied. However, the interaction between vitamin D and MS associated SNP has been studied thoroughly. Thirty-five of 57 SNP associated with an increased risk to develop MS contain a vitamin D binding element and are thus potentially modulated by vitamin D [176-178]. These vitamin D binding elements might affect chromatin remodelling. Interestingly, vitamin D regulates the expression of HLA-DR via a vitamin D binding element. Both up- and downregulation of HLA-DR upon vitamin D treatment in vitro have been observed. Treatment with the bioactive form of vitamin D in DC downregulated HLA-DR expression [179, 180], whereas HLA-DR expression on B-cells increased upon vitamin D stimulation. Interestingly, this only occurred in HLA-DRB1*1501 positive individuals [62]. In **chapter 4**, we found that treatment of monocyte-derived dendritic cells with vitamin D resulted in a significant downregulation of HLA-DR and CLEC16A, one of the other genes containing a vitamin D binding element.

Infection with EBV and infectious mononucleosis (M. Pfeiffer) are well known risk factors for MS. It would be very informative to assess the effect of EBV on chromatin remodelling in B-cells to assess whether EBV-induced epigenetic modifications also overlap with MS risk genes. Moreover, only a minority of persons infected with EBV will develop clinical mononucleosis. Assessing which genetic factors contribute to the control of the virus will give more insight in the risk to develop MS, as there

might be overlap in genes associated with the development of these two diseases. The development and the severity of infectious mononucleosis is associated with two microsatellite markers and two SNP in an HLA-A allele [181]. Interestingly, the HLA-A2 SNP (rs6457110) is also associated with MS, independent of HLA-DRB1*1501, which supports the notion that there might be shared genetics between MS and clinical mononucleosis [182].

Interestingly, in patients with high vitamin D levels, a lower EBV DNA-load was observed, providing a possible link between these two well-known environmental risk factors as well as with HLA-A2 and HLA-DRB1*1501 alleles [183]. More precisely assessing which genetic variants are associated with individual environmental triggers will shed more light on the complex etiology of MS.

Gene-gene interactions

It is increasingly being recognised that genes associated with independent modest effects on the risk to develop MS, might enhance or decrease MS risk in combination with each other. The most well-known gene-gene interaction in MS is the epistatic phenomenon in the HLA-locus. HLA-DRB1*15 is the strongest genetic risk factor for MS. Heterozygous carriers have an approximately two-fold increased risk to develop MS and homozygous carriers seven-fold. However, a person who is a heterozygous carrier of HLA-DRB1*15 and HLA-DRB1*10 has a very low risk to develop MS (relative risk approximately 0.3). This differential modulatory effect of HLA-alleles on the risk to develop MS is an example of epistasis. Several epistatic associations have been described for MS [184].

Currently, relatively little is known regarding non-HLA gene-gene interactions in MS. A study assessing gene-gene interactions found that two of the first GWAS SNP (IL-2Rα and IL-7Rα SNP) mediate Golgi N-glycosylation. The risk alleles of these two SNP downregulated the expression of N-acetylglucosaminyltransferase 1 (Mgat1) and the inhibitory cytotoxic T-lymphocyte antigen 4 (CTLA4). Interestingly, vitamin D, one of the well-validated environmental risk factors for MS, counteracts the effects of the IL-2Rα and the IL-7Rα risk SNP. This provocative study by Mkhikian et al. provides direct experimental evidence, rather than statistical evidence, for gene-gene and gene-environment interactions [185]. Alterations in this pathway have been linked to MS by a variety of EAE studies, both in neuroinflammation and neurodegeneration. A second study investigating gene-gene interactions in trio families found significant interactions in calcium-mediated regulation of the cytoskeleton, which may be important for neurodegeneration [186]. None of the associated genes is independently found in other GWAS.

In conclusion, some critical remarks should be noted regarding GWAS. For example, the real causal variants are not identified yet, the relatively common prevalence of these SNP, and the current lack of clinical applicability. However, concluding that the current knowledge gained by the GWAS is relatively little compared with the huge costs is quite preliminary, as translation from bench to bedside in general takes decades [187] and the first MS GWAS was published only six years ago. During the last couple of years, our understanding of the genetic architecture and the functional consequences of MS genetics greatly improved. Also aberrant pathways were identified, which are

promising pathways for future therapies. Nevertheless, still a lot of the complex heritability of MS needs to be discovered, in terms of identifying new genes, assessing the real causal variants and the functional consequences of these SNP, and experimentally determining the effects of gene-gene and gene-environment interactions. In addition, analysis of other genetic variants, like CNV, insertions-deletions, rare structural variants and epigenetics in large cohorts of patients and controls will contribute to our understanding of the genetics of MS and the pathogenesis of this complex disease (Table 2).

Table 2. Where are we standing currently with MS genetics?

Currently captured	Outstanding questions
HLA class I and II	Missing heritability
2007 - 2 SNP 2008 - 3 SNP 2009 – 7 SNP 2010 – 8 SNP 2011 – 57 SNP 2013 – 105 SNP	Rare variants Other structural variants
Epistasis in HLA locus One non-HLA gene-gene interaction (Effect of CD25 and CD127 SNP on Golgi N-glycosylation)	Gene-gene interactions Gene-environment interactions
Functional consequences of 8 SNP (CD6, CD25, CD58, CD120a, CD127, CLEC16A, IRF8 and TYK2)	Functional immunogenetics for remaining 97 SNP
Not clinically useful	Epigenetics/ miRNA/ lncRNA
	Pharmacogenomics

Part three

Integration of different types of genetic research and clinical immunology into clinical trials

Future perspectives

The current GWAS are now approaching a conundrum. Furhter increasing the sample sizes will only increase the number of newly associated SNP with very modest effects on disease risk [188]. Therefore, new research directions to further unravel MS genetic architecture are needed. These include assessing rare variants, other structural variants like copy-number variantion, insertion/deletion variants, whole exome or genome sequencing, assessing epigenetic alterations in MS including miRNA screening and expression quantitative trait loci (eQTL) assessment.

Currently, only two studies in MS identified rare variants (defined as <1% prevalent in the population instead of >5% for a SNP). One study identified rare variants in CBLB, the IL-7Rα and the vitamin D converting enzyme, CYP27B1. These rare variants were subsequently validated in a large cohort of patients and controls [189]. The other study identified a rare variant in TYK2 [190]. Interestingly, all the reported rare variants are in genes previously implicated in MS GWAS. Assessing rare variants in regions already associated with a disease by determining common variants has only a minor contribution to explain the missing heritability [191]. Although the studies on TYK2 and CBLB, IL-7Rα and CYP27B1 are the first published on whole exome sequencing in MS, one might be critical about this approach. This approach captures around 1.5% of the total genomic variation. Whole exome sequencing neglects that intronic regions contain regulatory sequences, which are important to control the expression of certain genes. Therefore, whole genome sequencing, which detects rare variants in both protein coding as well as regulatory sequences might be more promising [192]. It is estimated that a genome contains around 3.5 million variants. Whole genome sequencing results in larger amounts of data and therefore even more stringent criteria for multiple testing issues must be applied. These strict multiple testing criteria may result in more false negative results.

Assessing rare variants might help to clarify the missing heritability in MS under the assumption that these rare variants with quite high OR are less frequently observed. However, it should be noted that it is currently unknown how many of these rare variants are present in the human genome [193]. Some rare variants are only observed in a few persons [194], so it is questionable how likely it is that we are able to capture this in the current population-based MS studies. Additionally, the relevance of these very rare variants in the pathogenesis of a common disease is questionable.

Possible explanations for recent increase in MS prevalence

The recent increase in the incidence of autoimmune diseases, including MS, has classically been attributed to mechanisms supported by the hygiene hypothesis [195]. In this view, the pressure on the immune system by infectious diseases is lowered in the Western World due to a better hygiene. With this improved hygiene, the risk to develop autoimmunity would increase. In parallel with the increase in several autoimmune diseases, the human population has grown worldwide with a massive speed during the past 100 generations. This implies that the human population has departed from the genetic equilibrium. Particularly, this population explosion has led to an increase in rare variants. Due to this rapid increase, the selection pressure on these potential harmful variants was lower due to the relatively short time interval wherein these variants were introduced. SNP with a potential harmfull effects were introduced in the human genome much earlier in history and negative selection had much more time to eliminate potential harmful SNP. The introduction of these rare variants into the population may have a major impact on disease risk [196]. This phenomenon might contribute to the increase in autoimmune diseases during the last century. Importantly, these rare variants are more often in protein coding regions in the genome and therefore more likely contributing to the disease risk (advantages and disadvantages of assessing rare variants in MS are summarised in Table 3) [194]. These more recently introduced rare variants are now being detected with next generation sequencing. A recent study sequenced 15.585 protein coding genes in 2.440 persons and identified half a million variants, of which 86% were rare. Each individual carried approximately 13.500 variants, of which 2.3% was predicted to cause altered protein function [197]. The vast majority of variants are population specific and this may cause problems with validation across populations. Given the rarity of these variants, it will be necessary to study very large populations to fully identify disease associated variants [188]. Obtaining such large populations of patients from a single ethnic background to prevent population admixture may be difficult.

Table 3. Assessing rare variants in autoimmune diseases

Advantages	Disadvantages
More likely to contribute to the disease	Population specific; more subtle effects, more prone to population stratification => more false positive results; lack of replication across populations
Rapid increase in incidence of autoimmune diseases might be due to more recently inherited rare variants	Better prediction models necessary for functional annotation of the effect of the single nucleotide variants (SNV)
Might be more informative to study response on drugs	Time-consuming to perform
	Still costly

Lastly, besides the hygiene hypothesis and the increase in recently introduced rare variants, also environmental factors have changed dramatically during the last century. For instance, an increase

in salt intake might contribute to the higher incidence of MS. High salt concentrations induce the expression of the glucocorticoid kinase 1 (GSK1), subsequently inducing the expression of the IL-23 receptor and enhancing Th17 phenotype polarization [198-200].

In addition to the identification of new genetic associations with MS, other types of studies can be applied to further understand the complexity of the pathogenesis of MS. For example, combining different types of datasets may identify aberrant pathways in MS. An example of such an approach is to investigate eQTL. A SNP can alter gene expression, which can be a ***cis*** eQTL (local effects) or ***trans*** eQTL (distant effects). A study in MS assessed eQTL of the rs3135388 tagging SNP (proxy for HLA-DRB1*1501). The rs3135388 [A] allele correlated with abundant mRNA expression of HLA-DRB1, HLA-DRB5 and HLA-DQB1 in the presence of HLA-DRB1*1501 allele [201]. Additionally, these studies may reveal new genes to be involved in the pathogenesis of MS. An example is the identification of DEXI as a new candidate gene in type I DM. Assessment of eQTL for CLEC16A revealed that the CLEC16A SNP correlated with DEXI expression, a gene with an unknown function. Intron 19 contained a large number of transcription factor binding events and markers for enhancer activity. Intron 19 of CLEC16A was physically in close proximity to the DEXI promoter, but not to other promotors in this genomic region and thereby DEXI was identified as an interesting candidate molecule to be regulated by the CLEC16A SNP associated with DM type I [54].

Application of systems biology to MS

Additionally, systems biology can be applied to complex diseases. This approach 'holistically' combines different data sources, for example transcriptomics, miRNA screens, GWAS and proteomics, and integrates these into models about pathways and biological networks aberrantly expressed or predicted to have an altered function in a disease. Importantly, this 'holisitic' approach is suitable to generate hypotheses (comparable with GWAS), which should subsequently be tested experimentally to reveal whether or not the computational models were accurate. Currently, transcriptomics, proteomics and antigen arrays are mainly aimed to determine aberrantly expressed genes or proteins to be used as a clinical relevant biomarker. The discovery of biomarkers for MS has been challenging, because independent replication of interesting markers across studies or platforms failed. This is in clear contrast to GWAS, in which almost all genomic loci are replicated between studies (Table 2, **chapter 1**). Therefore, it might be intuitive to combine GWAS with other types of datasets to assess whether the outcomes relate to certain pathways aberrantly expressed in MS. Recently, Villoslada and Baranzini performed a study where they included 21 proteomics, lipidomics and antigen/antibody array studies comparing both MS and HC or RRMS, SPMS and PPMS and assessed which gene ontology and related proteins were over- and underrepresented in these studies. Significantly more genes were downregulated than upregulated in MS. The relative contribution of the different pathways also differed between the up- and downregulated pathways. Unfortunately, this study did not identify new candidate molecules or pathways other than original molecules published [202]. Another study included 164 large-scale gene expression studies of PBMC of MS versus HC and MS

patients in remission vs. patients having a relapse. The separation of MS patients versus healthy controls was more accurate than relapse vs. remission within MS patients. Additionally, several new candidate molecules were identified to study further, although functional experiments were not performed [203]. Lastly, one other study assessed human T-cell activation genes. Based on 20 genes associated with T-cell activation, 31 previously unknown interacting genes were identified. Subsequent experimental validation revealed that Jagged1 (not differently expressed between MS and HC), was very important for this T-cell activation network in MS patients. In EAE, Jagged1 agonist shifted the balance of T-cells towards Th2 [204]. Interestingly, in a completely independent proteomics study, Jagged1 was also identified as aberrantly expressed in CSF of PPMS compared with RRMS [205].

This systems biology approach can also be applied to the results of GWAS. Recently, protein-protein interactions of the SNP of three large GWAS were assessed. Several genes, not previously associated with MS, were predicted to be involved in a network of MS-associated risk genes. Unfortunately, no comparison of these genes between MS and HC nor risk allele carriers vs. non-risk allele carriers of these SNP was pursued [206].

Lessons to be learned from clinical trials in MS

The number of new drugs currently being developed for MS is very large and all are targeting the inflammatory component of MS. Phase II and III clinical trials are of course very important to establish safety, tolerability and efficacy of new drugs. However, much more can be learned from these studies. Currently, one of the gaps in clinical trials is the relative lack of followup studies using materials obtained longitudinally during clinical trials to assess the exact underlying immunological mechanisms altered by the drug, and compare these findings with patients treated with a placebo.

One of the ongoing debates in the immunology of MS is the CD4 T-cell 'subsetology'. The classical paradigm regarding different Th-subsets was Th1 (IFN-γ producing cells, associated with autoimmunity) versus Th2 (IL-4 and IL-13 producing cells, associated with allergy). Subsequently, regulatory T-cells controlling pathogenic responses were identified and these cells could be further subdivided as discussed previously. During the last decade, this concept was challenged. A fourth subset was identified producing high amounts of IL-17 and named therefore Th17, subsequently followed by Th9, Th22 and Tfh-cells. The master transcription factors responsible for the different subsets were established. During recent years, it became apparent that these subsets are not stable and given the situation in the micro-environment, a lineage-committed Th-cell can become another type of Th-cell. Lastly, also the stability of the transcription factors of the Th-subsets was challenged, especially because a functional Th-subset is able to express multiple lineage-specific master transcription factors (excellently reviewed in O'Shea and Paul [207]). Additionally to T-cells, B-cell phenotyping is increasingly be performed and the origin, function and trafficking of new B-cell subsets is now being elucidated [208]. Lastly, also more research is performed on the role of innate immune cell types in (auto)immunity, like NK- and MAIT cells. With expanding possibilies in immunophenotyping, pinpointing subtile differences in leucocyte subsets between patients and controls will increase the understanding of the immunopathogenesis of MS.

Incorporation of immunological studies in clinical trials

There are several advantages of incorporating functional immunological studies into clinical trials. First, clinical trials are randomized and double-blind. Patients receiving active treatment and those receiving placebo are matched for potential confounders (age, gender, smoking, disability at sampling and genotype, exposure to environmental factors). Because of this, the two groups of patients are (relatively) homogeneous, making it easier to detect subtle differences. Second, especially in phase III clinical trials a large number of patients is included and subdividing the patients into several phases is possible, so the initial findings can be validated in a secondary cohort of patients. Standardizing procedures regarding sample handling and read-out assays between different trial sites and countries is important for success of these types of studies. Importantly, one should develop a parallel study to collect samples from healthy controls.

It is interesting and valuable to assess the behaviour and expression of all different leukocyte subsets and to perform functional assays in aberrant subsets longitudinally nested within clinical trials. Especially in the placebo group, the stability of subsets and responses over a long period of time (as most clinical trials last several years) can be assessed and plasticity in vivo can be determined. Additionally, responses to treatment can be pinpointed to individual subsets, which can subsequently be studied thoroughly. It would be very informative to include genetics in these studies as well, although some concerns regarding ethics have prevented inclusion of genetic sampling into industry-sponsored clinical trials [209].

Even within a single clinical trial, discrepancies have been observed between different endpoints. This was mainly between clinical (annualized relapse rate, development of disability) and MRI endpoints (development of new gadolinium enhancing lesions, T2 lesion load, development of black holes etc.) [210], likely occurring from the clinic-radiological paradox. Also discrepancies for the same outcome between different clinical trials have occurred [211, 212]. From a pharmacological point of view, inclusion of immunological studies into clinical trials can be used to develop new biomarkers for response to treatment, which could be used in subsequent trials as a new secondary endpoint.

Examples of new and exciting immunological mechanisms emerging from the effects of treatment came from the Daclizumab and Rituximab trials (anti-CD25 and anti-CD20 depleting monoclonal antibodies resp). The original presumed mechanism of action of Daclizumab was that the blockade of CD25 (IL-2Rα) would inhibit the proliferation of recently activated T-cells [213]. However, anti-CD25 treatment only moderately reduced the number of CD4 and CD8 T-cells, but unexpectedly a marked increase in NK-cells, especially CD56 bright regulatory NK cells was observed. These CD56 bright NK-cells are capable of inhibiting CD4 T-cell responses, although the exact mechanism is currently unknown [214]. Interestingly, patients not responding to Daclizumab did not show an increase in CD56 bright cells [215]. Anti-CD25 treatment may also have effects on dendritic cells [216]. The role of CD56 bright cells was not investigated thoroughly previously and the Daclizumab effects on this subset highlight the importance of well-designed immunological studies nested into clinical trials. From the Rituximab phase II clinical trial, important immunological mechanisms in addition to antigen-

presentation by B-cells have been identified. B-cells are important activators of CD8, Th1 and Th17 responses. The activating effects were mediated by increased production of lymphotoxin and TNF-α and to a lesser extent diminished IL-10 production by B-cells [217]. Recently, also the importance of IL-6 producing B-cells to stimulate T-cell responses, again both Th1 and Th17, was recognised [218]. In a RA clinical trial with Rituximab, biomarkers for the response on Rituximab have been developed and it is interesting to assess whether the levels of IgJ mRNA expression in MS also correlates with the response upon Rituximab treatment [219].

Concluding remarks

Our understanding of the (immuno)pathogenesis and genetic architecture of MS has greatly improved. We have now entered an exciting era with several large scale studies aimed to unravel the complex genetic architecture of the human genome further (HapMap, 1000 Genome project, ENCODE, deCODE, IMSGC, WTCCC etc.). These projects will accelerate the discovery of disease-associated genetic variants. Additionally, with increasing technological possibilities, large scale studies assessing the transcriptome, metabolome, proteome and the human microbiome project will facilitate the identification of aberrant pathways and interactions between genes and gene-environment interactions. In addition, the use of human biobanks to assess genotype – phenotype associations will facilitate our understanding of immunogenetics, both in health and disease. Additionally, with new MRI techniques we will hopefully gain more insight into the relationships between white matter and grey matter demyelination and lesion formation, as well as the development of atrophy. This will provide further insights in the poorly understood neurodegenerative process. Lastly, regarding therapeutical options, in the coming years a large number of new drugs for MS will become available, all modulating the immune system. One of the goals from all ongoing research efforts will be the development of new tools to understand the underlying mechanisms of neurodegeneration and the subsequent development of drugs inhibiting the degenerative component of MS.

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Appendices

Summary

Multiple sclerosis is a complex neurological disease. The etiology includes both environmental and genetic factors. The strongest and most longstanding genetic association in MS is carriership of certain HLA class II alleles. During the last six years, the knowledge on the genetic component of MS has changed dramatically. The first genome wide association study in MS, published in 2007, identified two MS risk polymorphisms and a few suspected SNP. From this year onwards, the number of SNP increased dramatically up to 57 in 2011 and 105 in 2013. The majority of MS risk genes are involved in adaptive immunity. Although our knowledge about the identity of genetic risk factors for MS increased dramatically, the main outstanding question is ***how*** these SNP contribute ***functionally*** to the increased risk to develop MS. Therefore, the next critical step in MS genetics is to perform functional immunogenetic studies to understand the mechanisms how these polymorphisms exert their effect on MS risk.

In **chapter 2.1**, we determined the effects of the SNP in the interleukin 7 receptor alpha chain (IL-7Rα). The expression of IL-7Rα by distinct functional CD4 and CD8 T-cell subsets was assessed. The percentage of CD8 effector memory T-cells expressing IL-7Rα is increased in MS patients. Additionally, increased levels of expression of IL-7Rα on CD8 effector memory T-cells were found. After stimulation with IL-7, enhanced phosphorylation of STAT5, a downstream target of the IL-7Rα, was observed in MS patients. This activated transcription factor induced the expression of cytotoxic molecules, like perforin and granzymes in MS patients. CD8 T-cells co-expressing the IL-7Rα were present in pre-active MS lesions, suggesting that they contribute to MS immunopathogenesis. Interestingly, in these lesions, also the ligand IL-7 was produced. No differences were found in either the percentage of positive cells or the levels of expression between IL-7Rα MS risk SNP carriers compared with non-risk carriers.

The balance of activated and regulatory T-cells is important in the physiological immune response. Functionally active regulatory T-cells are characterised by the expression of CD25 (IL-2RA), the transcription factor FoxP3 and the absence of the IL-7Rα (CD127). FoxP3 is the master transcriptional repressor of the IL-7Rα. In addition to the well-studied CD4 Tregs, also CD8 Tregs exist. Previously, no numerical differences in CD8 Treg were found between MS patients and HC. Additionally, also no functional impairment of these cells was detected. However, the characterization was based on solely the expression of CD25 and FoxP3. Therefore, we assessed whether numerical differences exist between $CD25^{hi}FoxP3^{+}CD127^{-}$ CD8 Tregs. We found that these CD8 Tregs with presumably functionally fully active FoxP3 were decreased in MS patients (**chapter 2.2**).

In addition to important cellular functions of the membrane bound IL-7Rα described in **chapter 2**, also a soluble IL-7Rα is produced. Based on a transfection study, it was previously hypothesised that the MS risk SNP in the IL-7Rα alters the expression of the sIL-7Rα. Therefore, we and others have investigated this in large cohorts of MS patients and healthy controls. Indeed, in **chapter 3**

we observed that risk SNP carriers of the IL-7Rα MS SNP have increased levels of the sIL-7Rα. In MS patients, the levels of the sIL-7Rα were significantly decreased compared with HC. Additionally, the expression IL-7 is significantly decreased in MS. This might be due to increased consumption of IL-7, as the levels of the sIL-7Rα are important to prevent enhanced consumption of IL-7. From these studies, it is apparent that in MS patients, the balance in the IL-7/IL-7Rα pathway has shifted towards enhanced T-cell responses to IL-7Rα ligation.

A T-cell can only be activated when it receives multiple signals. The first signal is antigen-presentation via HLA molecules, the second signal is costimulation and the third signal is cytokine stimulation. In **chapter 4**, we investigated the C-type lectin CLEC16A and its functional role in antigen presentation. We found that CLEC16A functionally interacts with the HOPS/ RILP complex, mediating important functions for the transport of late endosomes. CLEC16A is important for the maturation and functioning of these endosomes. Knockdown of CLEC16A impaired the formation of MIIC, the HLA loading compartments, and thereby decreased HLA-II surface expression. Given the strong association between HLA class II alleles and MS risk, it is interesting that one of the novel MS risk genes is involved in the regulation of HLA-II expression.

Besides genetic factors in the etiology of MS, also environmental factors are important. The most well-validated environmental risk factors for MS are low serum levels of vitamin D, and Epstein Barr virus (EBV) infections or a history of infectious mononucleosis (M. Pfeiffer). In MS patients, humoral responses against EBV are enhanced, especially against the latency associated protein EBNA-1. In **chapter 5**, we investigated whether the enhanced IgG-responses against EBNA-1 are at least partially due to MS-associated immune genes. We found that the SNP in the NF-κB1, BATF and TMEM39A locus are associated with enhanced EBNA-1 IgG production. Additionally, we validated that HLA-DQA1/DRB1 risk allele carriers have increased EBNA-1 IgG levels compared with other HLA class II allele carriers. These risk SNP were independently associated with EBNA-1 IgG levels. They exerted an additive effect on humoral EBV responses. Interestingly, BATF and NF- κB1 are also important for establishing EBV latency.

In addition to the inflammatory component of MS, neurodegeneration is another important hallmark of the disease. Compared to the immunological component of MS, relatively little is known about the underlying mechanisms of neurodegeneration. Only a few MS risk genes have a presumed function within the CNS and thereby these genes are promising candidates for further study to increase our understanding of neurodegeneration. In **chapter 6**, we investigated the kinesin kif21b. Kinesins are important proteins involved in the transport of organelles and proteins within cells. These molecules are very important for the survival and function of neurons, since large distances need to be covered. We found that cortical kif21b expression was increased in Alzheimer's patients (AD, a classical neurodegenerative disease) younger than 62 years compared with MS patients and non-demented

controls (NDC). In the white matter, kif21b expression was significantly increased in MS patients compared with NDC. Increasing levels of cortical kif21b were observed in areas with more severe neuropathology in both MS and AD and enhanced kif21b expression was associated with a shorter disease duration. Additionally, accelerated progression to sustained disability (EDSS 6) in MS was found in patients with abundant kif21b expression. Lastly, kif21b protein was expressed in neurons as expected. Interestingly, kif21b protein was also found in astrocytes. Upon astrocyte activation, kif21b expression increased. This may explain the observed differences between the patients groups, as reactive astrocytosis is mainly found in young Alzheimer's patients and in the white matter of MS patients.

Currently, for the minority of MS-associated SNP functional immunogenetic studies have been performed. The majority of studies found functional alterations associated with the SNP. For the remaining SNP, it will be important to perform functional studies to increase our knowledge regarding the (immune)pathogenic mechanism underlying MS, to integrate these studies into clinical trials in order to indentify reliable biomarkers. This will facilitate the development of new more targeted drugs to efficiently treat MS patients and minimising the side effects caused by currently more general immunomodulatory drugs.

Samenvatting

Multiple sclerose (MS) is een complexe neurologische ziekte. Zowel omgevings- als genetische factoren dragen bij aan het ontstaan van MS. De sterkste genetische associatie met MS, die sinds de jaren 70 bekend is, is dragerschap van bepaalde HLA klasse II allelen. De afgelopen zes jaar is de kennis van MS-geassocieerde genen die niet behoren tot de HLA klasse II locus aanzienlijk gestegen. De eerste genoom-brede associatie-studie (GWAS), gepubliceerd in 2007, heeft twee MS risico polymorfismen geïdentificeerd. Tevens werden een paar verdachte genen gevonden. Vanaf 2007 is het aantal MS risico polymorfismen gestegen tot 57 in 2011 en 105 in 2013.

De meerderheid van de MS risicogenen is betrokken bij de verworven immuniteit. Alhoewel de kennis van de genetische risicofactoren betrokken bij het ontstaan van MS sterk is toegenomen, blijft de belangrijke vraag bestaan ***hoe*** deze risico polymorfismen ***functioneel*** bijdragen aan het verhoogde risico op het ontwikkelen van MS. Daarom zijn functionele immunogenetische studies in MS noodzakelijk om de onderliggende mechanismen beter te begrijpen.

In **hoofdstuk 2.1** hebben we het effect van het polymorfisme in de interleukine 7 receptor alfa keten (IL-7Rα) onderzocht. Ten eerste onderzochten we of de hoeveelheid IL-7Rα op verschillende functionele groepen van lymfocyten verschilt tussen MS patiënten en gezonde controles. Het percentage CD8-positieve effector geheugen cellen met een IL-7Rα was significant hoger in MS patiënten vergeleken met gezonde controles. Tevens was ook de hoeveelheid IL-7Rα per cel verhoogd op de CD8 effector memory T-cellen van MS patiënten. Na stimulatie met interleukine 7 (IL-7) vonden we in MS patiënten een versterkte fosforylering van het door de IL-7Rα gebruikte signaleringseiwit STAT5. Deze geactiveerde transcriptiefactor leidde in MS patiënten tot de productie van cytotoxische moleculen, zoals perforine en granzymen. CD8-positieve T-cellen die een IL-7Rα hebben, werden ook gevonden in MS brein weefsel. In gebieden met $CD8^{+}IL\text{-}7R\alpha^{+}$ cellen vonden we tevens het ligand IL-7. Dit maakt het aannemelijk dat deze cellen bijdragen aan het immunopathogene mechanisme van MS. Er werden geen verschillen gevonden in zowel het percentage positieve cellen als de hoogte van expressie van de IL-7Rα tussen dragers en niet-dragers van het IL-7Rα risico polymorfisme.

De balans tussen geactiveerde en regulatoire T-cellen is erg belangrijk in de normale fysiologische immuunrespons. Functionele regulatoire T-cellen worden gekarakteriseerd door de expressie van CD25 (IL-2RA), de afwezigheid van de IL-7Rα (CD127) en de transcriptie factor FoxP3 intracellulair. FoxP3 is een belangrijke negatieve regulator van de IL-7Rα. Naast de uitvoerig bestudeerde CD4 regulatoire T-cellen, bestaan er ook CD8 regulatoire T-cellen. In de literatuur werden tot op heden geen verschillen in de aantallen CD8 regulatoire T-cellen gevonden tussen MS patiënten en gezonde controles. De functionaliteit van deze cellen is tevens hetzelfde tussen patiënten en controles. Echter de karakterisering van deze cellen was alleen gebaseerd op hoge expressie van CD25 en FoxP3. Daarom hebben wij onderzocht of er verschillen zijn in de aantallen cellen met het fenotype $CD8^{+}CD25^{hoog}IL\text{-}7R\alpha^{-}FoxP3^{+}$ regulatoire T-cellen tussen MS en gezonde personen. MS patiënten

hebben significant minder CD8 regulatoire T-cellen zonder IL-7Rα vergeleken met gezonde controles (**hoofdstuk 2.2**).

Naast belangrijke cellulaire functies van de membraan-geassocieerde IL-7Rα beschreven in **hoofdstuk 2**, bestaat er ook een oplosbare IL-7Rα. Het risicopolymorfisme in de IL-7Rα zou leiden tot meer vrij oplosbare IL-7Rα. Deze hypothese is echter gebaseerd op een transfectie studie waarin alleen een cellijn en geen primair patiënten materiaal gebruikt is. Daarom hebben anderen en wij in een grote groepen MS patiënten en gezonde controles onderzocht of deze hypothese correct is. Mensen met het risico polymorfisme hebben inderdaad meer vrij oplosbaar IL-7Rα (**hoofdstuk 3**). MS patiënten hebben significant minder vrij oplosbaar IL-7Rα vergeleken met gezonde controles. Tevens onderzochten we of de hoeveel van het in het bloed aanwezige ligand IL-7 (dit gaat een functionele interactie met de IL-7Rα aan) verschilt tussen MS patiënten en gezonde controles. MS patiënten hebben significant verlaagde hoeveelheden IL-7 in het bloed ten opzichte van gezonde personen. Dit is mogelijk het gevolg van toegenomen IL-7 consumptie, aangezien de hoeveelheid vrij oplosbaar IL-7Rα belangrijk is in de regulatie van de consumptie van IL-7. Concluderend uit de studies in de **hoofdstukken 2 en 3** is het duidelijk dat in MS patiënten de balans in de IL-7Rα biologie verschoven is naar versterkte cellulaire responsen.

Voor T-cellen is het noodzakelijk om meerdere signalen te ontvangen voordat ze geactiveerd kunnen worden. Het eerste signaal krijgen T-cellen door het presenteren van een antigen in een HLA molecuul door een antigen presenterende cel, het tweede signaal is co-stimulatie door de antigeen presenterende cel en het derde signaal is cytokine stimulatie. In **hoofdstuk 4** hebben we onderzocht welke functionele rol het C-type lectine CLEC16A speelt in antigen presenterende cellen. We vonden dat CLEC16A functioneel een interactie aangaat met het HOPS/ RILP complex en daarmee belangrijk is voor het transport van endosomen. CLEC16A is cruciaal voor de maturatie en de functie van deze endosomen. Het uitschakelen van CLEC16A vermindert de vorming van de HLA klasse II beladings compartimenten (MIIC). Daardoor neemt na het uitschakelen van CLEC16A de hoeveel HLA klasse II op de celmembraan van de antigen presenterende cel af. Gegeven de lang bekende associatie van HLA klasse II allelen en het verhoogde risico op het ontwikkelen van MS, is het extra interessant dat een van de nieuwe MS risico polymorphismen betrokken is bij de regulatie van HLA klasse II expressie.

Naast genetische factoren in de etiologie van MS spelen ook omgevingsfactoren een belangrijke rol. De meeste bekende en gevalideerde omgevingsfactoren geassocieerd met een verhoogd risico op het ontwikkelen van MS zijn verlaagde hoeveelheden vitamine D in het bloed en het doormaken van een Epstein Barr virus (EBV) infectie, of het hebben doorgemaakt van de ziekte van Pfeiffer door EBV. MS patiënten hebben een versterkte humorale immuunrespons tegen EBV, met name tegen het EBNA-1 eiwit van EBV, wat erg belangrijk is voor latentie van het virus. In **hoofdstuk 5** hebben we

onderzocht of de versterkte humorale respons tegen EBV gedeeltelijk veroorzaakt worden door de MS-geassocieerde immunologische risicogenen. Polymorfismen in de transcriptie factoren NF-κB1 en BATF en in het gen TMEM39A (functie op dit moment onbekend) zijn geassocieerd met verhoogde EBNA-1 IgG levels. Daarnaast valideerden we dat dragers van het HLA-DQA1/DRB1 allel meer EBNA-1 IgG in het serum hebben vergeleken met dragers van andere HLA klasse II allelen. De polymorfismen en het HLA klasse II allel zijn onafhankelijk geassocieerd met versterkte humorale EBNA-1 responsen en tevens versterken ze elkaars effect. Zowel BATF en NF-κB1 zijn belangrijk voor de latentiefase van EBV.

Naast de inflammatoire component van MS is neurodegeneratie een belangrijk kenmerk van het ziektebeeld. Op dit moment is er relatief weinig bekend over de onderliggende mechanismen van de neurodegeneratieve component vergeleken met de immunologische component van MS. Een paar MS-risico polymorfismen hebben een veronderstelde functie in het centrale zenuwstelsel (CZS). Daarom zijn deze genen erg interessant om verder te bestuderen om de kennis over neurodegeneratie in MS uit te breiden. In **hoofdstuk 6** hebben we het kinesine kif21b onderzocht. Kinesines zijn intracellulaire eiwitten, betrokken bij het transport van organellen en eiwitten. Voor het overbruggen van grote afstanden zijn speciale eiwitten nodig en de kinesines die hierbij betrokken zijn, zijn dus cruciaal voor de functie en het overleven van neuronen. Wij vonden dat corticale kif21b expressie verhoogd is in jonge Alzheimer patiënten (Alzheimer is een klassieke neurodegeneratieve ziekte) ten opzichte van MS patiënten en niet-dementerende controles. In MS patiënten werden verhoogde kif21b levels gevonden in de witte stof ten opzichte van niet-dementerende controles. In zowel AD en MS werden toenemende hoeveelheden corticale kif21b gevonden in gebieden met meer neuropathologische afwijkingen. Hogere levels van kif21b waren ook geassocieerd met kortere ziekteduur. MS patiënten met veel kif21b hadden minder tijd nodig voor het ontwikkelen van blijvende invaliditeit (gedefinieerd als EDSS 6.0). Het eiwit kif21b werd zoals verwacht gevonden in neuronen. Tevens werd kif21b in astrocyten gevonden. Zodra we astrocyten activeerden, nam kif21b toe. De toename van kif21b in astrocyten na activatie verklaart de gevonden verschillen in kif21b expressie tussen de verschillende patiënten groepen, aangezien reactieve astrocytose met name gevonden wordt in jonge dementie patiënten en dit fenomeen ook is waargenomen in de witte stof van MS patiënten.

Op dit moment is voor de minderheid van de MS-risico polymorfismen een functionele immunogenetische studie uitgevoerd. Van de immunogenetica studies die zijn gedaan, heeft de meerderheid functionele verschillen gevonden tussen niet-risico en risicodragers. Het is erg belangrijk om voor alle SNP waar op dit moment nog geen functionele studies voor gedaan zijn, dit uit te voeren om zo de (immuno)pathogenese van MS beter te gaan begrijpen. Beter inzicht hierin zal de ontwikkeling van nieuwe meer gerichte geneesmiddelen bevorderen. Deze meer rationele en specifiekere geneesmiddelen zullen waarschijnlijk effectiever zijn en minder bijwerkingen hebben vergeleken met de meer algemene immunosupressieve middelen die op dit moment voorgeschreven worden aan MS patiënten.

List of abbreviations

AD	Alzheimer's disease
ALS	amyotrophic lateral sclerosis
APC	antigen presenting cell
APP	amyloid precursor protein
BBB	blood brain barrier
CD	cluster of differentiation
CD8 EM	CD8+ effector memory T-cell
CD8 EMRA	CD8+ effector memory CD45RA+ T cell
CFEOM1	congenital fibrosis of the extraocular muscles type 1
CI	confidence interval
CIS	clinically isolated syndrome
CLR	C-type lectin receptor
CNS	central nervous system
CSF	cerebrospinal fluid
DC	dendritic cell
DM	diabetes mellitus
dpi	days post infection
EAE	experimental autoimmune encephalomyelitis
EBNA-1	Epstein Barr nuclear antigen 1
EBV	Epstein Barr virus
ETP	early thymic progenitor
eQTL	expression quantitative loci
ER	endoplasmatic reticulum
FOXP3	forkhead box P3
GC	germinal center
GFAP	glial fibrillary acidic protein
GM	grey matter
GWAS	genome wide association studies
HC	healthy control
HLA	human leucocyte antigen
HOPS	homotypic fusion and protein sorting
HR	hazard ratio
IFN-γ	interferon gamma
IG	immunoglobulin
IL-7	interleukin 7
imDC	immature DC
(s)IL-7Rα	(soluble) interleukin 7 receptor α chain
IQR	interquartile range
IM	infectious mononucleosis (M. Pfeiffer)
JAK	janus kinase
Kif	kinesin
lncRNA	long non-coding RNA
MAG	myelin associated glycoprotein
MAP2	microtubule-associated protein 2
MBP	myelin basic protein
mDC	mature DC
MIIC	HLA class II loading compartment
miRNA	micro RNA
mtDNA	mitochondrial DNA
(mo)DC	(monocyte derived) dendritic cell
MOG	myelin oligodendrocyte glycoprotein
MRI	magnetic resonance imaging
MS	multiple sclerosis
NAWM	normal appearing white matter
NDC	non-demented control
NeuN	neuronal nuclear antigen
NFT	neurofibrillary tangles
OCB	oligoclonal bands
ORF	open reading frame
ORO	oil red O staining
PBMC	peripheral blood mononuclear cells
PHA	phytohemagglutinin
PLP	myelin proteolipid protein
PMD	post-mortem delay
PML	progressive multifocal leucoencephalopathy
PPMS	primary progressive multiple sclerosis
PRR	pattern recognition receptor
(p)STAT	(phosphorylated) signal transducers and activators of transcription
RA	rheumatoid arthritis
RILP	Rab7-interacting lysosomal protein
RRMS	relapsing remitting multiple sclerosis
SCID	severe combined immunodeficiency
SNP	single nucleotide polymorphism
SOCS1	suppressor of cytokine signalling 1
SPMS	secondary progressive multiple sclerosis
TCR	T-cell receptor
Tfh	follicular T helper cell
Th	helper T cell (CD4+)
TMEV	theiler's murine encephalomyelitis virus
TNF	tumour necrosis factor
Treg	regulatory T cell
Tyk2	tyrosine kinase 2
UTR	untranslated region
VLA-4	very late activation protein 4
WM	white matter

Dankwoord

Translationeel, multidisciplinair onderzoek is net als het lopen van een estafette:
zonder een goed op elkaar ingespeeld team, zal je nooit tot een goed onderzoek komen

Maar in het onderzoek, gaat het niet alleen over een groot team, maar ook over een gevarieerd team. En daarom zijn er vele mensen die hier aandacht verdienen, maar helaas is het niet mogelijk om iedereen hier persoonlijk te noemen. Daarom wil ik allereerst iedereen die aan de totstandkoming van dit boekje heeft bijgedragen, van harte bedanken!

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Translationeel onderzoek betekent ook dat je heel veel samples van patiënten nodig hebt. Dorine, Leonieke en Yvonne, jullie hebben me de afgelopen jaren veelvuldig geholpen met het verzamelen van al het patiëntenmateriaal, waarvoor mijn dank. Ook de overige collega's van het MS centrum wil ik graag bedanken voor alle samenwerking en discussies de afgelopen jaren. Josje, ook jij hebt me altijd geholpen bij al mijn vragen. Daarnaast waren de borrels en etentjes altijd erg gezellig en daarom ben ik ook erg blij dat jij mijn andere paranimf wil zijn!

Een immunogenetisch proefschrift kan uiteraard niet tot stand komen zonder hulp van mensen die veel weten van genetica. Linda, ik heb vaak je hulp gekregen bij het verzamelen van SNP data uit de MS GWAS. Ook wil ik graag Jeanette en Andy bedanken voor hun hulp bij het verwerken en opzoeken van alle samples, die ik gebruikt heb in alle studies. Julia, ook jij hebt me vaak geholpen met de lijstjes met SNP die ik graag ik wilde krijgen. Tevens hebben we een mooi hoofdstuk over de genetica van MS geschreven!

En dan hebben we nog het werk over de MS risico polymorfismen en de veranderde humorale immuunresponsen in MS patiënten. In deze studie hebben we heel veel samples getest. Gijs, zonder jouw hulp was het nooit gelukt om alle samples te testen en ook het verkrijgen van de nodige SNP gegevens van de gezonde controles was zonder jouw hulp erg lastig geworden. Dank je voor de fijne en gezellige samenwerking! Ook wil ik graag Sandra en Janniene van het serologie lab van de virologie bedanken voor jullie tips en trucs bij het bepalen van alle antistof levels. Georges, je hebt veel meegedacht in de opzet van de studie en het beoordelen van de data. Ik heb je kritische input altijd erg gewaardeerd.

Ook buiten het Erasmus MC zijn er veel mensen die hebben geholpen aan de totstandkoming van dit proefschrift. Prof. Neefjes, beste Sjaak, we hebben elkaar leren kennen tijdens een voordracht die je hield bij ons in het ziekenhuis. Toen we over ons CLEC16A werk met je in discussie gingen, was je onmiddellijk enthousiast. Je hebt ons de mogelijkheid geboden om dit werk verder uit te breiden met vele nieuwe experimenten in het Nederlands Kanker Instituut (NKI) in Amsterdam. Marlieke, je eindeloze geduld met de co-immunoprecipitatie experimenten voor CLEC16A en al je hulp bij het opzetten van de verschillende experimenten hebben een belangrijke bijdrage geleverd aan ons

werk, waarvoor ik je graag wil bedanken. Tevens wil ik graag de overige collega's van het NKI, Hans, Rik, Lennert, Bram, Lenny en Lauran bedanken voor jullie input en hulp. Het was altijd fijn om met jullie samen te werken!

Ook voor het kif21b werk heb ik met Amsterdamse collega's van de Nederlandse Hersenbank en het Nederlands Instituut voor Neuroscience samengewerkt. Dr. Huitinga, beste Inge, de discussies die we gehad hebben over ons kinesine werk hebben ervoor gezorgd dat het werk extra diepgang heeft gekregen. Dr. Lucchinetti, beste Sabina, de stadiëring van de grijze stof laesies en de correlatie met het kinesine werk was niet mogelijk geweest zonder jouw hulp en uitleg over de verschillende laesies en hoe je deze het beste kan aantonen. Prof. Hol, beste Elly, toen we met het kif21b werk verder wilden gaan over de rol van dit kinesine in astrocyten was je onmiddellijk bereid om mee te werken. Jouw enthousiasme, kritische blik op het manuscript en de geboden mogelijkheden hebben voor een goede verklaring van de gevonden effecten in neurologische patiënten gezorgd. Dr. van Strien, beste Miriam, jij hebt de astrocyten experimenten opgepakt, waardoor we snel door konden pakken om tot een mooie afronding van het onderzoek te komen, waarvoor mijn dank.

Dr. Priatel, dear John, we met in Vancouver, Canada. We had nice discussions about our CLEC16A work. You kindly provided us with monoclonal antibodies, labelled them for us and gave us constructs to further investigate the role of CLEC16A in antigen presentation. Additionally, we had extensive discussions and you provided us with additional updates about CLEC16A, which further sharpened our thoughts about this molecule. Thanks for the nice collaboration.

Zoals al gezegd, in het onderzoek is een groot team noodzakelijk en helaas kan niet iedereen hier persoonlijk genoemd worden. Ik wil daarom ook iedereen van de flowfaciliteiten FCCF, het Optical Imaging Centrum OIC, de digitale scan microscoop van de pathologie, alle patiënten en gezonde controle personen die bereid waren om bloed te geven voor dit onderzoek van harte bedanken voor hun medewerking aan het onderzoek!

Last, but absolutely not least, mam. Jij hebt me altijd gesteund in alles wat ik deed en stond onvoorwaardelijk voor me klaar. Je enthousiasme en luisterende oor zorgde ervoor dat ik altijd vol overgave kon vertellen wat we gedaan en gevonden hadden. Bedankt voor alles wat je altijd voor me hebt gedaan!

Curriculum vitae

About the author

Karim Léon Kreft was born October 25th, 1984 in Sousse, Tunesia. He attended secondary school at the Comenius College in Capelle aan den IJssel. In 2003, he started medical school at the Erasmus MC in Rotterdam. In his third year medical training, he also started with his Master of Science in Clinical Research (Netherlands Institute of Health Sciences). During his Master, he had an exchange visit with Harvard University, Boston, MA, USA. He conducted a research project on the predictive value of lesions in the corpus callosum in clinically isolated syndrome patients under the supervision of Prof. Dr. R.Q. Hintzen of the department of Neurology at the Erasmus MC, and his Master's thesis was rated as excellent. After obtaining his Master's degree in 2008, he started his PhD training on the immunogenetics of multiple sclerosis under supervision of Prof. Dr. R.Q. Hintzen and Prof. Dr. J.D. Laman (departments of Neurology and Immunology at the Erasmus MC). During his PhD research, he had a joint research project for one year with the Dutch Cancer Institute (Anthoni van Leeuwenhoek ziekenhuis/ Nederlands Kanker Instituut, Amsterdam), where he collaborated with Prof. Dr. J. Neefjes at the department of Cell Biology. Moreover, he established and contributed to several national and international collaborations, as described in this thesis. He completed and defended his PhD thesis during his internships to finish medical training. After completion of his medical training, he will become a resident in Neurology under the supervision of Prof. Dr. P.A.E. Sillevis Smitt (dept. Neurology, Erasmus MC).

List of publications

Jafari N*, **Kreft KL***, Flach HZ, Janssens AC and Hintzen RQ. Callosal lesion predicts future attacks after clinically isolated syndrome. ***Neurology, 2009, 73, 1837-41***

Kreft KL, Mellema SJ and Hintzen RQ. Spinal cord involvement in Balo's concentric sclerosis. ***Journal of the Neurological Sciences, 2009, 279, 114-7***.

Kreft KL, Verbraak E, Wierenga-Wolf AF, Laman JD and Hintzen RQ. Role of CD8 regulatory T-cells in multiple sclerosis. ***Annals of Neurology, 2011, 169, 593-4***

Kreft KL, Verbraak E, Wierenga-Wolf AF, van Meurs M, Oostra BA, Laman JD and Hintzen RQ. The IL-7Rα pathway is quantitatively and functionally altered in CD8 T-cells in multiple sclerosis. ***Journal of Immunology, 2012, 188, 1874-83***

Kreft KL, Verbraak E, Wierenga-Wolf AF, van Meurs M, Laman JD and Hintzen RQ. Decreased systemic IL-7 and soluble IL-7Rα in multiple sclerosis patients. ***Genes and Immunity, 2012, 13, 587-92***

Huizinga R, **Kreft KL**, Onderwater S, Boonstra JG, Brands R, Hintzen RQ and Laman JD. Endotoxin- and ATP-neutralising activity of alkaline phosphatase as a strategy to limit neuroinflammation, ***Journal of Neuroinflammation, 2012, 9, 266***

Mescheriakova JY, **Kreft KL** and Hintzen RQ. Genetics of multiple sclerosis, book chapter in: ***Multiple sclerosis immunology - a foundation for current and future treatments.*** Springer, 2013, ISBN 978-1-4614-7952-9, page 197-228

Singh V, Stoop MP, Stingl C, Dekker LJ, **Kreft KL**, Luider TM and Hintzen RQ. Cerebrospinal fluid derived immunoglobulin G of different multiple sclerosis patients share mutated sequences in complementarity determining regions, ***Molecular and Cellular Proteomics, in press***

Raj DDA, Jaarsma D, Olah M, Ferreira F, Holtman I, Schaafsma W, Brouwer N, Meijer M, de Waard MC, van der Pluijm I, Brandt R, **Kreft KL**, Laman JD, Dykstra B, de Haan G, Eggen BJL, Biber KPH, Hoeijmakers JHJ and Boddeke HWGM. Priming of microglia as response to neuronal dysfunction in a DNA repair deficient model of accelerated aging, ***manuscript submitted***

Kreft KL, van Meurs M, Wierenga-Wolf AF, Melief MJ, van Strien ME, Hol EM, Oostra BA, Laman JD and Hintzen RQ. Abundant kif21b is associated with accelerated progression in neurodegenerative diseases***, manuscript submitted***

Kreft KL, Siepman TAM, Wolvers-Teterro IL, Bennett JL, Owens GP, Sillevis Smitt PAE, van Dongen JJM, Langerak AW and Hintzen RQ. Cerebrospinal fluid abnormalities associated with inflammatory demyelination suggesting primary CNS lymphoma, ***manuscript submitted***

van Luijn MM*, **Kreft KL***, Jongsma MLM, Mes SW, Wierenga-Wolf AF, van Meurs M, Melief MJ, van der Kant R, Janssen L, Janssen H, Priatel JJ, Tan R, Neefjes J, Laman JD and Hintzen RQ. Multiple sclerosis-associated CLEC16A is a key regulator of late endosomal processing and surface expression of HLA class II in antigen-presenting cells, ***manuscript submitted***

Kreft KL*, van Nierop GP*, Scherbeijn SMJ, Klaasse J, Beersma T, Verjans GMGM and Hintzen RQ. Genetic determinants of enhanced EBNA-1 IgG production in MS, ***manuscript in preparation***

Kreft KL, Dijke IE, Ionita MG, van Meurs M, Strijder C, Pasterkamp G, de Kleijn D and Laman JD. MRP-8/14 expression segregated with lipid uptake function and alternative activation (M2) features in atherosclerosis and multiple sclerosis, ***manuscript in preparation***

Kreft KL, Wierenga-Wolf AF, Laman JD and Hintzen RQ. Endotoxin levels do not predict conversion from CIS to MS, ***manuscript in preparation***

Kreft KL, Jafari N, Hoppenbrouwers IA, Schreurs MWJ, Hooijkaas H and Hintzen RQ. MS risk genes predispose for the development of thyroid auto-antibodies, ***manuscript in preparation***

van Luijn MM, **Kreft KL**, Stoop MP, Jafari N, van Meurs M, Wierenga-Wolf AF, Laman JD and Hintzen RQ. Clusterin and chromogranin A are differentially expressed in white and grey matter of multiple sclerosis patients, ***manuscript in preparation***

* Shared first authorship

Portfolio

Summary of PhD training and teaching activities

Name PhD student: drs. K.L. Kreft, MSc Erasmus MC Departments: Neurology and Immunology Research School: MolMed	PhD period: 2008-2013 Promotors: Prof. Dr. R.Q. Hintzen and Prof. Dr. J.D. Laman

1. PhD training			
	Year	**Workload (ECTS)**	**Workload (Hours)**
General academic skills			
Biomedical English Writing and Communication	2009	4	
Research skills			
Statistics	MSc Clinical Research		
Methodology			
Biomedical Research Techniques	2008	1	
In-depth courses (e.g. Research school, Medical Training)			
SNP Course V	2008	1.5	
Teach the teacher	2008		16 h
Genetical analysis in clinical research	2009	1.5	
Erasmus MC Flowday	2009		8 h
BROK/GCP	2010	1.0	
Infinicyt workshop	2010		8 h
Mucosal immunology	2011		16 h
Confocal microscopy	2011		8 h
Image analysis	2011		8 h
Presentations			
Department of Immunology Research Meeting	2009, 2010		
Department of Neurology Patiënten demo	2010		
Klinische relevantie van genetische studies in MS, awarded with "Hollands MS talent 2010" award by stichting MS educatie Nederland (geaccrediteerd door de Nederlandse vereniging voor Neurologie)	2010	7	
Brain Research Meeting	2011		
Seminar UMCG	2012		
Genetisch onderzoek in MS, Nationale MS patiëntendag	2010		
Immunological research topics and HLA biology for MD in training	2009		
(Inter)national conferences			
ESNI	2009		
ECTRIMS			
3 poster presentations	2011		
poster presentation	2010		
2 poster presentations	2009		
UEPHA*MS meeting, invited speaker	2010		
COST, 2 poster presentations	2010	19	
FOCIS			
poster presentation	2012		
poster presentation	2011		
poster presentation	2010		
ISNI			
Oral presentation	2012		
poster presentation	2010		

1. PhD training Continued			
	Year	**Workload (ECTS)**	**Workload (Hours)**
(Inter)national conferences Continued			
MS Research days			
1 poster and 1 oral presentation	2011		
1 poster and 1 oral presentation	2009		
Wetenschappelijke vergadering voor Neurologie, 1 oral presentation	2011		
Seminars and workshops			
NVVI Lunteren Immunology course	2008, 2009, 2011-2013	3	
Mini-symposia department of immunology			
NK-cells	2008		
Brain Body Debate	2009		
Chronic antigen stimulation in leukemia/lymphoma development	2009	2	
Lipids, macrophages and immunity	2009		
Journal club	2008-2011	2	
Occasional referee for peer-reviewed journals			
Genes and Immunity			
Multiple Sclerosis Journal			
Plos One	2010-2012	1	
European Journal of Neurology			
Journal of Neurology			
Journal of Neuroimmunology			
2. Teaching activities			
Lecturing			
Immunology of multiple sclerosis (course for residents in Neurology by the Nederlandse Vereniging voor Neurologie)	2012, 2013		
Immunogenetics of multiple sclerosis (Master of Science Infection and Immunity)	2010-2013	8	
Research topics in Neuro-immunology (Master of Science Infection and Immunity)	2010-2012		
Provocative research topics in biomedical sciences and neuro-immunology (Minor 3rd year Medical students)	2011		
Supervising practicals and excursions	2008-2013	5	
Immunology practicals, 2nd year Medical students			
Supervising Bachelor's theses			
Dysregulated IL-7Rα pathway in multiple sclerosis, HLO, Saxxion Higher Education Enschede	2010		5 months
Genetic determinants of memory T-cell responses in multiple sclerosis, Biomedical sciences, VU mc	2010-2011		4 months
IL-7 pathway has a differential effect on Granzyme A, B and K expression in functional CD8 T-cell subsets, HLO, Hoge School Rotterdam. This thesis was awarded with the national Stichting HAS prize for best HLO thesis	2010-2011		7 months
Supervising Master's theses			
Th17 and Treg in MS, Biomedical sciences, University Utrecht	2008-2009		6 months
CLEC16A expression and function in multiple sclerosis, Medicine, Erasmus MC, Rotterdam. This thesis was nominated for the Gerrit Jan Mulder Prize	2011		9 months
Molecular characterisation of monoclonal B-cell populations in the CSF of (suspected) MS patients, Master of Science Infection and Immunity, MolMed	2012		6 months

3. Other activities			
	Year	**Workload (ECTS)**	**Workload (Hours)**
Junior Science Program	2009, 2010	1	
Member of the JongGR, official committee of the Health Council (Gezondheidsraad) of the Ministry of Health, Well-being and Sports in The Netherlands.	2011-2013	1	
MS goes Live, MS patiëntendag, discussieleider	2013		20 h
Participation in the IMC weekendschool Rotterdam	2009-2011	1	
Participation in the National Children MS day	2010, 2011, 2013	1	
First-Ambassador of the Erasmus MC	2008-2010		
One year joint research project with the Nederlands Kanker Instituut (NKI/AVL) Amsterdam to perform functional cellelular and biological assays	2012		
SMBWO Immunologist exam obtained	2013	1	
Total (supervision of students not included)		**61**	**84 h**

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